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Desarrollo y aplicación de marcadores genéticos para el estudio y gestión de la perdiz roja (*Alectoris rufa*)

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Desarrollo y aplicación de marcadores genéticos para el estudio y gestión de la perdiz roja (*Alectoris rufa*)

Memoria presentada por

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ÍNDICE

INTRODUCCIÓN GENERAL

Introducción a la genética de la conservación

La genética de la conservación es la aplicación de la genética en la preservación de la biodiversidad. Para ello utiliza la variación genética. La expresión del fenotipo en casi todos los rasgos de un individuo tiene una base genética, denominada genotipo, sobre el cual influye el medio ambiente al que el individuo está expuesto. El genotipo está compuesto por ácido desoxirribonucleico (ADN), éste puede encontrarse en el núcleo celular formando cromosomas. El ADN está compuesto por secuencias codificantes, genes, y no codificantes. La localización de cualquier secuencia de ADN en el genoma se denomina locus, en plural loci. Los alelos son la variación que tiene un locus.

En un individuo diploide (a nivel nuclear), en cada locus, un alelo proviene de la madre y el otro del padre. Si en un locus los dos alelos son iguales, el individuo se considera homocigoto para ese locus. Por el contrario, si los alelos son distintos, el individuo es heterocigoto. A nivel poblacional, la heterocigosidad describe la variación de un locus. La heterocigosidad está relacionada con el equilibrio de Hardy-Weinberg. Respecto al número de alelos de un locus, si el número es mayor de uno el locus es polimórfico, en caso contrario es monomórfico.

En vertebrados, el ADN también está presente en las mitocondrias, ADN mitocondrial (ADNmt), y se caracteriza por ser una molécula de ADN circular, de pequeño tamaño y con un número elevado de copias. Se hereda por vía materna, es haploide y pueden existir pseudogenes mitocondriales en el genoma nuclear. Se ha secuenciado en muchas especies el genoma mitocondrial, al ser una molécula sencilla, por lo que se han desarrollado numerosos oligonucleótidos. Carece o presenta muy poca recombinación, al contrario que de lo que sucede en el ADN del núcleo. El genoma mitocondrial de vertebrados tiene entre 16000 y 21000 nucleótidos, mostrando una

organización conservada en la mayoría de las especies estudiadas. Contiene diversos genes y la región control, que regula le replicación de la cadena H y la transcripción de todos los genes mitocondriales. La región control parece ser la parte más variable del genoma mitocondrial, presentando diferentes tasas de evolución, (Randi y Lucchini 1998).

Marcadores genéticos

Para conocer la variación genética y así poder resolver cuestiones aplicadas a biología de poblaciones, conservación y gestión de especies, se emplean marcadores genéticos. Antes de los años 60, fundamentalmente utilizaban variantes fenotípicas. El desarrollo de la electroforesis de proteínas en los años 60, permitió medir la diversidad genética de manera fácil y directa. Una de las desventajas de utilizar como marcador molecular proteínas, es necesidad de sacrificar al individuo para obtener tejido rico en proteínas (hígado, corazón). La aparición de la reacción en cadena de la polimerasa (PCR, del inglés, "Polimerase Chain Reaction"; Mullis *et al*. 1986) permitió utilizar marcadores moleculares de ADN sin necesidad de métodos destructivos ó invasivos para obtener una muestra.

Isoenzimas

Para obtener este marcador molecular se utiliza un extracto de proteínas de determinados tejidos, por ejemplo el hígado. Obtenido el extracto mediante

homogeneización, se transfiere al gel, por ejemplo de acetato de celulosa, y se realiza electroforesis. El genotipo de un individuo puede deducirse a partir de los patrones de bandas observadas en los geles. Bandas de diferente movilidad se corresponden con isoenzimas distintas, diferentes secuencias de aminoácidos. Se asume que estas diferencias tienen una base genética y son heredables. Individuos homocigotos pueden tener una banda y heterocigotos dos. Como desventajas, estos marcadores son poco numerosos, poco variables, es posible que enzimas con distinta secuencia de aminoácidos tengan la misma movilidad electroforética y puede existir variabilidad en la secuencia de ADN que no se vea reflejada en la secuencia de la proteína (debido al balanceo de la 3ª base, sustituciones en esta base pueden resultar silenciosas). Además, la obtención y conservación de las muestras suele ser muy exigente y suele implicar la muerte del animal, limitando el número de especies e individuos que se pueden usar así como la diversidad de estudios que se pueden abordar.

Marcadores de ADN

Al tratarse de ADN, estos marcadores pueden ser nucleares o mitocondriales dependiendo de donde se encuentre el locus. Estos marcadores son mas variables que las isoenzimas y suelen tener más resolución pudiendo discriminar entre individuos y poblaciones. La técnica de PCR permitió la amplia utilización de los marcadores de ADN, debido a la amplificación exponencial de un determinado locus, utilizando unos oligonucleótidos, una polimerasa y varios ciclos de temperatura, fundamentalmente. Además, esta técnica permite analizar muestras de baja calidad de ADN (muestras de museo). La PCR es la base de los marcadores de ADN que se describen a continuación.

ADN polimórfico amplificado al azar

El ADN polimórfico amplificado al azar (RAPD, del inglés "Random Amplified Polymorphic DNA"), es un marcador que emplea pequeños oligonucleótidos que se pueden unir a multitud de secuencias en el genoma, resultando así una gran variedad de fragmentos de distinto tamaño amplificados por PCR y distribuidos al azar. Los polimorfismos se pueden visualizar en geles de agarosa o acrilamida y se determinan mediante la presencia de un fragmento amplificado o su ausencia. Es un método sencillo y rápido para revelar niveles elevados de variación, sin ser necesarios conocimientos previos del genoma a estudiar. Sin embargo tiene como desventajas que son marcadores dominantes, los fragmentos amplificados son anónimos y son muy sensibles a las condiciones experimentales, resultando muy difícil la interpretación de resultados.

Microsatélites

Los microsatelites son ADN no codificante que se encuentra en el núcleo de los eucariotas y están formados por repeticiones en tándem de un nucleótido (mono-nucleótido), dos nucleótidos (di-nucleótido), tres nucleótidos (tri-nucleótido), cuatro nucleótidos (tetra-nucleótido) o cinco nucleótidos (penta-nucleótido). Microsatélites di-, tri- y tetra-nucleótidos aparecen en estudios de genética poblacional, siendo los di-nucleótidos los más utilizados. Además los microsatélites pueden clasificarse en tres tipos, microsatélites puros son aquellos cuya secuencia de repetición no se interrumpe, por ejemplo, $(CA)_{19}$, compuestos son microsatélites con distintas unidades de repetición $(CA)_4(GA_9)$ e imperfectos cuando las unidades de repetición son interrumpidas por una secuencia no repetitiva $(CA)_{10}ATCG(CA)_{15}$ (Jarne y Lagoda 1996). Son marcadores

codominantes con herencia mendeliana. Pueden presentar muchos alelos en una población, en un individuo presentan dos alelos. La frecuencia de aparición de microsatélites suele ser cada 5000-50000 pares de bases (Morin *et al*. 2004).

Los loci que contienen microsatélites puede ser amplificados mediante PCR, utilizando un par de oligonucleótidos específicos que flanquean el locus a amplificar. Son reproducibles y los productos amplificados presentan un alto grado de polimorfismo en su longitud debido a la variación en el número de unidades que se repiten, produciendo una gran variabilidad en alelos, incluso con un pequeño número de loci y un gran número de individuos. Es posible discriminar, establecer relaciones de parentesco, estructura y clasificación, a varios niveles, a nivel de especie con microsatélites diagnóstico o mediante análisis filogenéticos, a nivel poblacional mediante frecuencias alélicas y a nivel individual mediante microsatélites muy polimórficos. Tienen una eleva tasa de mutación y el proceso de mutación es controvertido.

Utilizando microsatélites no específicos de especie, algunos autores han descrito posibles fallos en la amplificación, poca variabilidad o nula y aparición de alelos nulos en otras especies, aún muy relacionadas (Reed *et al*. 2000; Inoue-Murayama *et al*. 2001; Kayang *et al*. 2002; Sevane *et al*. 2011). Estos problemas pueden ocurrir debido a que los microsatélites se suelen encontrar en regiones no codificantes donde la tasa de sustitución nucleotídica es mayor que en regiones codificantes, resultando el diseño de oligonucleótidos menos "universal" que en una región conservada, pudiendo afectar en el caso de producirse mutaciones a la unión entre el oligonucleótido y su secuencia de ADN. Esto ocasiona que algunos oligonucleótidos no se unan a su secuencia complementaria y parejas de oligonucleótidos no amplifiquen o que fallen en la amplificación de ciertos alelos en otras especies (alelos nulos).

Otra desventaja de los microsatélites es la homoplasia, es decir, dos alelos que son idénticos no necesariamente tienen el mismo origen, pueden ser el resultado de diferentes procesos de mutación (Jarne y Lagoda 1996). Por ejemplo, una mutación puede añadir una unidad de repetición y luego quitar una unidad de repetición a un alelo, mientras que otro alelo no muto, ambos tendrían la misma secuencia. También existe homoplasia en el tamaño de los alelos, alelos que tienen el mismo tamaño pueden no ser iguales en secuencia, debido a inserciones o delecciones en la zona que flanquea el microsatélite ó por sustitución nucleotídica en la zona de repetición. La homoplasia en el tamaño de los alelos es uno de los principales problemas de la utilización de microsatélites en análisis filogenéticos. La elevada tasa de mutación y la posibilidad de homoplasia en el tamaño de alelos podría producir falta de resolución en un conjunto de datos de microsatélites a nivel poblacional (Hedrick 1999).

Polimorfismos en un único nucleótido

Polimorfismos en un único nucleótido (SNPs, del inglés, "Single Nucleotide Polymorphisms") es una sustitución, inserción ó delección en un nucleótido del genoma de un individuo. En el genoma nuclear, presentan herencia mendeliana, codominancia, la mayoría tienen dos alelos (Brown 1999) y un individuo tiene dos alelos. Los SNPs ocurren cada 300-1000 pares de bases (Morin *et al*. 2004). Debido a su alta frecuencia, facilidad para su identificación (alineamiento de secuencias del mismo locus) y genotipado es una marcador eficaz para la conservación de especies. Los SNPs también se encuentran en el genoma mitocondrial, en este caso se caracterizan por tener herencia materna y presentar un único alelo, denominado haplotipo.

Secuenciación de ADN, RFLP, SSCP y electroforesis

Una vez elegido y genotipado el marcador de ADN hay que revelar la variación genética que contiene. Para ello existen diversas técnicas, su elección dependerá del objetivo del estudio, financiación y equipamiento. Algunas de las técnicas se describen brevemente a continuación.

La electroforesis consiste en aplicar una corriente eléctrica a través un gel (agarosa, acrilamida), donde migran las moléculas en función de su carga eléctrica. Este método se utiliza en RAPDs, RFLPs, SSCP y puede ser utilizado en microsatélites, si los alelos presentan una diferencia de tamaño tal que puedan ser diferenciados con la resolución de esta técnica.

El polimorfismo en la conformación de hebra simple (SSCP, del inglés, "Single Stranded Conformation Polymorphism"), parte de un fragmento de ADN amplificado mediante PCR, el ADN es desnaturalizado, resultando sus cuatro hebras y estás migran en electroforesis a través de un gel de acrilamida, adquiriendo distintos patrones en función de cualquier diferencia en la secuencia de ADN. Esta técnica resulta útil cuando no hay información previa de polimorfismos, pudiendo identificar SNPs.

Los polimorfismos en la longitud de fragmentos de restricción (RFLP, del inglés, "Restriction Fragment Length Polymorphism"), se basan en un fragmento de ADN amplificado por PCR y la existencia de diferentes sitios de corte para una determinada enzima de restricción, resultando fragmentos de ADN de distinta longitud, que finalmente son visualizados mediante electroforesis. Este método se puede utilizar para visualizar SNPs.

La secuenciación de ADN, consiste en conocer la secuencia completa de ADN (nuclear o mitocondrial) que se ha amplificado previamente mediante PCR. Para ello se

utilizan los nucleótidos marcados con fluorescencia, que después de una electroforesis son leídos por un laser y finalmente interpretados por un programa informático, en un secuenciador automático. Es una técnica muy informativa y de alta resolución, pero resulta cara. Está técnica es utilizada para secuenciar cualquier fragmento de ADN , mitocondrial o nuclear, y también para verificar un SNP de un fragmento amplificado de ADN (resultando así una técnica poco informativa). Mediante un secuenciador automático también se puede visualizar únicamente el tamaño de los microsatélites mediante otro procedimiento y programa informático, donde la molécula que está marcada con fluorescencia es uno de los oligonucleótidos que flanquean el fragmento amplificado.

Aplicaciones en biología

El desarrollo de los marcadores moleculares de ADN, análisis y el muestreo no invasivo han permitido el aumento de análisis.

Nivel de individuo

Los marcadores de ADN pueden ser utilizados para detectar exogamia, cruzamiento de individuos no emparentados, ó endogamia, cruzamiento entre individuos genéticamente relacionados. Aunque estos procesos son diferentes, tienen en común su posible efecto en la reducción de la eficacia biológica del individuo. Los marcadores de ADN también pueden identificar un individuo y establecer relaciones de parentesco.

Nivel intra específico

La genética de poblaciones estudia cómo se distribuye la variación genética entre poblaciones e individuos. Puede evaluarse los procesos históricos que han dado lugar a la distribución geográfica de las poblaciones, filogeografía, la cual utiliza la distribución geográfica de las poblaciones y el patrón resultante de su genealogía de genes. Desde un punto de vista ecológico, pueden conocerse los factores ambientales (climáticos, geográficos) que actúan modulando la distribución de las especies y sus poblaciones.

Nivel taxonómico e hibridación

Cuando se estudia la distribución de la variación genética entre especies, entramos en el campo de la genética evolutiva. A nivel evolutivo puede haber diferencias entre especies en secuencias codificantes y no codificantes de ADN, en función de su proximidad filogenética.

Los marcadores moleculares pueden aportar información adicional que ayude a resolver interrogantes taxonómicos a nivel de especies o subespecies, incluyendo unidades de significado evolutivo (ESUs, del inglés "Evolutionary Significant Units") y unidades de gestión (MUs, del inglés "Management Units"). Las ESU se consideran unidades ecológicas y evolutivas independientes, especies. Desde el punto de vista genético, ESU es cuando todos los individuos tienen un ancestro común (reflejado en análisis filogenéticos del ADNmt) y presentan diferencias de frecuencias alélicas en el ADN nuclear (Moritz 1994). El tema es controvertido respecto a los aspectos a considerar para identificar estas unidades de significado evolutivo. En las MU no es

necesario que presenten un ancestro común a nivel ADNmt, sí pueden tener divergencia en frecuencias alélicas en ADN nuclear (Moritz 1994). Las MU se refieren a grupos de poblaciones con restricciones en el intercambio de individuos entre grupos.

La información genética también nos ayuda a detectar e interpretar las consecuencias de la hibridación. La hibridación es un proceso natural que ocurre cuando se cruzan dos especies diferentes dando lugar a una descendencia viable. La hibridación ocurre en muchos organismos, aproximadamente el 10% de especies animales y el 25% en plantas (Mallet 2005), contribuyendo a la adaptación y especiación en plantas, peces e insectos. En escenarios de hibridación, donde se producen retro-cruzamientos sucesivos (Mallet 2005), puede ocurrir la integración estable de material genético de una especie a otra, produciendo introgresión.

En las últimas décadas se está produciendo un aumento de hibridación introgresiva mediada por la mano del hombre, ocurriendo ésta entre una especie autóctona y otra doméstica o criada en cautividad. Este proceso de hibridación introgresiva puede ocasionar la homogeneización de poblaciones, pérdida de diversidad y perdida diferenciación genética. Además la pérdida de características genéticas, morfológicas, comportamentales o ecológicas que le han permitido a la especie adaptarse a distintos ambientes, puede ocasionar en las poblaciones híbridas una peor adaptación (Rhymery & Simberloff 1996; Allendorf 2001).

Para evitar que esta hibridación artificial se extienda y poder establecer estrategias de conservación y gestión, es fundamental la identificación de híbridos. Esta tarea puede resultar más difícil cuando la hibridación se produce entre especies cercanas y no se poseen marcadores genéticos diagnóstico (Vilá *et al*. 2003).

Los marcadores de ADN mencionados pueden clasificarse en categóricos y probabilísticos, en función de su capacidad para diferenciar especies, en consecuencia,

identificar híbridos ante escenarios de hibridación entre distintas especies. Los marcadores categóricos tienen polimorfismos diagnóstico o fijados para cada especie. Por el contrario, los marcadores probabilísticos, no poseen alelos diagnóstico y se basan en complejos modelos estadísticos bayesianos para identificar los individuos híbridos (Vähä y Primmer 2006).

Género *Alectoris*: distribución e hibridación natural en zonas de contacto

La perdiz roja (*Alectoris rufa* Linnaeus 1758, orden Galliformes, familia Phasianidae) comparte género con las perdices *A. graeca*, *A. chukar*, *A. magna*, *A. melanocephala*, *A. philbyi* y *A. barbara*. La distribución del género *Alectoris* incluye Europa, Asia y norte de África (del Hoyo *et al*. 1994). Las especies de este género tienen en su mayoría una distribución alopátrica, excepto por la existencia de simpatría entre *A. melanocephala* y *A. philbyi* en el sur de Arabia. Existe hibridación natural en zonas de contacto en la montaña Liupan (China) *A. chukar* x *A. magna* (Liu *et al*. 2006), en Tracia (Grecia) *A. graeca* x *A. chukar* y en los Alpes franceses, donde la población de *A. rufa* x *A. graeca* está localizada en un área específica sugiriendo que posibles factores genéticos (depresión por exogamia) y no genéticos (ambientales) pudieran estar impidiendo la difusión de perdices híbridas en los Alpes (Randi y Bernard-Laurent 1998, 1999; Barilani *et al*. 2007 b ,Randi 2008).

Perdices mediterráneas: situación actual

Las perdices mediterráneas son especies cinegéticas sometidas a intenso manejo. Durante el último siglo se ha producido el declive en abundancia de poblaciones de perdiz (Tucker y Health MF 1994), como consecuencia de las alteraciones en su hábitat (el abandono de la agricultura tradicional y el aumento de la agricultura intensiva) y el aumento de la presión cinegética. En zonas donde la especie es cazada de forma intensiva, se ha intentado contrarrestar esta disminución en abundancia con sueltas masivas de perdices criadas en cautividad. Varios autores han encontrado hibridación con otras especies de *Alectoris* (Barbanera *et al*. 2005; Barbanera *et al*. 2007; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Barbanera *et al*. 2015; Randi *et al*. 2003; Barilani *et al*. 2007a; Barilani *et al*. 2007b; Negri *et al*. 2013; Martínez-Fresno *et al*. 2008; Blanco-Aguiar *et al*. 2008; Tejedor *et al*. 2007), apareciendo híbridos en poblaciones de perdiz donde no había zona de contacto natural entre las especies de *Alectoris* hibridadas.

La perdiz roja es endémica del mediterráneo occidental donde su distribución natural comprende Portugal, España, Francia, noroeste de Italia, Elba y Córcega (del Hoyo *et al*. 1994). Estudios previos han demostrado la aparición de introgresión de *A. chukar* en poblaciones de *A. rufa* (Barbanera *et al*. 2005; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Negri *et al*. 2013; Blanco-Aguiar *et al*. 2008; Tejedor *et al*. 2007). La distribución natural de la perdiz griega (*A. graeca*) comprende Francia, Suiza, Austria, Italia, Yugoslavia (Croacia, Eslovenia, Rumania) Albania, Grecia y Bulgaria. Randi *et al*. 2003, Barilani *et al*. 2007a, Barilani *et al*. 2007b y Barbanera *et al*. 2009a han encontrado introgresión de *A. chukar* y *A. rufa* en perdices griegas. El rango de distribución original de la perdiz chukar (*A. chukar*) se extiende desde los Balcanes a

través de Asia central hasta Manchuria. Algunos autores (Barbanera *et al*. 2007; Panayides *et al*. 2011) han observado introgresión de *A. rufa* en perdices chukar.

Especie de estudio: la perdiz roja

La perdiz roja es una especie de importancia ecológica, biológica y socio-económica. Las perdices son aves con un papel importante en los ecosistemas, al ser componente en la dieta de numerosos depredadores: córvidos, reptiles, roedores, mamíferos carnívoros y aves rapaces (Calderón 1977, Duarte y Vargas 2001, Herranz 2000).

Ha sido introducida en Europa (Inglaterra), islas Atlánticas (Azores, Canarias, Madeira) y, con poco éxito, en Estados Unidos y Nueva Zelanda (del Hoyo *et al.* 1994).

Se han descrito 3 subespecies en *A. rufa*: *A. r. rufa* (Linneaus, 1758) distribuida a través de Francia, noroeste de Italia, Elba y Córcega, *A. r. hispanica* (Seoane, 1894) en el norte y oeste de la Península Ibérica y *A. r. intercedens* (A. E. Brehm, 1857) en el este, sur de la Península Ibérica e islas Baleares (Cramp y Simmons 1980). Una cuarta subespecie, *A. r. laubmanni* fue reconocida por Jordans (1928) en las islas Baleares. Esta clasificación de subespecies se basa en sutiles diferencias morfológicas y fenotípicas (Cramp y Simmons 1980; Villafuerte y Negro 1998).

Un estudio con ADN mitocondrial sugirió que una población del norte de España (Palencia) estaba genéticamente diferenciada del resto de poblaciones (Martínez-Fesno *et al*. 2008), mientras que recientemente otros autores no han encontrado tal patrón (Barbanera *et al*. 2010). En base a los estudios publicados, la

posible estructura genética de la especie y su relación con las subespecies descritas queda pendiente de evaluar.

La perdiz roja es una especie sedentaria, cuyo rango de dispersión tiene una media de aproximadamente 500 metros y un máximo de casi 1800 metros, en individuos procedentes de suelta en la península Ibérica (Carvalho *et al*. 1998; Pérez *et al*. 2004; Pérez *et al*. 2005). Si bien mayores distancias han sido observadas (Cramp y Simmons 1980). En cuanto a requerimientos de hábitat, la perdiz roja está aparentemente menos especializada que otras especies del mismo género, pudiendo habitar desde el nivel de mar hasta los 2000 metros de altitud, aunque raramente superan los 1500 metros. Por lo que en las regiones más eurosiberianas de la cornisa cantábrica (País Vasco, sur de Cantabria, Asturias y Galicia) se encuentra ausente o es rara. Las poblaciones de perdiz roja pueden ocupar una gran variedad de hábitats, seleccionando áreas con campos de cultivo y alta heterogeneidad espacial, incluyendo bosques mediterráneos así como áreas de matorral bajo (Blanco-Aguiar *et al*. 2003; Buenestado *et al*. 2008; Buenestado *et al*. 2009; Casas *et al*. 2009).

Debido a su plasticidad ecológica la perdiz roja está adaptada a diversidad de ambientes, desde zonas mediterráneas a zonas templadas húmedas, evitando ambientes extremos, muy fríos o muy cálidos y secos. Años extremadamente secos y calurosos reducen la producción de vegetación, supervivencia de la nidada (Rands 1987; Pescador y Peris 2007), supervivencia en juveniles (Doxa *et al*. 2012), la cría en perdices rojas criadas en campo (Casas *et al*. 2009) y la densidad de perdices (Lucio 1990; Villanúa 2008), además se incrementa el riesgo de parasitismo (Calvete *et al*. 2003). Inviernos muy fríos, con temperaturas bajas extremas y nevadas pueden disminuir la supervivencia y densidad en perdices del mismo género (Bernard-Laurent y Leonard

2000) y en numerosas especies de aves en Europa (Forsman y Monkkonen 2003; Ontiveros y Pleguezuelos 2003).

La distribución de las especies, abundancia y diversidad están en gran medida influenciadas por la heterogeneidad medioambiental (e.g., Wakeley y Aliacar 2001; Hanski y Ovaskainen 2003). Los gradientes medioambientales contribuyen realmente a la adaptación local de poblaciones estructuradas espacialmente (Doebeli y Dieckmann 2003). Sin embargo, variaciones en el clima pueden en gran medida complicar el patrón de distribución y el escenario genético en poblaciones naturales (Jenkins y Hoffmann 2001; Carrascal y Seoane 2009). Uno de los principales factores climáticos es la temperatura. Fluctuaciones en la temperatura han tenido un profundo impacto en la distribución de organismos, lo que es visible a través del tiempo (Taberlet *et al*. 1998; Hewitt 2000; Jenkins y Hoffmann 2001; Walther *et al*. 2002; Carrascal y Seoane 2009; Provan 2013). Los efectos de la temperatura también son aparentes en los patrones genéticos internos de cada especie en un amplio grupo de animales (e.g., a high dispersal terrestrial species: *Canis lupus*, Geffen *et al*. 2004; avian species: *Alectoris chukar*, Randi y Alkon 1994; Huang *et al*. 2005; Randi *et al*. 2006; *Alectoris magna*, Huang *et al*. 2007).

Otros factores como la localización geográfica de las poblaciones puede afectar a la diversidad genética de una especie. La hipótesis de `abundancia central´ sugiere que poblaciones localizadas en el centro del rango de distribución de una especie tienen una mayor abundancia que las poblaciones más alejadas del centro (periféricas) (Vucetich y Waite 2003; Eckert *et al.* 2008), lo que predice una mayor diversidad genética en poblaciones centrales respecto a periféricas. Variaciones latitudinales y longitudinales pueden también afectar el patrón de diversidad genética de una especie, posiblemente a consecuencia de procesos de expansión después de periodos glaciares (Hewitt 1996;

Hardie y Hutchings 2010; Ferrero *et al*. 2011) o por condicionantes ambientales(Geffen *et al*. 2004), aunque otros escenarios más complejos pueden ocurrir (Petit *et al.* 2003).

Esta tesis está fundamentalmente enfocada en la península Ibérica, la cual se encuentra situada en las regiones biogeográficas/climáticas eurosiberianas y mediterráneas (Peinado Lorca y Rivas-Martínez 1987) y se caracteriza por tener una alta heterogeneidad ambiental, favoreciendo la diversificación de varios taxones endémicos y la evolución de sus particulares comunidades bióticas (Benayas y Scheiner 2002). Esta amplia variedad de climas y microclimas podría haber promovido la aparición de sub-refugios dentro del refugio que fue la península Ibérica, albergando múltiples especies durante el máximo glacial (Gómez y Lunt 2007).

La península Ibérica fue una de las áreas sin cobertura glacial más importantes en Europa durante el Pleistoceno (Hewitt 2000; Gómez y Lunt 2007), posiblemente debido a su localización latitudinal entre áreas secas y templadas y a su posicionamiento geográfico entre el océano Atlántico y el mar Mediterráneo. Varios estudios mediante análisis filogeográficos han mostrado como la península Ibérica ha sido un refugio con sub-refugios en su interior (por ejemplo, mamíferos, Branco *et al.* 2002; Centeno-Cuadros *et al.* 2009; anfibios, Martínez-Solano *et al.* 2006; insectos, Vila *et al.* 2005; reptiles, Paulo *et al.* 2008). Sin embargo, hay pocos estudios en aves que hayan evaluado esta posibilidad (Gómez y Lunt 2007; pero ver en norte América Shafer *et al*. 2010), probablemente a causa de su alta capacidad de dispersión.

Complejos procesos ocurridos en los refugios podrían haber afectado a las especies de forma diferente y en distinto momento, dependiendo de su historia vital. Para realmente poder entender sus efectos se deben examinar varios taxones. El género *Alectoris* está formado por siete especies de aves ampliamente distribuidas en la región paleártica, caracterizadas por una baja dispersión, favoreciendo de este modo el

aislamiento geográfico durante los ciclos glaciares. La perdiz griega (*A. graeca*) y la perdiz chukar (*A. chukar*) son perdices mediterráneas cuya estructuración genética poblacional se atribuye a procesos de aislamiento durante el último ciclo del Pleistoceno y posterior expansión durante el Holoceno (Randi *et al*. 2003, 2006). Sin embargo, los patrones filogeográficos en la perdiz roja (*A. rufa*) aún no han sido evaluados.

El tamaño poblacional de la perdiz roja es importante, Purroy 1997 estimo entre más de 3 y más de 7 millones de perdices. La mayor parte de su población natural se encuentra en España, donde las mayores densidades se encuentran en el centro y sur peninsular, siendo menos abundante en la costa mediterránea y en el norte de la península Ibérica (País Vasco, sur de Cantabria, Asturias y Galicia). El declive en abundancia de poblaciones de perdiz roja en su habitat natural comenzó durante el último siglo, al final de los años 60 y se acentuó especialmente durante los años 80. Alteraciones en su hábitat (el abandono de la agricultura tradicional, aumento de la agricultura intensiva, repoblaciones forestales en terrenos agrícolas) y el aumento o la inadecuada gestión de la actividad cinegética son las principales causas (Blanco-Aguiar *et al*. 2003; 2004; 2008). La perdiz roja está considerada como una especie amenazada por las leyes europeas y está clasificada como vulnerable en la lista SPEC (BirdLife Itrenational 2004). Datos más recientes indican que el declive de la perdiz roja persiste (SEO/Birdlife 2013).

En España, la perdiz roja es el ave más apreciada en la caza menor. La importancia económica de esta actividad ha sido estimada en cinco mil millones de euros, considerando cinco países de la Unión Europea: Portugal, España, Francia, Reino Unido y Finlandia (Martínez *et al*. 2002). En zonas de caza intensiva, la disminución en abundancia de perdices se ha intentado contrarrestar con sueltas masivas de perdices criadas en cautividad, que han resultado ser perdices híbridas entre *A. rufa* x *A. chukar*

(Barbanera *et al*. 2005; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Negri *et al*. 2013; Blanco-Aguiar *et al*. 2008; Tejedor *et al.* 2007), a pesar de que en España la utilización de perdices híbridas ha sido prohibida por ley desde 1975 (Orden 15 julio 1975, Instituto de Conservación de la Naturaleza, ICONA, Ministerio de Agricultura Pesca y Alimentación) y sucesivas leyes nacionales (Ley 4/89 de Conservación de los Espacios Naturales y la Flora y Fauna Silvestres, Ley 42/2007 de Patrimonio Natural y Biodiversidad y Real Decreto 1628/2011 de Especies Exóticas e Invasoras) e internacionales (79/409/CEE relativa a la Conservación de Aves y 92/43/CEE referente a la Conservación de Habitats, Fauna y Flora). El uso ilegal de perdices híbridas permite reducir costes de producción en las granjas, debido a que las perdices híbridas criadas en granja tienen un mayor tamaño de puesta, más fácil manejo y crianza (Padrós 1991; Blanco-Aguiar *et al*. 2008).

La identificación de híbridos es fundamental para establecer estrategias de conservación y gestión. Los parentales de cada especie, primera (F1) y segunda (F2) generación de híbridos podrían ser identificados fenotípicamente, sin embargo, el primer retrocruzamiento y sucesivos no podrían identificarse debido a la similitud con sus parentales. Siendo necesaria la utilización de marcadores genéticos diagnóstico.

El cariotipo de la perdiz roja está formado por 78 (2n) cromosomas, un par de cromosomas sexuales (hembras ZW, machos ZZ) y 38 pares de autosomas, siendo 30 pares micro-cromosomas y 9 pares macro-cromosomas (Arruga *et al*. 1996). El idiograma de perdiz es muy similar al de gallina (*Gallus gallus*), dos aves muy cercanas evolutivamente y se ha observado una sintenia casi total entre ambos genomas (Kasai *et al*. 2003). El genoma de gallina se ha secuenciado (International Chicken Genome Sequencing Consortium 2004) y se puede acceder a él a través de la página Web http://www.ensembl.org/Multi/blastview?species=Gallus_gallus. Está herramienta

resulta de gran utilidad al permitir situar los marcadores de perdiz en la secuencia de gallina, así de este manera obtener información relativa al cromosoma, posición y su proximidad o no a un determinado gen.

En la península Ibérica se ha descrito introgresión de ADN mitocondrial de perdiz chukar en perdiz roja (Tejedor *et al*. 2007; Blanco-Aguiar *et al*. 2008; Martínez-Fresno *et al*. 2008; Tejedor *et al*. 2008; Barbanera *et al*. 2009; Barbanera *et al*. 2010; Rodríguez-García y Galián 2014). Debido a la herencia materna del genoma mitocondrial es posible que poblaciones de perdiz con manejo intensivo (sueltas masivas de perdices) no tengan evidencia de introgresión o sea subestimada con este tipo de marcador. Algunos estudios han empleado marcadores nucleares, microsatélites no específicos de especie con alelos solapantes en puntuales localidades de muestreo, obteniendo nulos o bajos niveles de introgresión nuclear (Tejedor *et al*. 2007; Tejedor *et al*. 2008; Sevane *et al*. 2011). Es necesario evaluar el nivel de introgresión nuclear de perdiz chukar en perdiz roja, utilizando marcadores nucleares codominantes con alelos diagnóstico, mediante un amplio estudio a través de España y Portugal.

Objetivo de la tesis

Ante el problema de hibridación introgresiva que existe en las especies de perdiz roja, chukar y griega, es necesario el desarrollo de marcadores genéticos para diferenciar individuos con introgresión, debido a que fenotípicamente esto no es posible. Se desconocen marcadores nucleares codominantes con alelos diagnóstico para estas especies.

El grado de introgresión nuclear que existe en la perdiz roja se desconoce, tanto a nivel de campo como de granja, debido a que se han empleado marcadores de ADN con un reducido poder de resolución para detectar hibridación.

Respecto a la estructuración genética de la especie, los pocos estudios genéticos que hay publicados (ADN mitocondrial) emplean un tamaño muestral bajo, no representativo de la distribución de la especie en la península Ibérica e incluyen localidades en las que han encontrado híbridos mitocondriales (excluyendo únicamente los híbridos en posteriores análisis; Barbanera *et al*. 2010), es decir es probable que hayan incluido en sus análisis localidades con perdices de suelta, no autóctonas de la zona de muestreo. Actualmente en la especie se desconoce su estructura genética, los procesos demográficos que la han llevado a ocupar la distribución geográfica actual, tanto como sus posibles refugios durante las glaciaciones.

Desde una aproximación ecológica y en base a los resultados obtenidos de expansión demográfica de la perdiz roja y su posible relación con los cambios climáticos ocurridos en la península Ibérica durante las glaciaciones del Pleistoceno (Capítulo 3), se desconoce la influencia del clima en la diversidad y diferenciación genética de las localidades de perdiz roja. Del mismo modo, en esta especie no se ha evaluado la hipótesis de `abundancia central´ ni su posible relación con la diversidad genética de la especie.

Por tanto, el **objetivo general** de esta tesis es:

Describir cómo se ha estructurado en el espacio la diversidad genética de la perdiz roja, con el fin último de ayudar a la conservación de esta especie.

Para conseguir este objetivo general se han utilizado una serie de aproximaciones que se describen a continuación:

1ª. Desarrollo de marcadores genéticos mediante la construcción de una librería de ADN de perdiz roja enriquecida en microsatélites (Capítulo 1A, 1B).

2ª. Caracterización de una serie de marcadores de ADN, microsátelites y polimorfismos de un único nucleótido (SNP) en intrones, para las especies de perdiz roja, griega y chukar. Desarrollo de marcadores diagnóstico, microsátelites sin solapamiento de alelos y SNPs en intrones con alelos fijados, para las tres especies de perdiz (Capítulo 1B).

3ª. Evaluación de la introgresión de perdiz chukar en el genoma nuclear de perdiz roja, en localidades de campo y granja, mediante muestras de perdices distribuidas en España y Portugal (Capítulo 2).

4ª. Estudio de estructuración genética en la perdiz roja, hipótesis de aislamiento en sub-refugios dentro del refugio que fue la península Ibérica y procesos demográficos que posiblemente influenciaron la presente estructura y distribución poblacional (Capítulo 3).

5ª. Influencia del clima en la diversidad y diferenciación genética entre localidades de perdiz roja. Evaluación de la hipótesis de `abundancia central´ así como su predicción referente a la diversidad genética (Capítulo 4).

6ª. Medidas de gestión para que las repoblaciones o sueltas alteren lo menos posible las características genéticas de las poblaciones naturales de perdiz roja.

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CHAPTER 1

The development of molecular markers for the study of red-legged partride (*Alectoris rufa*) and related species

PART A) Sixteen new polymorphic microsatellite markers isolated for red-legged partridge (*A. rufa*) and related species

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PRIMER NOTE

Sixteen new polymorphic microsatellite markers isolated for red-legged partridge (*Alectoris rufa*) and related species

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Abstract

We developed and tested 16 new polymorphic microsatellite markers for the red-legged partridge (*Alectoris rufa*): four dinucleotide, two trinucleotide, eight tetranucleotide and two pentanucleotide repeat loci. The number of alleles per locus ranged from two to 21, observed heterozygosity was from 0.03 to 1.00 and expected heterozygosity was comprised between 0.18 and 0.91. Cross-specific amplification in others members of the Phasianidae family highlighted the potential usefulness of these molecular markers for the study of related species.

Keywords: *Alectoris chukar*, *Alectoris rufa*, cross-specific amplification, *Gallus gallus*, microsatellite, *Perdix perdix*

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The red-legged partridge (*Alectoris rufa*) is an endemic species of the western Mediterranean whose natural distribution includes France, northwestern Italy and Iberian Peninsula. There are three recognized *A. rufa* subspecies: *A. r. rufa* (Linnaeus, 1758) throughout France, northwestern Italy, Elba and Corsica; *A. r. hispanica* (Seoane, 1894) in northern and western Iberian Peninsula and *A. r. intercedens* (Brehm A. E. 1857) in eastern and southern Iberian Peninsula and Balearic Islands (del Hoyo *et al*. 1994).

Alectoris spp. are valuable game birds that have shown general declines in their distribution ranges, and are thus considered threatened by European Union laws and are classified as Species of European Conservation Concern (SPEC) (BirdLife International 2004). Due its general decline, local populations have been reinforced with captive-bred individuals. If captive stocks are used, they must be the closest available subspecies or population to the original stock (IUCN 1998). Here we report new highly polymorphic microsatellite markers in *A. rufa* that might allow the assessment of the potential genetic structure in red-legged partridge populations.

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Blood samples were collected from wild populations in Álava (Spain, $N = 33$) and Cádiz (Spain, $N = 36$), which were preserved at room temperature in 100% ethanol. Genomic DNA was extracted from blood samples using a standard phenol–chloroform method (Sambrook *et al*. 1989) and enriched for $(AGC)_8$, $(TGGA)_7$, $(CA)_{13}$, $(GTG)_7$, $(GACA)_7$, $(GAGAA)_6$, $(CT)_{13}$, $(AAAG)_7$, $(GAT)_8$, $(GAA)_8$, $(GATA)_7$ and $(TAA)_{14}$ following a procedure modified from Hamilton *et al*. (1999). Briefly, 50 µg of DNA was digested with 150 U each of *Alu*I, *Bsp*LI, *Hpy*8I, *Rsa*I, *Bse*LI, *Taa*I, *Bsu*RI, *Mbo*I, *Tru*1I, *Bme*1390I (Fermentas) and *Dde*I (New England BioLabs). Restriction fragments of 300–700 base pairs (bp) were excised from an agarose gel, dephosphorylated with calf intestinal alkaline phosphatase and ligated to SNX linkers (Hamilton *et al*. 1999). It was then hybridized with biotinylated microsatellite oligonucleotides and the DNA with microsatellites was captured with streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen) and subsequently amplified by polymerase chain reaction (PCR) with SNX-F primer. Amplified DNA was ligated into the *Xba*I site of pUC19 (Fermentas) and plasmid constructs were used to electroporate ElectroTen-Blue electroporation-competent *Escherichia coli* cells (Stratagene). Positive recombinants were replated on LB agar medium and lifted onto nylon membranes, which were probed with DIG-labelled microsatellite motives used in the enrichment. Insert DNA from screen

Table 1 Characteristics of new microsatellite loci isolated from red-legged partridge (*Alectoris rufa*)

						Population				
						Cádiz/Álava				
Locus GenBank Accession no.	Primer sequence 5′– 3′	Repeat motif	Type of repeat	Allele size range (bp)	T_a (°C)	*N*	N_a	H_O	H_E	Dev. from HWE
Aru 1A1 EF546637	F [N]: GGAAGCCAGATGAACCAAGG R [N]: ATGCATGCGTGGAGGCTGAG	$(CCAT)_{19}$	I	232–252	56	36/33	3/4	0.47/0.73	0.55/0.67	NS/NS
Aru 1B3 EF546638	F: GCTCTATAGACTCCCCTTGA R [F]: CAGCTCACTGGCTTTGCA	$(GAGAA)_{33}$	S	235–360	59	35/32	13/14	0.51/0.72	0.89/0.88	**/NS
Aru 1D39 EF546639	F [N]: ATGTGGAGACCTGGCGAGGA R: AACACTCTGCTGCTCACCAG	$(GT)_{16}$	S	150–160	58	36/33	4/5	0.50/0.67	0.53/0.68	NS/NS
Aru 1E7 EF546640	F: CCACTTCACATCAACACCCA R [F]: GACACTGGATTTCGGTTTGG	$(GGAT)_{12}$	S	176–212	55	36/33	7/8	0.72/0.76	0.80/0.78	NS/NS
Aru 1E66 EF546641	F: CCTGTTTCCAGAAATACTCCG R [F]: GAAGTTGGACCAGATGGACC	$(CA)_{17}AAA$ $(CAAA)_6$	C	226–280	58	36/33	9/14	0.64/0.52	0.71/0.87	NS/**
Aru 1E93 EF546642	F: CACGTCAATATGCTAGAGCG R [F]: ATTGGGCTATTAAGTCCTCC	$(CCAT)_{11}$	S	82–126	56	36/33	7/7	0.64/0.73	0.69/0.77	NS/NS
Aru 1E97 EF546643	F [N]: GCTGTGATTCCATTGGAGAC R: GGCGTATGTCTGCATGTAAG	$(GACA)_{32}$	I	207–435	58	35/33	21/15	0.74/0.49	0.91/0.87	**/**
Aru 1E102 EF546644	F: AGGATCTCCACTGAAGAGTG R [N]: CTCCATGGCACAAGAGAATAG	$(GGAT)_7$	S	159–199	58	36/33	10/8	0.69/0.85	0.73/0.86	NS/NS
Aru 1F25 EF546645	F: CCTAATGCTGTGTCCACCTC R [N]: GCTCACACTGTCAACAGGTG	$(CCAT)_{18}$	I	174–246	60	36/33	10/7	0.75/0.70	0.77/0.78	NS/NS
Aru 1F114 EF546646	F: TTAATTCTGAGGAGAACTCCA R [V]: GTCGGTATGATTAGACTGATC	$(CCAT)_{15}$	S	184–212	56	36/33	8/7	0.61/0.91	0.80/0.78	*/NS
Aru 1G4 EF546647	F [F]: CTGCAGTCACACAAGGCTAC R: AGTGGGTCAAGGATGAGTGG	$(CCAT)_{13}$	S	129–161	58	36/33	7/7	0.83/0.94	0.79/0.79	NS/NS
Aru 1G49 EF546648	F: GGGAACCAAACTATGCCTCC R [P]: GCACTTCTTCAGGCGCAGCT	$(GAGAA)_{31}$	S	233–333	62	36/33	15/15	1.00/0.82	0.91/0.86	NS/NS
Aru 1I121 EF546649	F [F]: TTCTACGATCTGGTCAGCCTCC R: TTGCCTGCTAAGGAGCCCAC	$(GCT)_8$	S	91–97	62	36/33	2/3	0.33/0.52	0.31/0.45	NS/NS
Aru 1J76 EF546650	F: GGTATGATTCAGCTGGGAAGT R [P]: GCAGAACTTGGTTTCCTAACG	$(TG)_{18}$	S	186–216	63	36/33	10/11	0.81/0.91	0.84/0.82	NS/NS
Aru 1K10 EF546651	F: GTGGAGAAGATATTGTTTTGGAC R [V]: GGGAGCATTCATTTATTGTCAAG	$(TG)_{14}$	S	91–97	52	36/33	2/4	0.03/0.36	0.18/0.40	**/NS
Aru 1M127 EF546652	F [P]: GCTGTGACTGATGACTATGGCT R: CAAAGACGCAAGCAGCAAGA	$(GCT)_{44}$	I	273–309	56	36/33	5/5	0.64/0.58	0.55/0.56	NS/NS

[N], NED; [F], FAM; [V], VIC and [P], PET indicate fluorescent label used; S, simple repeats; I, imperfect repeats; C, compound repeats; T_a, annealing temperature; *N*, number of individuals genotyped; N_a, number of alleles; H_o, observed heterozygosity; H_E, expected heterozygosity; Dev. from HWE, deviations from Hardy–Weinberg equilibrium: NS, not significant ($P > 0.01$); *, significant ($P < 0.01$); **, significant ($P < 0.001$).

positives with inserts in the range of 300–700 bp were sequenced with forward M13 primer and BigDye Terminator version 3.1 in an ABI PRISM 3130xl sequencer (Applied Biosystems).

Sequences were edited in BIOEDIT 7.0.4.1 (Hall 1999) and primers for those sequences containing microsatellites were designed by eye. Clone sequences were deposited in GenBank (Accession nos EF546637–EF546652). PCR amplifications were optimized for each locus and consisted of an initial denaturation of 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature (Table 1) and 30 s at 72 °C, plus a final extension of 5 min at 72 °C. All PCRs were realized on a 2720 thermalcycler (Applied Biosystems). Reaction mix (20 μL final volume) contained 16 mM $(NH_4)_2SO_4$, 67 mM TrisHCl pH 8.8, 0,01% Tween-20, 2.5 mM $MgCl_2$, 0.5 U of *Taq* DNA polymerase (BIORON), 0.5 μM of each primer, 0.2 mM of each dNTP and 50 ng of genomic DNA. The PCR products of nine DNA samples were screened for variability by visualization on 10% acrylamide gels stained with ethidium bromide. Primer pairs that reliably amplified polymorphic loci were tagged with VIC, FAM, PET or NED fluorescent labels (Applied Biosystems). Allele sizes were determined according to GENESCAN 500-LIZ Size Standard and GENEMAPPER version 4.0. (Applied Biosystems).All loci were genotyped in an ABI PRISM 3130xl sequencer (Applied Biosystems).

Twenty-one positive clones were sequenced, and all contained microsatellites. We designed primer pairs for

Table 2 Cross-specific amplification of new microsatellite loci develop for red-legged partridge (*Alectoris rufa*)

	Species								
	Alectoris chukar			*Gallus gallus*			*Perdix perdix*		
Loci	Allele sizes (bp)	*N*	N_a	Allele sizes (bp)	*N*	N_a	Allele sizes (bp)	*N*	N_a
Aru 1A1	196–236	2	3	220–256	2	2	204–232	2	3
Aru 1B3	280–285	2	2	125	3	1	120	2	1
Aru 1D39	156–158	2	2	146	3	1	142	2	1
Aru 1E7	184–196	2	3	NA			NA		
Aru 1E66	238–252	2	3	206–218	3	4	224–240	2	3
Aru 1E93	114–126	2	3	90–106	1	2	118	1	1
Aru 1E97	251–283	2	2	227	1	1	227–275	1	2
Aru 1E102	199–223	2	2	167–191	3	4	223–255	2	3
Aru 1F25	290–294	2	2	158–174	3	2	218–222	2	2
Aru 1F114	184–200	2	3	256	1	1	196–204	2	3
Aru 1G4	149–157	2	3	97–101	3	2	145–205	2	2
Aru 1G49	248–268	2	2	118	1	1	123	2	1
Aru 1I121	91–100	2	3	91	3	1	91–139	2	2
Aru 1J76	182–196	2	2	196	1	1	NA		
Aru 1K10	97	2	1	123–125	2	2	93–97	2	2
Aru 1M127	303	1	1	200–303	1	2	114–195	2	2

N, number of individuals genotyped; N_a, number of alleles; NA, no amplification. Primers were optimized for *A. rufa* only.

17 loci. Sixteen microsatellites were polymorphic in *A. rufa* (Table 1) and one was monomorphic; it was not possible to design primers for four cloned sequences. Hardy–Weinberg equilibrium and linkage disequilibrium were performed in GENEPOP version 1.2 on the Web (Raymond & Rousset 1995). Observed and expected heterozygosities were estimated with GENALEX 6 (Peakall & Smouse 2006). After Bonferroni correction (Rice 1989), there was no evidence of genotypic disequilibrium. There was no homology between our microstellites and the published *A. rufa* microsatellites (González *et al*. 2005), except for a partial homology between Aru 1J76 and AY837853.

Successful amplifications were achieved for 16 loci in chukar partridge (*Alectoris chukar*), 14 loci in grey partridge (*Perdix perdix*) and 15 loci in chicken (*Gallus gallus;* Table 2). Polymorphism was detected in 14 loci in chukar partridge, 10 loci in grey partridge and eight loci in chicken, highlighting the potential usefulness of their application in Galliformes.

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CHAPTER 1

The development of molecular markers for the study of red-legged partride (*Alectoris rufa*) and related species

PART B) The development of biparental codominant DNA markers with diagnostic alleles for *A. rufa*, *A. chukar* and *A. graeca* species identification

The development of biparental codominant DNA markers with diagnostic alleles for *Alectoris rufa*, *A. chukar* and *A. graeca* species identification.

Abstract

In the genus *Alectoris* the process of introgressive hybridization is widespread in most of its Mediterranean species, *Alectoris rufa*, *A. graeca* and *A. chukar*. For assessing this anthropogenic process it have been employed DNA markers with some limitations: maternal inherited mitochondrial DNA, dominant Random Amplified Polymorphic DNA and non-diagnostic microsatellites. However, codominant nuclear markers with diagnostic alleles of introgression among these species are unknown. Here, from *A. rufa* enriched genomic DNA library and intronic sequences obtained from other avian species, we tested and developed diagnostic microsatellite and intronic loci in these three species. We found eight microsatellites with diagnostic alleles and ten intronic single nucleotide polymorphism with fixed alleles in *A. rufa*, *A. graeca* and/or *A. chukar* species. The developed markers were tested with *A. graeca* (n = 111), *A. chukar* (n = 101) and museum *A. rufa* (n = 229) reference sample sets. These markers with diagnostic alleles would be useful to detect introgressed populations. However to identify pure and advanced backcross partridges more diagnostic markers are necessary.

Keywords: *A. rufa*, *A. graeca*, *A. chukar*, microsatellites, ancient DNA, single nucleotide polymorphism

Introduction

In the genus *Alectoris* the process of introgressive hybridization is widespread in most of its Mediterranean species, *Alectoris rufa*, *A. graeca* and *A. chukar*. From the biological point of view, the recent speciation, closely related species and no evidences of reproductive incompatibilities (Randi 1996; Randi & Lucchini 1998; Randi *et al*. 2003) make possible this process. Natural *A. chukar* x *A. magna* hybrid parapatric contact zones exist in the Liupan Mountain (China) (Liu *et al*. 2006), *A. graeca* x *A. chukar* in Thrace (Greece) and there is a third hybrid contact zone in the southern French Alps, where an *A. rufa* x *A. graeca* population is distributed over a narrow overlapping area, which suggests that genetic (outbreeding depression) and no genetic factors (environment) could restrict the diffusion of hybrid partridges in the Alps (Randi & Bernard-Laurent 1998, 1999; Barilani *et al*. 2007b; Randi 2008).

The mentioned *Alectoris* species suffer intensive management. The decline in abundance of *A. rufa* and *A. graeca* throughout their native range, especially during the last century, due to habitat changes (abandonment of traditional agricultural practices with increasing levels of intensive agriculture) and overhunting led to this diminution of partridge populations (Tucker and Health 1994). In areas where these species are actively hunted, there have been attempts to counter population decline through massive releases of captive-reared partridges, which often included hybrids with *A. chukar*, thus hybrids appear in partridge populations where there is no natural parapatric contact zone. Furthermore, farm-reared hybrid partridges can interbreed with wild populations (Casas *et al*. 2012).

The red-legged partridge (*A. rufa*) is an endemic species of the western Mediterranean which natural distribution includes France, NW Italy, Iberian Peninsula and Sicily (del Hoyo *et al*. 1994), and it has been introduced in the UK and the Mediterranean islands of Corsica, Elba and the Balearics (Alonso 1994). Previous studies have indicated the introgression of *A. chukar* in *A. rufa* populations (Barbanera *et al*. 2005; Tejedor *et al*. 2007; Blanco-Aguiar *et al*. 2008; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Negri *et al*. 2013). The natural distribution range of rock partridge (*A. graeca*) encompasses Greece, Albania, Balkans, Dinaric Alps, Alps, Italian Apennines and Sicily. Randi *et al*. 2003, Barilani *et al*. 2007a, Barilani *et al*. 2007b and Barbanera *et al*. 2009a have showed introgression of *A. chukar* and *A. rufa* in *A. graeca* partridges. The native range of the chukar partridge (*A. chukar*) extends from the Balkans across central Asia up to Manchuria. Several authors (Barbanera *et al*. 2007; Panayides *et al*. 2011) have found a few instances of introgression from *A. rufa* in *A. chukar* populations.

The identification of hybrids is a subject of primary concern for the development of conservation and management strategies, but it can be difficult when the hybridizing species are closely related and do not possess diagnostic genetic markers (Vilá *et al*. 2003). The morphological identification of *A. rufa, A. graeca, A. chukar,* first generation hybrids (F1) and second generation hybrids (F2) is possible, however first backcross or more advanced backcrosses cannot be tell apart from the parental species. Thereby, it is necessary the use of genetic markers to disclose such hybrids.

For detecting hybrids, few independent diagnostic markers (four or five) are adequate for classification of genotypes into parental, F_1 hybrids and simple backcrosses. To identify adequately pure individuals in advanced backcrosses

population is required a large number of markers (upwards 70, Boecklen & Howard 1997).

Previous studies have employed genetic markers that suffer of one or other drawbacks: invasive allozymes (Randi & Bernard-Laurent 1998, 1999); mitochondrial DNA markers of maternal inheritance (Barbanera *et al*. 2005; Barbanera *et al*. 2007; Barilani *et al*. 2007a; Barilani *et al*. 2007b; Tejedor *et al*. 2007; Blanco-Aguiar *et al*. 2008; Martínez-Fresno *et al*. 2008; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Negri *et al*. 2013; Barbanera *et al*. 2015); random amplified polymorphic DNA (RAPD) markers of nuclear, dominant inheritance and unknown nature variation, that makes difficult the interpretation of the results (Barbanera *et al*. 2005; Barbanera *et al*. 2007; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Barbanera *et al*. 2015); and low number of no species-specific microsatellite markers with overlapping alleles among species (Baratti *et al*. 2004; Barilani *et al*. 2007a; Barilani *et al*. 2007b; Tejedor *et al*. 2007; Barbanera *et al*. 2009a; Barbanera *et al*. 2010; Negri *et al*. 2013; Barbanera *et al*. 2015).

Another classification of employed DNA markers is possible depending on the method to reveal hybrids: categorical (mtDNA, RAPD) or probabilistic (microsatellite with overlapping alleles). The categorical markers have diagnostic or fixed haplotypes, alleles or fragment patterns for each species. On the other hand, probabilistic markers without diagnostic alleles rely on Bayesian statistical techniques to identify hybrid individuals, remaining to be determined the power of resolution of such markers and models in empirical case-studies (Vähä & Primmer 2006), and resulting uncertain the definite assignment of some samples. Furthermore, the identification of backcrosses is problematic, which could underestimate the true proportion of introgressed partridges analysed (Barilani *et al*. 2007a; Barilani *et al*. 2007b; Randi 2008). Indeed, the

development of diagnostic biparental codominant inherited DNA markers in *A. rufa*, *A. graeca* and *A. chukar* species is needed.

Therefore, the first aim of the present study was to characterize a set of species specific nuclear DNA markers, with codominant Mendelian inheritance, microsatellite and intronic loci in *A. rufa*, *A. graeca* and *A. chukar* species. The second aim was to discover diagnostic microsatellite and intronic loci, with non-overlapping alleles and single nucleotide polymorphisms (SNPs) with fixed alleles, respectively, among the *A. chukar*, *A. graeca* and *A. rufa* species. To test our markers, we employed reference samples of the three related *Alectoris* species (museum individuals for *A. rufa*, supposed no managed populations of *A. graeca* and *A. chukar* from diverse provenances).

Two approaches were tested for partridge sequence characterization. On the one hand, we sequenced random clones obtained from a red-legged partridge enriched genomic DNA library to identify length polymorphisms in microsatellite loci and to genotype with fluorescent labeled primers in an automatic sequencer. On the other hand, we tested PCR primer pairs (based on the sequences obtained from other avian species) designed to amplify intronic regions, from which SNPs could be screened. Interspecific SNPs were analysed by restriction fragment length polymorphism (RFLP).

Materials and methods

Reference sample collection and DNA extraction

For a species identification assay, we used reference samples of the three related *Alectoris* species: *A. rufa*, *A. chukar* and *A. graeca*. The first set of reference samples corresponded to muscle and toe pads tissue taken from 229 *A. rufa* specimens held in the Estación Biológica de Doñana museum collection (Sevilla, Spain), collected from wild individuals in the Iberian Peninsula between 1960 and 1980 (Blanco-Aguiar *et al*. 2008; see Table S2), earlier than supplemental stocking was common management practice. The second set of reference samples was composed of muscle tissue, liver and blood of 101 *A. chukar* samples that were collected from the wild in China (Gansu province = 8), Greece (Ikaria island = 19; Kos island = 5; Karpathos island = 8; Crete island = 3; Andros island = 2), Armenia (Ehegnadzor = 3), Lebanon (Aammiq = 4) and from private bird collections (n = 49). 85 *A. chukar* samples employed in Blanco-Aguiar *et al*. 2008 were included in this second set. The third set was made up of muscle tissue of 111 *A. graeca* individuals sampled from the wild in 2003 in Greece (Peloponnese = 20; Thessaly = 35; Epirus = 24; Central Greece = 8; West Macedonia = 3; East Macedonia = 19) and Italy (Sicily = 2). 98 *A. graeca* samples used in Blanco-Aguiar *et al*. 2008 were included in this third set.

Reference partridge samples were classified using diagnostic morphologic traits (del Hoyo *et al*. 1994). Samples were preserved at room temperature in absolute ethanol (except feathers which remained drie) and at -20ºC once in the laboratory. Genomic DNA was extracted from blood, liver or tissue samples using a standard phenol-chloroform method (Sambrook *et al*. 1989). For museum and feather samples DNA was isolated using the DNeasy Tissue Kit (Quiagen). Museum extractions were carried out

in a separate room specially conditioned for working with ancient DNA, which was UV irradiated before use. A negative control containing no partridge tissue was included in each group of DNA extractions.

Mitochondrial PCR-RFLP analysis of cyt-b

We analysed part of the mitochondrial cytochrome b gene through PCR-RFLP technique (Blanco-Aguiar *et al*. 2008). For museum *A. rufa* reference samples, a 186 base pair fragment of cytochrome b was amplified using primers Aroja-Cytb F and Aroja-Cytb R. The amplified product was digested with BsrGI and Tsp45I. For *A. chukar* and *A. graeca* reference samples, a 964 base pair fragment was amplified using primers Aru-Cytb F and Aru-Cytb R. In *A. chukar* reference samples, the amplified fragment was digested with BsrGI and in *A. graeca*, with BsrGI and Tsp45I restriction enzymes (Blanco-Aguiar *et al*. 2008).

Developing a DNA method for species identification: microsatellite characterization

To identify interspecific length polymorphisms in microsatellite loci among *A. rufa*, *A. chukar* and *A. graeca* species, we sequenced random clones obtained from a red-legged partridge enriched genomic DNA library developed by Ferrero *et al*. (2007). 500 new positive clones were sequenced with forward M13 primer and BigDye Terminator v 3.1 in an ABI PRISM 3130xl sequencer (Applied Biosystems). Sequences were edited and

primers were designed by eye for those sequences containing microsatellites. For each PCR primer pair we tested a gradient PCR assay, with two *A. rufa*, two *A. chukar* and two *A. graeca* samples, to determine the optimum annealing temperatures. PCR amplifications were optimized for each locus and consisted of an initial denaturation of 2 min at 94ºC followed by 35 cycles of 30 s at 94ºC, 30 s at the annealing temperature and 30 s at 72 ºC, plus a final extension of 5 min at 72ºC. PCRs were carried out in a 2720 thermal cycler (Applied Biosystems). Reaction mix (20µL final volume) contained 16 mM $(NH_4)_2S0_4$, 67 mM TrisHCl pH 8.8, 0,01 % Tween-20, 2.0 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (BIORON), 0.5 µM of each primer, 0.2 mM of each dNTP and 50 ng of genomic DNA. Negative control PCRs (without template DNA) were routinely conducted to rule out sample contamination. PCR products were run on 1.5 % agarose gels, along with a 100 base-pair ladder that was used as a molecular weight marker and gels were stained with ethidium bromide.

In order to screen for sequence polymorphism, we amplified each microsatellite locus in 10 individuals per partridge reference species. The PCR products were visualized on 10% acrylamide gels stained with ethidium bromide. Primer pairs that amplified interspecific locus were tagged with VIC, FAM, PET or NED fluorescent labels and all loci were genotyped in an ABI PRISM 3130xl sequencer (Applied Biosystems). Allele sizes were determined according to GeneScan 500-LIZ Size Standard and GeneMapper v.4.0. (Applied Biosystems). Several individuals per microsatellite and species were sequenced, both DNA strands, for verifying the repeat motif and microsatellite size.

Ancient DNA was manipulated in a laminar-flow hood kept ultraviolet light irradiated before use.

Genetic diversity analyses

Microsatellites. Observed, unbiased expected heterozygosities (Nei 1978), FIS values, mean number of alleles per locus, gene diversity and allelic richness for each sample set were determined using the program FSTAT 2.9.3.2 (Goudet 1995). Deviations from Hardy–Weinberg equilibrium (HWE), heterozygote deficits, heterozygote excess and linkage equilibrium were tested in GENEPOP 4.0.10 on the Web with default settings (Rousset 2008). Sequential Bonferroni correction was used to test for significance in multiple comparisons (Rice 1989). The presence of null alleles was tested using MICRO-CHECKER 2.2.3. (Van Oosterhout *et al*. 2004). We calculated the nuclear differentiation Fst among species in GENALEX 6 (Peakall & Smouse 2006).

Developing a DNA method for species identification: SNP-RFLP characterization

To identify interspecific SNPs in *A. rufa*, *A. chukar* and *A. graeca* species, we tested 24 PCR primer pairs designed to amplify intronic regions based on the sequences obtained from other avian species. For each PCR primer pair we tested a gradient PCR assay, with one *A. rufa*, one *A. chukar* and one *A. graeca* samples, to determine the optimum annealing temperature. Loci for which potentially homologous PCR products were observed in agarose gel electrophoresis were then optimized using PCR conditions (2 min at 94ºC followed by 35 cycles of 30 s at 94ºC, 30 s at one of various annealing temperatures and between 30-45 s at 72 ºC, plus a final extension of 5 min at 72ºC) and

adjustment of $MgCl_2$ (1.5-3 mM) concentration. In order to screen for sequence polymorphism, we amplified each intronic locus for 6 individuals per each three partridge species using reference samples. Amplified introns were purified with Sephadex TM G-50 medium using CENTRISEP Spin Columns (Princeton Separations) and sequenced with forward and reverse primers in an ABI PRISM 3130xl sequencer using BigDye ® Terminator v 3.1 chemistry (Applied Biosystems).

Sequences were edited in BIOEDIT 7.0.4.1 (Hall 1999). For each intron, we checked the alignment by eye to identify potential SNPs. To minimize false SNPs (sequencing artefacts; Ewing & Green 1998; Wang *et al.* 1998), we considered a SNP when the base was sequenced in both directions, when the base was observed in more than one individual and when the possible polymorphism occurred in a region of high sequence quality.

SNPs in sequences target of a restriction enzyme were chosen among all identified interspecific SNPs. The PCR-RFLP interspecific pattern for each locus was verified by agarose gel electrophoresis. Finally, to enable the amplification of degraded DNA extracted from museum samples, we designed internal specific PCR primer pairs for each intron locus containing the SNP which differentiated between *A. chukar*, *A. graeca* and/or *A. rufa* species. These internal specific primer pairs were optimized and were used to genotype all reference samples by PCR-RFLP.

DNA amplification with internal specific primer pairs and restriction enzyme analysis

PCR amplifications consisted of an initial denaturation of 2 min at 94ºC followed by 35 cycles of 30 s at 94ºC, 30 s at annealing temperature (see results) and 30 s at 72 ºC, plus a final extension of 5 min at 72ºC. PCRs were performed in a 2720 thermal cycler (Applied Biosystems). Reaction mix (20µL final volume) contained 16 mM $(NH_4)_2S0_4$, 67 mM TrisHCl pH 8.8, 0,01 % Tween-20, 2.0 mM $MgCl_2$ (except for RDPSN internal primers: 1.5mM), 0.5 U of Taq DNA polymerase (BIORON), 0.5 µM of each primer, 0.2 mM of each dNTP and 50 ng of genomic DNA. Negative control PCRs (without template DNA) were routinely conducted to rule out sample contamination. All the manipulations with ancient DNA were made in a laminar-flow hood that remained UV irradiated before use. The PCR products were run on 1.5 % agarose gels, along with a 100 base-pair ladder that was used as a molecular weight marker, stained with ethidium bromide.

For each intron locus, amplification products were digested overnight with a restriction enzyme following instructions provided by the suppliers. The resulting fragments were subjected to electrophoresis on 2.5 % NuSieve agarose gels at 4V/cm (constant voltage), along with a 100 base-pair ladder, stained with ethidium bromide for visualization the fragments from digestion. In addition, we included positive control in all digestions to verify the resulting fragment pattern.

Results

Mitochondrial PCR-RFLP analysis of cyt-b

In museum *A. rufa* reference samples, we did not find recognition sites for BsrGI but all amplified fragments were digested with Tsp45I (fragment sizes: 43, 143 pb). All amplified fragments from *A. chukar* reference samples were digested with BsrGI (fragment sizes: 146, 818 pb). For *A. graeca* samples, we did not find recognition sites for BsrGI and all amplified fragments were digested with Tsp45I (fragment sizes: 681, 283 pb).

Interspecific microsatellite characterization

Of 500 sequenced clones, 262 contained microsatellites and we designed primer pairs in 130. 85 out of 130 primer pairs were tested, being polymorphic all but 6 . Primer sequences, PCR annealing temperatures, chromosome location and characteristics of 8 interspecific microsatellite loci are showed in Table1.We detected 8 microsatellite loci with not-overlapping specific alleles per species. Three of those loci were located on the *Gallus* Z chromosome (Aru 1F32, Aru 1I68 and Aru 1V16) and the same location on the sexual chromosome of *Alectoris* was corroborated checking female partridges (ZW) were homozygote for these microsatellite loci.

We analysed our three sets of reference samples to test the 8 interspecific microsatellite loci are actually diagnostic markers. All rare or anomalous alleles were sequenced, both DNA strands, for verifying the repeat motif and microsatellite size.

No loci departed significantly from linkage equilibrium after Bonferroni correction (Rice 1989). Probability tests for departure from HWE, performed in each population and by locus were in equilibrium after Bonferroni correction. No evidence of null alleles, stuttering or small allele dominance was found in the data set.

The eight microsatellite loci were variable in *A. rufa* species, showing an average of 11 alleles per locus.

In museum *A. rufa* set of reference samples, the 8 interspecific microsatellite loci verified this partridge species. None of the 8 microsatellite loci showed neither *A. chukar* nor *A. graeca* alleles in any museum reference sample, even though *A. rufa* reference samples were from widely distributed locations on the Iberian Peninsula (see Table S2 and Figure 1 in Blanco-Aguiar *et al*. 2008).

The *A. chukar* set of reference samples was corroborated by the 8 interspecific microsatellite loci, with the exception of the following samples: Aru1F32 locus, two samples from Greece, Andros island, had one allele from *A. graeca* or *A. rufa*.

In *A. graeca* reference samples, the 8 interspecific microsatellite loci verified this partridge species. One partridge sample exhibited discordant profile: Aru1H15 locus, one sample from Italy, Sicily, showed one allele from *A. chukar*.

For each microsatellite locus, specific, shared alleles per species and identified species are showed in Table 2. The Aru1E45 locus presented specific allelic pattern for *A. chukar* and some specific alleles for *A. rufa*. There was not specific pattern for *A. graeca* owing to *A. graeca* alleles were included in the pattern of *A. rufa*. The Aru1E78 locus had specific allelic pattern for *A. rufa*. The *A. chukar* and *A. graeca* species presented different pattern from *A. rufa* but the same allelic pattern between *A. chukar* and *A. graeca* species. The Aru1F32, Aru1G47, Aru1H15 and Aru1I68 loci showed

specific allelic patterns or some specific alleles for *A. chukar*; *A. graeca* and *A. rufa*. The Aru1F138 locus presented specific allelic pattern for *A. rufa* and some specific alleles for *A. chukar* and *A. graeca*. The Aru1V16 locus had some specific alleles for *A. chukar, A. graeca* and *A. rufa*.

Interspecific RFLP characterization

Of 24 primer pairs tested, high-quality sequences were obtained for 15 loci. PCR annealing temperatures and $MgCl_2$ concentrations are shown in Table 3. The total number of base pairs screened was 8787. We identified 104 SNPs (intraspecific and interspecific), including eight indels (Table 3). In four loci we did not observe interspecific SNPs and one locus had an interspecific SNP that were not target of any restriction enzyme. We found 10 intron loci containing a SNP able to be used for diagnostic purpose by means of PCR-RFLP differentiating among *A. chukar*, *A. graeca* and/or *A. rufa* species. These intronic loci had scattered distribution on *Gallus gallus* genome (Table 4). The ALDB locus was situated in Z sexual chromosome.

We designed internal specific PCR primer pairs for these 10 intronic sequences that amplify shorter fragments and were used on ancient DNA from museum samples. PCR conditions of internal specific PCR primer pairs are shown in Table·4. Description of the SNP position, diagnostic restriction enzyme, restriction fragments produced per species for each intron loci and identified species is shown in Table 5.

For the ATP-citrate lyase locus (ACL), the restriction enzyme HphI produced diagnostic RFLPs for all three partridge species under consideration. Restriction fragment sizes in base pairs (bp): *A. chukar* 84/81/35; *A. graeca* 119/81; *A. rufa* 200.

The Adenylate kinase locus (AK) showed RFLP specific pattern to *A. chukar* with the restriction enzyme AvaI. Restriction fragment sizes in bp: *A. chukar* 113/86; *A. graeca* 199; *A. rufa* 199.

In the Aldolase B locus (ALDB), the restriction enzyme NcoI produced diagnostic RFLPs for all three partridge species under consideration. Restriction fragment sizes in base pairs (bp): *A. chukar* 209; *A. graeca* 95/86/28; *A. rufa* 114/95.

The Alpha enolase locus (αENOL) had RFLPs specific pattern to *A. graeca* with the restriction enzyme RsaI. Restriction fragment sizes in bp: *A. chukar* 192; *A. graeca* 137/55; *A. rufa* 192.

The Beta fibrinogen locus (βFIB) presented RFLPs specific pattern to *A. rufa* with the restriction enzyme RsaI. Restriction fragment sizes in bp: *A. chukar* 203; *A. graeca* 203; *A. rufa* 115/88.

For Hepatocyte nuclear factor 1α locus (HNFAL), the restriction enzyme HphI showed RFLPs specific pattern to *A. chukar*. Restriction fragment sizes in bp: *A. chukar* 195; *A. graeca* 101/94; *A. rufa* 101/94.

In the L-lactate dehydrogenase locus (LDH) the restriction enzyme HphI had RFLPs specific pattern to *A. graeca*. Restriction fragment sizes in bp: *A. chukar* 176; *A. graeca* 91/86; *A. rufa* 176.

For the Myelin proteolipid protein locus (MPP) the restriction enzyme NlaIII presented RFLPs specific pattern to *A. rufa*. Restriction fragment sizes in bp: *A. chukar* 112/70; *A. graeca* 112/70; *A. rufa* 182.

In the Ornithine decarboxylase (OD) the restriction enzyme AvaI had RFLPs specific pattern to *A. graeca*. Restriction fragment sizes in bp: *A. chukar* 109/87; *A. graeca* 196; *A. rufa* 109/87.

The Rhodopsin locus (RDPSN) presented RFLPs specific pattern to *A. rufa* with the restriction enzyme NlaIV. Restriction fragment sizes in bp: *A. chukar* 132/56; *A. graeca* 132/56; *A. rufa* 188.

To test these interspecific intron loci, we analysed our three reference samples with the 10 RFLP markers selected. Restriction pattern results are shown in Table 6. Taking into account the lack of *A. graeca* haplotypes within the extant wild *A. rufa* samples studied in Blanco-Aguiar *et al*. 2008, we did not analyse the intron loci with RFLPs specific pattern to *A. graeca* (αENOL, LDH, OD) in museum *A. rufa* reference samples. All rare or anomalous digestion patterns, visualized by agarose gel electrophoresis, were sequenced and the SNPs were verified on the chromatogram.

In museum *A. rufa* reference samples, the 10 nuclear intron loci verified the RFLP pattern of this species. We did not find restriction patterns of different *Alectoris* species (*A. chukar* and *A. graeca*, see Table 6) in any museum samples, even though these were from widely distributed locations on the Iberian Peninsula (see Table S2 and Figure 1; Blanco-Aguiar *et al*. 2008).

The *A. chukar* RFLP pattern was corroborated in the *A. chukar* references samples in the 10 loci, with the exception of the following samples (see Table 6): ACL

locus, six samples (six from Greece, Ikaria island) showed the restriction patterns of heterozygote *A. chukar* / *A. rufa*, and one sample from Greece, Ikaria island showed the restriction pattern of *A. rufa*; ALDB locus, four samples from Lebanon presented the restriction patterns of *A. rufa*; αENOL locus, two samples from Greece, Ikaria island showed the restriction patterns of heterozygote *A. chukar* or *A. rufa* / *A. graeca*.

In *A. graeca* samples the 10 RFLP intron loci verified this partridge species. A few partridges samples exhibited discordant profiles (see Table 6): αENOL locus, four samples from Greece, East Macedonia (Serres) showed the restriction patterns of heterozygote *A. graeca* / *A. rufa* or *A. chukar*; βFIB locus, one sample from Greece, Thessaly (Trikala) had the restriction pattern of heterozygote *A. graeca* or *A. chukar* / *A. rufa*; HNFAL locus, one sample from Italy, Sicily showed the restriction pattern of heterozygote *A. graeca* or *A. rufa* / *A. chukar*.

Discussion

The mitochondrial PCR-RFLP analysis of cytb (Blanco-Aguiar *et al*. 2008) is an effective tool for maternal inherited DNA identification in *Alectoris* species, *A. rufa*, *A. chukar* and *A. graeca*, providing a cheaper and less time-consuming alternative to sequencing. Nonetheless, the development of nuclear diagnostic markers for the identification of introgression among these *Alectoris* species was necessary.

This study reveals diagnostic nuclear DNA markers, eight microsatellite and ten intronic loci with diagnostic alleles and SNPs with fixed alleles, respectively, in *A. chukar*, *A. graeca* and/or *A. rufa* species. Diagnostic alleles definitely characterize a particular species genome without probabilistic assessment, whereas markers with no diagnostic alleles employ complex mathematical models, and the results of the assignment of some samples is uncertain. Although depending on the divergence level of hybridized populations, an efficient detection of F1 hybrids is necessary using at least 12 probabilistic markers (without diagnostic alleles; Vähä & Primmer 2006), while only one diagnostic marker can detect F1 hybrids.

We observed instances of shared alleles among *Alectoris* species, in out of the 8 microsatellite loci of the present study. These shared alleles are not informative to detect introgression (Table 2), owing to their presence in two or three set of reference partridge species. The largest number of shared alleles was between *A. rufa* and *A. graeca*, and the lowest between the *A. rufa* and *A. chukar* species (see Table 2). The homoplasy (Jarne & Lagoda 1996), co-occurrence of some alleles those were identical in size though not descend from the same ancestral allele, might explain the existence of common alleles. However, the different number of shared alleles among pair of species and common alleles could be explained according to the cytochrome b phylogeny, see Figure 5b of Randi 1996 , in which the species *A. rufa* and *A. graeca* could be phylogenetically closer related than *A. rufa-A. chukar*. Likewise, in Aru1V16 locus there is an allele (153 pb) shared by the three *Alectoris* species, that could descend from the same ancestral allele without mutation or consequence of homoplasy.

The no homogeneous allelic pattern per species is an interesting microsatellite issue. In Aru1G47 locus occurred the following pattern: 111bp *A. chukar* allele, 113bp *A. rufa* allele (widely present in museum *A. rufa* data set), 115bp *A. chukar* allele (Table

2). This heterogeneous allelic pattern was also present in Aru1E78 locus: 164bp *A. rufa* allele (some samples from a geographic area), 166bp *A. chukar* or *A. graeca* allele, 168bp *A. chukar* or *A. graeca* allele. On the one hand, we believe that these alleles are from *A. rufa* species because of these alleles did not appear in our *A. chukar* or *A. graeca* data set. Furthermore, we did not find neither *A. chukar* nor *A. graeca* genome in any museum *A. rufa* samples, after analysing 19 DNA markers (one mitochondrial, eight microsatellites and ten intronic loci), even though *A. rufa* reference samples were from widely distributed locations on the Iberian Peninsula (see Table S2 and Figure 1; Blanco-Aguiar *et al*. 2008). The 164bp *A. rufa* allele could be a phylogeographic signal owing to the samples with this allele are from the same geographic area in North-western cluster (Ferrero *et al*. 2011). On the other hand, an increase in sample size could corroborate the origin of these alleles. Following the evolutionary dynamics of microsatellite, small number of repeats could generate less variability in species owing to the repeat number is an important factor in microsatellite mutation rates (Rose & Falush 1998; Schlötterer 2000). The Aru1E78 locus, in *A. chukar* and *A. graeca* species is very little variable (only two alleles,166-168 pb; see Table 2), this microsatellite is small in length (166 pb CA_{10}, 168 pb CA_{12}), hardly ever mutes and is fixed in these species. However in *A. rufa* species, the microsatellite began the process of mutation, increasing in length, and in consequence this locus is more variable than in *A. chukar* and *A. graeca* species.

Comparative studies between red-legged partridge and chicken (*Gallus gallus domesticus*) chromosomes (Dias *et al*. 1995; Ramos *et al*.1999; Kasai *et al*. 2003), revealed a high homology and similar close conservation between both species, which have a 2n = 78 karyotype. For this reason, the genetic markers developed herein were localized on *Gallus gallus* genome and we discovered that most of them were scattered

distribution through the genome, which entails that these markers could offer wide information about the partridge genome.

We obtained few loci (three microsatellites: Aru 1F32, Aru 1I68, Aru 1V16; and one intron: ALDB) situated on Z sexual chromosome. Due to partridge karyotype, the genotype resulting from these sexual loci depends on the sex of the sample analysed. In females (ZW), these loci were homozygote and we had information only the one of the parents' alleles, as a result of that, these markers might be less informative for introgression detection in females. In the case of male partridges (ZZ), these loci were heterozygote or homozygote depending on parents' alleles and we had complete information about its parents with these sexual loci in males.

In the analyses of reference samples, the origin of museum *A. rufa* set was corroborated by all DNA markers, however in the other sets there were samples with discordant profiles, *i.e*. samples showed alleles and/or restriction patterns of the other *Alectoris* species. On the one hand, chukar partridges presented alleles and restriction patterns of *A. rufa* and *A. graeca* species. One of the chukar private bird collections presented some *A. rufa* alleles and restriction patterns of heterozygote *A. chukar* / *A. rufa* species, possibly as a consequence of no authentic *A. chukar* origin. In Greece, Andros island, two samples had one allele from *A. graeca* or *A. rufa*. In Greece, Ikaria island, partridges showed the restriction patterns of heterozygote *A. chukar* / *A. rufa* (6), *A. chukar* or *A. rufa* / *A. graeca* (2) and the restriction pattern of *A. rufa* (1). In Lebanon, Aammiq, four partridges presented the restriction patterns of *A. rufa*. Barbanera *et al*. 2007 found introgression with *A. rufa* in *A. chukar* species, in Italy. Our study did not detect this introgression in Italian *A. chukar* population because of we did not sample this population. Neither of the markers employed in Barbanera *et al*. 2007, a mtDNA Control Region and four RAPDs, detected introgression in Greek

chukar partridges. Our mitochondrial results were in accordance with their results, but no our nuclear results, which showed introgression of *A. rufa* and *A. graeca* in Greek chukar partridges. The probability of identifying hybrid partridges increases along with increase of the number of markers. Barbanera *et al*. 2007 employed lower number of markers (a mtDNA Control Region and four RAPDs) than the present study (a mtDNA cyt-b, eight microsatellites and ten intronic loci). Furthermore, since the last century *A. rufa* and *A. graeca* partridges have been introduced in the Aegean islands (Papaevangelou *et al*. 2001; Barbanera *et al*. 2007; Barbanera *et al*. 2009b). Moreover, *A. rufa* and *A. graeca* partridges also have been introduced in Lebanon, in which has been found mtDNA introgression of *A. rufa* species in wild Lebanese chukar partidges (Barbanera *et al*. 2009b; Panayides *et al*. 2011), that results were in accordance with our nuclear Lebanese results.

On the other hand, rock partridges showed allele and restriction patterns of *A. chukar* and *A. rufa* species. In Italy, Sicily, one partridge showed an allele from *A. chukar* and restriction pattern of heterozygote *A. graeca* or *A. rufa* / *A. chukar*; in Greece, East Macedonia (Serres), four partridges had the restriction patterns of heterozygote *A. graeca* / *A. rufa* or *A. chukar*; and in Greece, Thessaly (Trikala), one partridge presented the restriction pattern of heterozygote *A. graeca* or *A. chukar* / *A. rufa*. Our results in rock partridge species were in accordance with the studies realized in *A. graeca* species (Randi *et al*. 2003; Barilani *et al*. 2007a; Barilani *et al*. 2007b; Barbanera *et al*. 2009a). In which have been detected hybrids with *A. chukar* or *A. rufa* in Greek and Italian wild *A. graeca* individuals, and in an Italian farm of *A. graeca* species (Sánchez-Barbudo 2007).

The interspecific nuclear DNA markers developed in the present study will be widely used in genetic conservation studies to assess the extent of introgressive

hybridisation in *A. chukar*, *A. graeca* and *A. rufa* species. We found markers specific of the chukar partridge species, which could be very useful to assess the extent of introgressive hybridisation of *A. chukar* in red-legged partridge distribution range or in rock partridge species from France, Sicily, Apennines, Greece and French Alps (natural red-legged x rock partridge hybrid zone), in which some authors have detected hybridization with chukar species (Randi *et al*. 2003; Barilani *et al*. 2007a; Barilani *et al*. 2007b; Barbanera *et al*. 2009a). We also obtained markers specific of the rock partridge species, which could be interesting in the study of natural red-legged x rock partridge hybrid zone in the French Alps. Furthermore, we got markers specific of the red-legged partridge species that could be used to evaluate the extent of introgression of *A. rufa* genome in rock partridge populations in Apennines, Greece and in natural red-legged x rock partridge hybrid zone in the French Alps; or in chukar partridge species.

Several factors influence the detection of genetic introgression between two species, the type of hybrid could be one of them. The nuclear method with diagnostic alleles developed in the present study can detect introgressed populations, however to differentiate between pure individual and advanced backcross would be needed upwards of 70 markers (Boecklen & Howard 1997; Halbert *et al*. 2005), a considerable genotyping effort per individual.

Recent advances in DNA sequencing and microarray technologies (Meldrum 2000 a, b) could make possible the identification of sequence polymorphisms of a hundred markers with a reduced amount of laboratory work. The SNaPshot® Multiplex System can analyze more than 15000 SNPs per day in an ABI PRISM 3130xl (Applied Biosystems). This could make possible the replacement of microsatellite by SNPs markers, avoiding the problematic shared alleles and size homoplasy of microsatellites in the identification of species origin.

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Table 1 Characterization of eight interspecific microsatellite loci for the study of introgression in *A. chukar, A. graeca* and *A. rufa* species.

Locus GenBank Accesión no.	Primer sequence 5´- 3´	Repeat motif	Type of Repeat	Allele size range (bp)	T^a (ºC)	*Gallus* Chr.	Population *A.chukar / A.graeca/ A.rufa* H_O	Population *A.chukar / A.graeca/ A.rufa* H_E
Aru 1E45	F^N: ACTGCATTGTGGACCTTCTG	$(CA)_{22}$	S	133-173	62	4	0.22/ 0.56/ 0.78	0.33/ 0.58/ 0.83
	R: TACAGCAGTGGAGGTGGTTG							
Aru 1E78	F: CCTTATGCCACGGAGTATGT	$(CA)_{25}$	S	164-214	62	13	0.06/ 0.04/ 0.57	0.06/ 0.20/ 0.58
	R^P: CTTTTGTGTCTACTAGCCCTG							
Aru 1F32	F: GCACAGCTATGCCTGACACA	$(CA)_{15}$	S	170-202	62	Z	0.13/ 0.08/ 0.27	0.31/ 0.77/ 0.56
	R^F: GACTGACTCTGGCACTTACG							
Aru 1F138	F: CCTCCATTCACTCATTCACC	$(CCAT)_{14}$	I	214-498	56	random	0.33/ 0.43/ 0.43	0.89/ 0.61/ 0.51
	R^F: GAGATCAGAAGGAAGATGGA							
Aru 1G47	F: AAATGAACCTAAACCAGAGAAC	$(CA)_{16}$	I	101-125	56	4	0.59/ 0.12/ 0.58	0.75/ 0.11/ 0.63
	R^F: GTGTTACATTTTGATACTCACC							
Aru 1H15	F^N: CTGGGAAGTTCCTTGTTCTGG	$(GT)_{30}$	I	191-261	61	1	0.32/ 0.40/ 0.42	0.58/ 0.48/ 0.78
	R: CCTGCTACTGAAGGAATCAAGG							
Aru 1I68	F: TCTTACACGTTTTACCACAGGC	$(TG)_{25}$	S	225-271	62	Z	0.06/ 0.40/ 0.28	0.25/ 0.88/ 0.64

	R^V: CTGACTCCACATCTACAGGCAG							
Aru 1V16	F: GCCTACTTTGTGCTGAGATC	$(GGT)_{24}$	S	147-186	56	Z	0.14/ 0.24/ 0.25	0.40/ 0.69/ 0.43
	R^V: TTAACATGTTCTGTCAGGAC							

^{N}, NED; ^{F}, FAM; ^{V}, VIC and ^{P}, PET indicate fluorescent label used; S. simple repeats; I. imperfect repeats; T^a. annealing temperature; Gallus Chr.*Gallus gallus* chromosome location; H_o. observed heterozygosity; H_E. expected heterozygosity.

Table 2 Cross-species comparisons for eight interspecific microstellite loci examined. Range of allele sizes and shared alleles among species tested. Identified species (ID) by locus are marked.

	Species						
	Alleles sizes						
Loci	*A.chukar* (bp)	*A.graeca* (bp)	*A.rufa* (bp)	Shared alleles (sp/ bp)	*A. chukar*	*A. graeca*	*A. rufa*
Aru1E45	133-137	143, 147-153, 161	143-169, 173	Aru/Agr: 143,147-153, 161	ID	-	*
Aru1E78	166-168	166-168	164, 174-200, 204-214	Ach/Agr: 166-168	-	-	ID
Aru1F32	170,174	180, 184-186, 190-200	182, 186-202	Aru/Agr: 186, 190-200	ID	*	*
Aru1F138	250-446	410-414, 422-434, 446-498	214, 222-234, 242	Ach/Agr: 410-414, 422-434, 446	*	*	ID
Aru1G47	111, 115-125	101, 107	107-109, 113	Aru/Agr: 107	ID	*	*
Aru1H15	191, 195, 199	225-229, 235-239, 243-245, 249	241, 245-247, 251-255, 259-261	Aru/Agr: 245	ID	*	*
Aru1I68	225, 233-235	241-269	239-267, 271	Aru/Agr: 241-267	ID	*	*
Aru1V16	147-153, 159-165	150-159	153,162-186	Ach/Agr: 150, 159 Aru/Ach/Agr: 153 Aru/Ach: 162,165	*	*	*

* some specific alleles for this species, - no identified species.

Table 3 Summary of 15 intronic sequences amplified and SNPs identified within and among studied species.

Locus	Ref.*	Primer sequences 5´-3´	$MgCl_2$ (mM)	T^a (C)	bp screened	Nº SNPs
ACL	1	F: GCTCTGCTTATGACAGCACT	2	59	482	7
		R: CAGCAATAATGGCAATGGTG				
AK	2	F: ATTGACGGCTACCCTCGCGAGGTG	2	61	650	10
		R: CACCCGCCCGCTGGTCTCTCC				
ALDB	3	F: ATCATCAAAGAAAAAGGCATGGTGGTGGG	1.5	61	523	6
		R: AGCACCATCTTTCTTGTACTGGGCACAGCG				
αENOL	3	F: TGGACTTCAAATCCCCCGATGATCCCAGC	2	63	320	9
		R: CCAGGCACCCCAGTCTACCTGGTCAAA				
βFIB	6	F: GGAGAAAACAGGACAATGACAATTCAC	2	58	≈900	7
		R: TCCCCAGTAGTATCTGCCATTAGGGTT				
GAPD	3	F: ACCTTTAATGCGGGTGCTGGCATTGC	2	63	466	4[1]
		R: CATCAAGTCCACAACACGGTTGCTGTA				
HNFAL	1	F: GCAGCCCTCTACACCTGGTA	2	60	950	5
		R: CAATATCCCCTGACCAGCAT				
LDH	4	F: GGAAGACAAACTAAAAGGAGAAATGATGGA	2	61	654	6[2]
		R: TTCCTCTGAAGCAGGTTGAGACGACTCTC				
MPP	4	F: TACATCTACTTTAACACCTGGACCACCTG	2	61	438	5
		R: TTGCAGATGGAGAGCAGGTTGGAGCC				
MYH2	5	F: GAACACCAGCCTCATCAACC	2	67	311	1
		R: TGGTGTCCTGCTCCTTCTTC				
OD	4	F: GACTCCAAAGCAGTTTGTCGTCTCAGTGT	2	63	684	5
		R: TCTTCAGAGCCAGGGAAGCCACCACCAAT				
RDPSN	1	F: TGCTACATCGAGGGCTTCTT	2	59	900	20[3]
		R: CGAGTGACCAGAGAGCGATT				
RP40	4	F: GGGCCTGATGTGGTGGATGCTGGC	2	67	363	6
		R: GCTTTCTCAGCAGCAGCCTGCTC				
TGFB2	1	F: GAAGCGTGCTCTAGATGCTG	2	60	618	10
		R: AGGCAGCAATTATCCTGCAC				
Tropomyosin	1	F: AATGGCTGCAGAGGATAA	2	59	528	3
		R: TCCTCTTCAAGCTCAGCACA				

bp - base pairs; *1, Primmer *et al*. 2002; 2, Shapiro & Dumbacher 2001; 3, Friesen *et al*. 1997; 4, Friesen *et al*. 1999; 5, Lyons *et al*. 1997, Dolman & Phillips (2004); 6, Prychitko & Moore (1997); [1] Includes two indel polymorphisms; [2] Includes one indel polymorphism; [3] Includes five indel polymorphisms.

Table 4 Characterization of 10 interspecific nuclear intron loci for the study of introgression in *A. chukar, A. graeca* and *A. rufa* species.

Locus name Assay name	Amplified intron	Internal primer sequences 5´-3´	Tª (ºC)	Fragment size (bp)	*Gallus* Chr
ATP-citrate lyase ACL	16	F: AACTTGGTCTCCCGACTTCC R: GGTGTCCTCTCAGCAGTCTC	60	200	27
Adenylate kinase AK	5	F: TGGTGATGGCACTATGTGAG R: TRTCCCCATGTCTCCTCCAG	60	199	17
Aldolase B ALDB	3	F: ACCTCTGGCAGGGACAAATG R: CGCTCAGCCAGTTTATCAAG	60	209	Z
Alpha enolase αENOL	8	F: CCATGTAAGTTCCAGGACCA R: CACCACTGCAGAGGGACAGA	60	192	21
Beta fibrinogen βFIB	7	F: CTAGTCAAGAACTTACCCTGCTCTGG R: CACCATCGACCTCTGATAATGAGTAT	66	203	4
Hepatocyte nuclear factor 1α HNFAL	2	F: CATTTCTGCCTGAGCACCAG R: AGATTCCAGGCAGATTCTTG	60	195	15

L-lactate dehydrogenase LDH	3	F: ATGAAATGCTAGTACCTCTG R: CTGAGTAGCCTCTGTACTGT	59	177	1
Myelin proteolipid protein MPP	4	F: CAAGACCACTGCCAGCATCG R: TACCCTGCACTGCCTGCGTT	60	182	4
Ornithine decarboxylase OD	6	F: AGTGATGTAGCTGGCAGTAG R: GGAGTTCCAGATTAAACAGA	60	196	3
Rhodopsin RDPSN	1	F: AGCAGAGCCGACTCCACTGT R: GCTCTGAGCTGCAGCACTGC	63	188	12

Gallus Chr.*Gallus gallus* chromosome location; Tª (ºC)-Annealing temperature

Table 5 Ten interspecific SNPs for *A. chukar*, *A. graeca* and *A. rufa* species. Amplified fragments with internal specific PCR primer pairs, restriction enzyme used and the resulting fragment pattern. Identified species (ID) by locus are marked.

		A. chukar	*A. graeca*	*A. rufa*	*A. chukar*	*A. graeca*	*A. rufa*	*A. chukar*	*A. graeca*	*A. rufa*
Locus	Restriction enzyme	SNP position:identity	SNP position:identity	SNP position:identity	Restriction fragment sizes (bp)	Restriction fragment sizes (bp)	Restriction fragment sizes (bp)			
ACL	HphI	96: C 107: G	96: T 107: G	96: T 107: A	84,81,35	119,81	200	ID	ID	ID
AK	Ava I	116: G	116: A	116: A	113,86	199	199	ID	-	-
ALDB	NcoI	89: C 115: G	89: T 115: C	89: C 115: C	209	95,86,28	114,95	ID	ID	ID
αENOL	RsaI	136: A	136: G	136: A	192	137, 55	192	-	ID	-
βFIB	RsaI	258: A	114: A	260: G	203	203	115,88	-	-	ID
HNFAL	HphI	102: C	102: T	102: T	195	101,94	101,94	ID	-	-
LDH[1]	HphI	78: -	78: A	78: -	176	91,86	176	-	ID	-
MPP	NlaIII	68: A	68: A	68: G	112,70	112,70	182	-	-	ID
OD	AvaI	114: G	114: A	114: G	109,87	196	109,87	-	ID	-
RDPSN	NlaIV	533: C	134: C	448: T	132,56	132,56	188	-	-	ID

SNP positions are numbered following the Genbank accession numbers in table3; [1] Includes one indel polymorphism.

Table 6 Result of the analyses of our three reference samples with the 10 RFLP intron loci selected. Restriction patterns and the percentage of samples.

	Reference samples		
Locus	*A. chukar* (n = 101)	*A. graeca* (n = 111)	museum *A. rufa* (n = 229)
ACL	88.1% 84/81/35pb, 6.9% 84/81/35/200pb, 1% 200pb, 4% na	98.2% 119/81 pb, 1.8% na	95.2% 200pb, 4.8 % na
	n = 89(Ach), n = 6(Ach/Aru), n = 1(Aru), n = 4	n = 109(Agr), n = 2	n = 218(Aru), n = 11
AK	96% 113/86pb, 4% na	96.4% 199 pb, 3.6% na	87.3% 199pb, 12.7% na
	n = 96(Ach), n = 4	n = 107(Agr or Aru), n = 4	n = 200 (Aru or Agr), n = 29
ALDB	94% 209pb, 4% 114/95pb, 2% na	100% 95/86/28pb	96.5% 114/95pb, 3.5% na
	n = 95(Ach), n = 4(Aru), n = 2	n = 111(Agr)	n = 221(Aru), n = 8
αENOL	84.1% 192pb, 2% 192/137/55pb, 13.9% na	96.4% 137/55pb, 3.6% 137/55/192pb	No analysed, see text
	n = 85(Ach or Aru), n = 2(Ach or Aru/Agr), n = 14	n = 107(Agr), n = 4(Agr/Aru or Ach)	
βFIB	98% 203pb, 2% na	10.8% 203pb, 0.9% 203/115/88pb, 88.3% na	91.3% 115/88pb, 8.7% na
	n = 99(Ach or Agr), n = 2	n = 12(Agr or Ach), n = 1(Agr or Ach/Aru), n = 98	n = 209(Aru), n = 20
HNFAL	96% 195pb, 4% na	99.1% 101/94pb, 0.9% 101/94/195pb	88.6% 101/94pb, 11.4% na
	n = 96(Ach) n = 4	n = 110(Agr or Aru), n = 1(Agr or Aru/Ach)	n = 203(Aru or Agr), n = 26
LDH	97% 176pb, 3% na	99.1% 91/86pb, 0.9% na	No analysed, see text
	n = 98(Ach or Aru), n = 3	n = 110(Agr), n = 1	
MPP	97% 112/70pb, 3% na	94.6% 112/70pb, 5.4% na	95.2% 182pb, 4.8% na

	n = 98(Ach or Agr), n = 3	n = 105(Agr or Ach), n = 6	n = 218(Aru), n = 11
OD	81.2% 109/87pb, 18.8% na	98.2% 196pb, 1.8% na	No analysed, see text
	n = 82(Ach or Aru), n = 19	n = 109(Agr), n = 2	
RDPSN	93.1% 132/56pb, 6.9% na	94.6% 132/56pb, 5.4% na	92.6% 188pb, 7.4% na
	n = 94(Ach or Agr), n = 7	n = 105(Agr or Ach), n = 6	n = 212(Aru), n = 17

Aru - *A. rufa*, Ach - *A. chukar*, Agr - *A. graeca*, na - no amplified, n - number of genotyped partridges, bp - base pairs.

CHAPTER 2

Assessment of reliable introgressive hybridization with diagnostic nuclear markers in red-legged partridge species in Spain and Portugal

Assessment of reliable introgressive hybridization with diagnostic nuclear markers in red-legged partridge species in Spain and Portugal

Abstract

Anthropogenic introgressive hybridization is one of the major threats to species conservation. The red-legged partridge (*Alectoris rufa*, Galliformes) is more vulnerable to this artificial process as a consequence of being a game species. The introgression of *A. chukar* into *A. rufa* have been observed, however the study of reliable nuclear introgression employing codominant nuclear markers with diagnostic alleles is unknown. In the present study we assess the introgrogressive hybridization in the red-legged partridge species with a mtDNA PCR-RFLP analysis of cyt-b, eight diagnostic non-overlapping microsatellites and seven diagnostic intronic single nucleotide polymorphisms (SNPs) which have fixed alleles in *A. chukar*, *A. graeca* and *A. rufa* species, across a wide study in Spain and Portugal with wild and farm partridges. Our markers detected nuclear introgressive hybridization, being higher and more widespread than previous studies. Breeding farms are hybrid swarms and the main source of the hybrids found in the wild. There are available genetic markers, the implication of government is necessary to avoid the continuous releasing of hybrids in red-legged partridge distribution range. We recommend that the localities without introgressive hybridization must not received released partridges, in order to avoid the real chukar introgression and possible mixture of differentiated genotypes.

Keywords: *Alectoris chukar*, *Alectoris rufa*, *Alectoris graeca*, codominant, diagnostic microsatellites, intronic SNPs with fixed alleles

Introduction

One of the main threats of biodiversity is the anthropogenic introduction of allochthonous species, through competence, predation or hybridization processes (Lodge and Shrader-Frechette 2003). If there is not ecological, behavioural or reproductive barriers, the introduction of allochthonous species may produce artificial hybridization between autochthonous and allochthonous species. In fact, this occurs in a large number of game species. For example, the native red deer (*Cervus elaphus*) has hybridized with the introduced sika deer (*Cervus nippon*) in Scotland (Goodman *et al*. 1999; Senn & Pemberton 2009; Biedrzycka et al 2012), the genetic introgression from domestic pigs on European wild boar (Goedbloed *et al.* 2013) or the domesticated quail *Coturnix coturnix japonica* may threaten the wild quail *Coturnix coturnix coturnix* (Barilani *et al*. 2005).

In the genus *Alectoris* (Galliformes), anthropogenic hybridization occurs between allopathic species, *e.g*. the red-legged partridge (*Alectoris rufa*), which is an endemic species of the western Mediterranean ecosystem which natural distribution includes France, NW Italy and Iberian Peninsula including the Mediterranean islands of Corsica, Elba and the Balearics, and the chukar partridge (*A. chukar*), which the native range extends from the Balkans across central Asia up to Manchuria (del Hoyo *et al.* 1994).

The red-legged partridge is the most important game bird species on the Iberian Peninsula. The decline in abundance throughout its native range began during the last century, at the end of the 1960s, due to habitat changes (abandonment of traditional agricultural practices with increasing intensive agriculture) and overhunting, which led

to this diminution of partridge populations (Blanco-Aguiar *et al*. 2004). The red-legged partridge species is listed as threatened under European legislation (79/409 CEE Ap. 2/1, 3/I; BERN Ap. 3) and is classified as Species of European Conservation Concern (BirdLife International 2004). In areas where the species is actively hunted, there have been attempts to counter hunting bag decline through massive releases of captive-reared partridges. It has been shown that released partridges are *A. rufa* x *A. chukar* hybrids (Barbanera *et al*. 2005; Barbanera *et al*. 2009; Barbanera *et al*. 2010; Negri *et al*. 2013; Blanco-Aguiar *et al*. 2008; Tejedor *et al.* 2007), in spite of this practice in Spain has been banned under law since 1975 (Instituto de Conservación de la Naturaleza, ICONA, Ministerio de Agricultura, Pesca y Alimentación) and successive national (4/89; 42/2007; Real Decreto 1628/2011) and international (79/409/CEE; 92/43/CEE) laws. The illegal use of hybrids might be a way of reducing production costs, due to hybrid partridges breed in farms have larger clutch sizes and easier animal handling and rearing (Padrós 1991; Blanco-Aguiar *et al*. 2008).

Previous studies have indicated the introgression of *A. chukar* in *A. rufa* populations, employing mitochondrial DNA markers of maternal inheritance (Barbanera *et al*. 2005; Barilani *et al*. 2007; Tejedor *et al.* 2007; Blanco-Aguiar *et al*. 2008; Martínez-Fresno *et al*. 2008; Tejedor *et al.* 2008; Barbanera *et al*. 2009; Barbanera *et al*. 2010; Negri *et al*. 2013; Rodríguez-García & Galián 2014; Barbanera *et al*. 2015); random amplified polymorphic DNA (RAPD) markers of nuclear, dominant inheritance and unknown nature variation, that make difficult the interpretation of the results (Barbanera *et al*. 2005; Barbanera *et al*. 2009; Barbanera *et al*. 2010; Barbanera *et al*. 2015) and low number of no species-specific microsatellite markers with overlapping alleles among species, which employed complex mathematical models of difficult comprehension (Baratti *et al*. 2004; Barilani *et al*. 2007; Tejedor *et al.* 2007; Barbanera

et al. 2009; Barbanera *et al*. 2010; Sevane *et al*. 2011; Negri *et al*. 2013; Barbanera *et al*. 2015). The use of species-specific nuclear codominant markers with diagnostic alleles for this species is unknown.

In Spain and Portugal there are studies that show the maternally introgression of *A. chukar* lineages in red-legged partridge populations (see above). However, as uniparental inheritance of mitochondrial genome, it is possible for a red-legged partridge with intensive management and constant releases to contain no mtDNA evidence of introgression. Some studies employed nuclear markers, no species-specific microsatellite markers with overlapping alleles in particular locations, that showed very low or no nuclear introgression (Tejedor *et al*. 2007; Tejedor *et al*. 2008; Sevane *et al*. 2011). Therefore, it is necessary to evaluate nuclear introgression levels of chukar in red-legged partridge species, employing species-specific nuclear codominant markers with diagnostic alleles in a wide sampled study across Spain and Portugal.

In the present study, we analysed wild (N = 1328) and farm (N = 572) red-legged partridges samples, employing a set of 16 species specific DNA markers: a mtDNA, eight diagnostic non-overlapping microsatellites and seven diagnostic intronic single nucleotide polymorphisms (SNPs) which have fixed alleles in *A. chukar*, *A. graeca* and *A. rufa* species. The maternally introgression of A. chukar lineages in red-legged partridge populations, intensive management and red-legged partridge releases, have been used as a basis to test two hypotheses. First, we hypothesised the *A. chukar* introgression into the nuclear genome of *A. rufa* wild sampled species. Second, following the nuclear introgression found in wild localities, a previous mtDNA study (Blanco-Aguiar *et al*. 2008) and that introgression come from farm facilities, we also expected *A. chukar* introgression into the nuclear genome of *A. rufa* partridges bred in farms, and a higher number of hybrid individuals in farm than in the wild.

Materials and methods

Sample collection and DNA extraction

There were two sets of *A. rufa* muscle tissue samples depending on their origin. First, 1328 legally hunted wild partridges from 1997 to 2006 years were collected in the Iberian Peninsula and Balearic Islands, from 89 localities in 27 Spanish and 4 Portuguese regions (each region with one or more localities; Table 1), with adult and chick individuals. 581 wild samples employed in Blanco-Aguiar *et al*. (2008) were included in this study. And second, 572 breeding farm partridges from six Spanish farms (Table 2) were taken during 2004-2007 years. The muscle tissue samples collected were preserved at room temperature in absolute ethanol and at -20ºC once in the laboratory. Genomic DNA was extracted according to the methods of Sambrook *et al*. (1989).

Laboratory procedures:

Mitochondrial PCR-RFLP analysis of cyt-b

We analysed part of the mitochondrial cytochrome b gene through PCR-RFLP (Blanco-Aguiar *et al*. 2008) in wild and farm individuals (wild, n = 1328; farm n= 572) to detect maternal introgressive hybridization. A 186 base pair fragment of cytochrome b gene

was amplified and digested with BsrGI and Tsp45I restriction enzymes to identify the restriction patterns of *A. chukar*, *A. graeca* and *A. rufa* species.

Microsatellite analyses

In all partridge samples (wild, n = 1328; farm n= 572) we genotyped 8 interspecific microsatellite markers, Aru 1E45, Aru 1E78, Aru 1F32, Aru 1F138, Aru 1G47, Aru 1H15, Aru 1I68 and Aru 1V16, with diagnostic alleles for *A. chukar*, *A. graeca* and *A. rufa* species. These loci were analysed as in Chapter 1.

Observed, unbiased expected heterozygosities (Nei 1978), FIS values, mean number of alleles per locus, gene diversity and allelic richness for each sampling location (n>) were determined using the program FSTAT 2.9.3.2 (Goudet 1995). Deviations from Hardy–Weinberg equilibrium (HWE), heterozygote deficits, heterozygote excess and linkage equilibrium were tested in GENEPOP 4.0.10 on the Web with default settings (Rousset 2008). Sequential Bonferroni correction was used to test for significance in multiple comparisons (Rice 1989). The presence of null alleles was tested using MICRO-CHECKER 2.2.3. (Van Oosterhout *et al*. 2004).

Intronic SNP-RFLP analyses

In wild (n = 866) and farm (n = 572) samples, we employed seven diagnostic intronic loci, ACL, AK, ALDB, βFIB, HNFAL, MPP and RDPSN, with RFLP specific patterns to *A. chukar*, *A. graeca* and *A. rufa* species. Furthermore, in four farms we also used three nuclear intronic markers with specific alleles of *A. graeca* species, αENOL, LDH, OD, using methods outlined in Chapter 1.

Negative controls were routinely conducted to rule out sample contamination. We included positive controls in both PCR and digestions. Rare or anomalous alleles or restriction patterns were corroborated by sequencing.

Results

Mitochondrial DNA

We found a total of 41 (3.1 %) partridges with *A. chukar* mtDNA among 1328 sampled Iberian wild partridges (Table1). We found *A. chukar* mtDNA in 18 out of 89 wild localities (20.2%). In 572 farm partridges from six sampled farms, we detected 20 (3.5 %) partridges with *A. chukar* mtDNA in four farms (66.7 %). The percentage of detected hybrid partridges varied from 0 % to 10.8 % (Table 2).

Microsatellite DNA

No loci departed significantly from linkage equilibrium after Bonferroni correction (Rice 1989). Probability tests for departure from HWE, performed in each population and by locus were in equilibrium after Bonferroni correction. No evidence of null alleles, stuttering or small allele dominance was found in the data set.

Microsatellites detected a total of 154 (11.6 %) *A. rufa* x *A. chukar* hybrids among 1328 sampled Iberian wild partridges (Table 1). We detected hybrids in 40 out of 89 wild localities (44.9 %). In 572 farm partridges from six sampled farms, we

detected 96 (16.8 %) *A. rufa* x *A. chukar* hybrids in six farms (100 %;). The percentage of detected hybrids varied from 8.3% to 59.5% (Table 2).

Intronic DNA

We found a total of 67 (7.7 %) *A. rufa* x *A. chukar* hybrids among 866 sampled Iberian wild partridges analysed with introns (Table 1). We detected hybrids in 15 out of 51 wild localities (29.4 %). In 572 farm partridges from six sampled farms, we detected 80 (14 %) *A. rufa* x *A. chukar* hybrids in five farms (83.3 %). The percentage of detected hybrids varied from 0 % to 48.6 % (Table 2).

Overall genetic results

The overall genetic profile (mtDNA, microsatellites and introns) for all wild Spanish and Portuguese red-legged partridges is reported in Table 1. With all markers, at least 215 individuals (16.2 %) were hybrids, in 44 out of 89 wild localities (49.4 %; 51 out of 89 localities were analysed with introns). In a locality with 15 wild sampled chicks four were hybrids. We found 45 (50.6 %) partridge localities without hybrids (Table 1). The percentage of *A. chukar* alleles per locality varied from 0.2 % in Tavira (Faro n = 20) and 5.4 % in Solana de los Barros (Badajoz n = 12; there were higher percentages but with low sample size n=2; Table 3), with an average of 2.6 %.

The overall genetic results found in partridge farms are shown in Table 2. All partridge farms 6 (100%) were *A. chukar* introgressed, with 154 (26.9 %) hybrids found. The percentage of *A. chukar* alleles per locality varied from 1 % (n = 241, n =

96) and 7.6 % (n = 37, Table 4; 0.01 between BC5 and BC6; 0.08 between BC2 and BC3), with an average of 2.9 % (0.03 is BC4).

For the overall results, the percentages of detected hybrids, introgressed localities and *A. chukar* alleles per locality, were higher in partridge farm localities than wild localities. No *A. graeca* mtDNA lineage or allele was found in our data.

In wild localities, a mtDNA marker detected 19.1 % of total hybrids (Table 3), while all nuclear markers (15loci) detected at least 87.4 % (some localities were not analysed with introns). Microsatellite (8 loci) markers detected 71.6 % of total hybrids in 89 localities and intronic markers (7 loci) detected 56.8 % in 51 wild localities analysed with intronic loci (118 total hybrids). In farm localities, the mtDNA marker found 13 % of total hybrids (Table 4), while all nuclear markers (15loci) detected 96.8 %. Microsatellite (8 loci) markers detected 62.3 % of total hybrids and intronic markers (7 loci) detected 52 %.

There were introgressed localities detected with only one kind of marker, for example, Mourela (Viana do Castelo) by mtDNA; Cangas de Narcea (Asturias) and farm locality (Cuenca) by microsatellites; Tavira (Faro) by introns (Table 3, 4). Also, only nuclear markers found introgressive hybridization in some localities, for example, Ossa de Montiel (Albacete); Poblete (Ciudad Real); Cadaqués (Gerona) (Table 3); farm locality (Soria; Table 4).

Among the microsatellite loci used, Aru 1V16, Aru 1I68 and Aru 1F32 were the microsatellite loci with less hybrid detection (1, 10 and 19 hybrids, respectively), these three loci were situated in Z sexual chromosome. The microsatellite loci Aru 1H15 and Aru 1G47 were the loci with the most hybrid detection (102 and 76 hybrids, respectively). Although Aru 1E45 and Aru 1G47 loci were situated in 4 chromosome, the information of hybrid detection was not the same (46 and 76 hybrids, respectively).

In intronic loci, the loci with less hybrid detection were ALDB, AK and RDPSN (6, 7 and 14 hybrids, respectively), the ALDB locus was situated in Z sexual chromosome. The loci with the most hybrid detection were MPP and HNFAL (62 and 38 hybrids, respectively). Although βFIB and MPP loci were situated in 4 chromosome, the information of hybrid detection was different (34 and 62, respectively).

Discussion

No *A. graeca* introgression was detected in this study. However, widespread nuclear and mitochondrial *A. chukar* introgressive hybridization was observed in red-legged partridge farm and wild localities. Our results showed higher percentages of detected hybrids, introgressed localities and *A. chukar* alleles per locality in farm than in wild localities (Blanco-Aguiar *et al*. 2008). Breeding farms are hybrid swarms and the source of the hybrids found in the wild. However, the *A. chukar* introgression in red-legged partridge localities may also extend by released hybrid partridges, which breed in wild (Casas *et al*. 2012; and this study), despite the low survival reported in released partridges (Duarte & Vargas 2004; Alonso *et al*. 2005). The releases of *A. rufa* x *A. chukar* hybrids has been banned under law since 1975 in Spain and 1979 in CEE. The inadequate regulation, monitoring and implication of government have allowed the widespread of introgressive hybridization in this country (Blanco-Aguiar *et al*. 2008).

The only use of mtDNA marker, maternal inheritance, may underestimate the number of hybrid individuals and thus, the overall impact of hybridization. Analyzing with nuclear markers, the percentage of total hybrid individuals and the percentage of

localities with introgressive hybridization increased respect to mtDNA marker (Blanco-Aguiar *et al*. 2008; this study Tables 1, 2). However, mtDNA marker can complement to other markers, mainly when mean introgression is very diluted (Goodman *et al*., 1999). For instance, in a locality analysed with mtDNA marker and 15 nuclear loci, only the mtDNA locus detected introgression (Mourela, Portugal; Table 1).

The percentage of *A. chukar* mtDNA individuals in Blanco-Aguiar *et al*. 2008 is higher (5.9 % farm, 4.3 % wild) than our mtDNA results (3.5 % farm, 3.1 % wild). However this decrease could be apparent, first because we increased sampling effort in wild partridges, and second because farm samples were collected after Blanco-Aguiar *et al*. (2008) hybrid detection technique was available. We have notice of some efforts to eliminate allocthonous mtDNA hybrids from farms. This effort to reduce *A. chukar* mtDNA hybrids is not useful to restore the genetic integrity of farm partridges because of applying nuclear markers the introgression continues higher in farm than in wild localities.

The kind of markers employed, codominant nuclear loci with diagnostic alleles that detected reliable *A. rufa* and *A. chukar* DNA, and high number of markers (mitochondrial and nuclear) made possible to detect more hybrids from partridge releases and thus, the overall and widespread impact of hybridization in wild localities. That showed an introgression levels higher than previous studies in Spain and Portugal (Tejedor *et al*. 2007; Blanco-Aguiar *et al*. 2008; Martínez-Fresno *et al*. 2008; Tejedor *et al*. 2008; Barbanera *et al*. 2009; Barbanera *et al*. 2010; Rodríguez-García & Galián 2014). Introgressed partridges were widespread across 49.4 % of sampled localities in Spain and Portugal. The highest percentage of hybrid partridges per wild locality was observed in central Spain (76.7 % , Picón, Ciudad Real), an area where there are more intensive restocking activities (Blanco-Aguiar *et al*. 2008), showing the important

consequences of the game management activities in the genetic integrity of wild populations.

Although this set of markers is a powerful tool to identify introgressed populations, the identification of advanced backcross in a partridge individual is a complicated task which need more analytical efforts. To calculate the power of detection of nuclear introgression with our markers, we employed a equation developed by Halbert *et al*. (2005; equation 2), with the assumptions that all our nuclear markers are autosomal (15 markers) and the backcrosses (BC) were stopped in BC4. For one individual, the power of detecting introgression is 62.0 %, increasing the sample size increases the power of detection, eight individuals, 99.9 %. For one individual and 99.9 % power of detecting introgression, it would be necessary 110 nuclear markers. In more advanced backcrosses (*e. g*. BC5) it should be increase the number of sample size or markers to obtain 99.9% of detection (for BC5, one individual, the power of detection is 37.9 %; 16 individuals, 99.9 %; one individual, 99.9 % of detection need 220 markers).

In the wild, F1 hybrids are absent, appearing backcrosses. The easy detection of F1 hybrids could be a reason for this lack. The observed backcross pattern indicates significant rates of introgressive hybridization as a result of high levels of backcrossing between pure and introgressed individuals (Campton 1987).

Patterns of introgression may vary across the genome (Bull *et al*. 2006; Baack & Rieseberg 2007). Our results showed less introgression of Z (sexual chromosome) linked loci compared to autosomal loci, which could be related with genomic incompatibilities on sexual chromosomes (Payseur *et al*. 2004; Macholan *et al*. 2007). No there is evidence that selection has acted to promote the persistence of chukar partridge alleles in the microstellite loci with higher detection, as neither demonstrates significant deviations from HWE. The high frequencies of chukar partridge alleles are

more likely the result of related hybrid founders with introgression in the same genomic region or random drift during the establishment of these populations when effective population sizes were very low.

The red-legged partridge species has undergone intensive game restocking during the last few decades, which is causing significant rates of introgressive hybridization with unknown consequences in *A. rufa* conservation. Furthermore, releases with partridges breed in farms do not seem to be efficient, having a negative effect in partridge abundance (Díaz-Fernández *et al*. 2013). In spite of the species management, it has been possible to find localities without restocking events, where DNA markers did not detect hybrids (Martínez-Fresno *et al*. 2008; Negri *et al*. 2013; this study Table 1). However, as governmental administration has not been receptive to this problem, we suspect that hybridization landscape is getting worse. Identify valuable partridge localities without introgression could be of particular conservation and research value (Ferrero *et al*. 2011). We recommend that these localities must not received released partridges, in order to avoid the real chukar introgression and possible mixture of differentiated genotypes. These partridge localities without restocking signals could be used as stock sources of red-legged partridges for restocking programs with conservation goals in close localities.

The establishment of reference partridge farms, funded by regional governments and strict genetic controls, could be a first step to reduce or eliminate introgressed partridges in commercial farms and could be an excellent tool for getting cancel the main source of genetic pollution. Finally, to monitoring populations and genetic quality trends, banding of all captive and released partridges should be mandatory. Unfortunately, economic lobbyist have avoid on different occasions that this proposal was implemented in environmental laws.

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Table 1 Wild red-legged partridge sampling locations in Spain and Portugal, sample size and summary of detected introgressed partridge localities employing one mitochondrial and 15 nuclear markers (microsatellites and introns).

			Partridges with A. chukar DNA			
Regions	Localities	N	mtDNA	micros.	introns	All markers
			(cyt-b)	(8 loci)	(7 loci)	
Spain						
Álava	Salinas de Añana,Espejo	43	0	0	0	0
Álava	Turiso, Villabezana	40	2 (5%)	7 (17.5%)	7 (17.5%)	12 (30%)
Álava	El Ciego	37	5 (13.5%)	12 (32.4%)	10 (27%)	16 (43.2%)
Albacete	Ossa de Montiel	41	0	8 (19.5%)	7 (17.1%)	14 (34.1%)
Albacete	Ossa de Montiel[(1)]	15	0	2 (13.3%)	3 (20%)	4 (26.7%)
Albacete	La Gineta	18	0	0	0	0
Alicante	Vall de Gallinera	8	0	0	0	0
Alicante	Confrides	7	0	0	0	0
Alicante	Castell de Castells	10	0	0	0	0
Almería	Purchena	13	0	0	0	0
Almería	Sierro	7	0	0	0	0
Almería	Oria	3	0	0	0	0
Almería	Velefique	8	0	0	0	0
Asturias	Cangas del Narcea[(2)]	4	0	1 (25%)	0	1 (25%)
Asturias	Cangas del Narcea[(3)]	6	0	0	0	0
Badajoz	Solana de los Barros	12	4 (33.3%)	4 (33.3%)	na	7 (58.3%)
Badajoz	Llerena	2	0	1 (50%)	na	1 (50%)
Badajoz	Alange	9	0	4 (44.4%)	na	4 (44.4%)
Balearic Is.	undetermined	16	0	0	0	0
Burgos	Pedrosa del Príncipe	8	0	0	na	0
Burgos	undetermined	6	0	0	na	0
Burgos	Torresandino	3	0	0	na	0
Burgos	Villadiego	40	0	2 (5%)	na	2 (5%)
Burgos	Arauzo de la Torre	20	0	5 (25%)	na	5 (25%)
Cádiz	Medina Sidonia	36	0	0	0	0
Cádiz	Medina Sidonia	13	0	1 (7.7%)	na	1 (7.7%)
Ciudad Real	Moral de Calatrava	34	0	0	0	0
Ciudad Real	Alcolea de Calatrava	34	2 (5.9%)	6 (17.7%)	1 (2.9%)	8 (23.5%)
Ciudad Real	Picón	30	4 (13.3%)	18 (60%)	15 (50%)	23 (76.7%)
Ciudad Real	Poblete	30	0	1 (3.3%)	6 (20%)	7 (23.3%)
Ciudad Real	Alhambra	30	0	2 (6.7%)	na	2 (6.7%)
Ciudad Real	Terrinches	30	1 (3.3%)	12 (40%)	na	13 (43.3%)
Ciudad Real	Sta Cruz de Mudela	23	1 (4.3%)	10 (43.5%)	na	11 (47.8%)
Cuenca	La Almarcha	31	0	0	0	0
Gerona	Cadaqués	10	0	1 (10%)	2 (20%)	2 (20%)
Gerona	Palau Saverdera	21	1 (4.8%)	4 (19.1%)	5 (23.8%)	8 (38.1%)
Granada	Baza	30	1 (3.3%)	7 (23.3%)	na	7 (23.3%)
Huelva	Villalba de Alcor	6	0	0	na	0

Jaén	Santisteban del Puerto	9	0	0	0	0
Jaén	Segura de la Sierra	1	0	0	0	0
Jaén	undetermined	3	0	1 (33.3%)	0	1 (33.3%)
Jaén	Vilches	2	0	1 (50%)	na	1 (50%)
Jaén	Montizón	2	1 (50%)	1 (50%)	na	1 (50%)
Jaén	Castellar	1	0	0	na	0
Jaén	Linares	2	0	1 (50%)	na	1 (50%)
Jaén	Torres	2	0	2 (100%)	na	2 (100%)
Jaén	Aldeaquemada	2	0	1 (50%)	na	1 (50%)
Jaén	Santisteban del Puerto	1	0	0	na	0
Jaén	Quesada	1	0	0	na	0
La Coruña	Boimorto	1	0	0	0	0
León	Villafranca del Bierzo	15	0	0	0	0
León	Palacios del Sil	20	0	0	0	0
Lugo	Cervantes	5	0	0	0	0
Madrid	Algete	21	0	0	0	0
Madrid	Valdetorres de Jarama	8	0	0	0	0
Madrid	Fuente el Saz de Jarama	14	5 (35.7%)	4 (28.6%)	2 (14.3%)	7 (50%)
Murcia	Lorca	19	0	2 (10.5%)	na	2 (10.5%)
Murcia	Moratalla	5	0	0	na	0
Navarra	Barbarin	33	0	0	0	0
Navarra	Ablitas	21	2 (9.5%)	3 (14.3%)	na	5 (23.8%)
Navarra	Fitero	10	1 (10%)	0	na	1 (10%)
Navarra	Ribaforada	2	1 (50%)	0	na	1 (50%)
Navarra	Lerín	1	0	0	na	0
Navarra	Corella	1	0	0	na	0
Navarra	Fontellas	5	1 (20%)	1 (20%)	na	2 (40%)
Navarra	Estella	42	0	0	0	0
Sevilla	undetermined	26	0	3 (11.5%)	na	3 (11.5%)
Soria	Castillejo de Robledo	34	0	4 (11.8%)	na	4 (11.8%)
Tarragona	Pontills	25	0	0	0	0
Tarragona	Vilanova de Padres	8	0	3 (37.5%)	4 (50%)	4 (50%)
Tarragona	Vimbodí	8	0	3 (37.5%)	1 (12.5%)	4 (50%)
Toledo	Méntrida	29	0	0	0	0
Toledo	Huecas	25	0	3 (12%)	na	3 (12%)
Toledo	Villacañas	30	7 (23.3%)	8 (26.7%)	na	15 (50%)
Toledo	Quintanar de la Orden	23	1 (4.3%)	1 (4.3%)	na	2 (8.7%)
Valladolid	Medina de Rioseco	25	0	0	0	0
Valladolid	Renedo de Esgueva	5	0	0	0	0
Valladolid	Mayorga	11	0	0	na	0
Valladolid	S. Pedro de Latarce	3	0	0	na	0
Valladolid	Villabrágima	1	0	0	na	0
Zaragoza	Azuara	18	0	0	0	0
Zaragoza	Moneva	8	0	0	0	0
Portugal						
Bragança	Sendim	2	0	2 (100%)	2 (100%)	2 (100%)
Bragança	Bragança	24	0	0	0	0
Faro	Castro Marim	3	0	1 (33.3%)	0	1 (33.3%)
Faro	Tavira	20	0	0	1 (5%)	1 (5%)

Guarda	Sabugal	10	0	0	0	0
Viana do C.	Valença	4	0	1 (25%)	1 (25%)	2 (50%)
Viana do C	Mourela	3	1 (33.3%)	0	0	1 (33.3%)
	Total	1328	41 (3.1%)	154 (11.6%)	67(7.7%)[4]	215 (16.2%)

[1] - chicks breed in the wild; [2] partridges collected from 2000-2001years; [3] partridges collected from 2004-2005years; [4] the total samples analysed with introns were 866 partridges; na - no analysed; micros - microsatellites; Viana do C. - Viana do Castelo.

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Table 2 Red-legged partridge farms in the Iberian Peninsula, sample size and summary of detected introgressed individuals employing 16 DNA markers (one mitochondrial and 15 nuclear).

		Partridges with A. chukar DNA			
Localities	N	mtDNA	micros.	introns	All markers
		(cyt-b)	(8 loci)	(7 loci)	
Albacete	241	9 (3.7 %)	37 (15.4 %)	1 (0.4 %)	38 (15.8 %)
Cuenca	8	0	3 (37.5 %)	0	3 (37.5 %)
Jaén	97	5 (5.2 %)	12 (12.4 %)	21 (21.6%)	25 (25.8%)
Navarra	37	4 (10.8 %)	22 (59.5 %)	18 (48.6 %)	29 (78.4 %)
Soria	96	0	8 (8.3 %)	18 (18.8 %)	25 (26 %)
Zaragoza	93	2 (2.2 %)	14 (15.1 %)	22 (23.7 %)	34 (36.6 %)
Total	572	20 (3.5 %)	96 (16.8 %)	80 (14 %)	154 (26.9 %)

Table 3 Wild *A. chukar* introgressed red-legged partridge localities in Spain and Portugal. Number of detected hybrids, hybrids identified per each type of marker (mtDNA, microsatellites and introns) and *A. chukar* alleles per locality.

				Hybrids identified per each type of marker				*A. chukar*
Regions	Localities	N	Hybrid[T]	mtDNA	micros.	introns	nuclear	Alleles[T]
				(cyt-b)	(8 loci)	(7 loci)	(15 loci)	
Spain								
Álava	Turiso,Villabezana	40	12 (30%)	2 (17%)	7 (58.3%)	7 (58.3%)	12 (100%)	20 (1.6%)
Álava	El Ciego	37	16 (43.2%)	5 (31.3%)	12 (75%)	10 (63%)	15 (93.8%)	43 (3.7%)
Albacete	Ossa de Montiel	41	14 (34.1%)	0	8 (57.1%)	7 (50%)	14 (100%)	19 (1.5%)
Albacete	Ossa de Montiel[(1)]	15	4 (26.7%)	0	2 (50%)	3 (75%)	4 (100%)	6 (1.3%)
Asturias	Cangas del Narcea	4	1 (25%)	0	1 (100%)	0	1 (100%)	1 (0.8%)
Badajoz	Solana de los Barros	12	7 (58.3%)	4 (57.1%)	4 (57.1%)	na	4 (57.1%)	11 (5.4%)
Badajoz	Llerena	2	1 (50%)	0	1 (100%)	na	1 (100%)	1 (2.9%)
Badajoz	Alange	9	4 (44.4%)	0	4 (100%)	na	4 (100%)	4 (2.6%)
Burgos	Villadiego	40	2 (5%)	0	2 (100%)	na	2 (100%)	2 (0.3%)
Burgos	Arauzo de la Torre	20	5 (25%)	0	5 (100%)	na	5 (100%)	5 (1.5%)
Cádiz	Medina Sidonia	13	1 (7.7%)	0	1 (100%)	na	1 (100%)	1 (0.5%)
Ciudad Real	Alcolea de Calatrava	34	8 (23.5%)	2 (25%)	6 (75%)	1 (13%)	6 (75%)	12 (1.1%)
Ciudad Real	Picón	30	23 (76.7%)	4 (17.4%)	18 (78.3%)	15 (65.2%)	22 (95.7%)	49 (5.3%)
Ciudad Real	Poblete	30	7 (23.3%)	0	1 (14.3%)	6 (86%)	7 (100)	8 (0.9%)
Ciudad Real	Alhambra	30	2 (6.7%)	0	2 (100%)	na	2 (100%)	2 (0.4%)
Ciudad Real	Terrinches	30	13 (43.3%)	1 (7.7%)	12 (92.3%)	na	12 (92.3%)	17 (3.3%)
Ciudad Real	Sta Cruz de Mudela	23	11 (47.8%)	1 (9.1%)	10 (90.9%)	na	10 (90.9%)	13 (3.3%)
Gerona	Cadaqués	10	2 (20%)	0	1 (50%)	2 (100%)	2 (100%)	4 (1.3%)
Gerona	Palau Saverdera	21	8 (38.1%)	1 (12.5%)	4 (50%)	5 (62.5%)	7 (87.5%)	15 (2.3%)
Granada	Baza	30	7 (23.3%)	1 (14.3%)	7 (100%)	na	7 (100%)	8 (1.6%)
Jaén	undetermined	3	1 (33.3%)	0	1 (100%)	0	1 (100%)	1 (1.1%)
Jaén	Vilches	2	1 (50%)	0	1 (100%)	na	1 (100%)	1 (2.9%)
Jaén	Montizón	2	1 (50%)	1 (100%)	1 (100%)	na	1 (100%)	3 (8.8%)
Jaén	Linares	2	1 (50%)	0	1 (100%)	na	1 (100%)	1 (2.9%)
Jaén	Torres	2	2 (100%)	0	2 (100%)	na	2 (100%)	4 (11.8%)
Jaén	Aldeaquemada	2	1 (50%)	0	1 (100%)	na	1 (100%)	2 (5.9%)
Madrid	Fuente el Saz de Jarama	14	7 (50%)	5 (71.4%)	4 (57.1%)	2 (28.6%)	4 (57.1%)	15 (3.5%)
Murcia	Lorca	19	2 (10.5%)	0	2 (100%)	na	2 (100%)	3 (0.9%)
Navarra	Ablitas	21	5 (23.8%)	2 (40%)	3 (60%)	na	3 (60%)	8 (2.2%)
Navarra	Fitero	10	1 (10%)	1 (100%)	0	na	0	1 (0.6%)
Navarra	Ribaforada	2	1 (50%)	1 (100%)	0	na	0	1 (2.9%)
Navarra	Fontellas	5	2 (40%)	1 (50%)	1 (50%)	na	1 (50%)	2 (2.4%)
Sevilla	undetermined	26	3 (11.5%)	0	3 (100%)	na	3 (100%)	3 (0.7%)
Soria	Castillejo de Robledo	34	4 (11.8%)	0	4 (100%)	na	4 (100%)	7 (1.2%)
Tarragona	Vilanova de Padres	8	4 (50%)	0	3 (75%)	4 (100%)	4 (100%)	11 (4.4%)
Tarragona	Vimbodí	8	4 (50%)	0	3 (75%)	1 (25%)	4 (100%)	8 (3.2%)
Toledo	Huecas	25	3 (12%)	0	3 (100%)	na	3 (100%)	3 (0.7%)
Toledo	Villacañas	30	15 (50%)	7 (46.7%)	8 (53.3%)	na	8 (53.3%)	22 (4.3%)

Toledo	Quintanar de la Orden	23	2 (8.7%)	1 (50%)	1 (50%)	na	1 (50%)	2 (0.5%)
Portugal								
Bragança	Sendim	2	2 (100%)	0	2 (100%)	2 (100%)	2 (100%)	4 (6.5%)
Faro	Castro Marim	3	1 (33.3%)	0	1 (100%)	0	1 (100%)	1 (1.1%)
Faro	Tavira	20	1 (5%)	0	0	1 (100%)	1(100%)	1 (0.2%)
Viana do C.	Valença	4	2 (50%)	0	1 (50%)	1 (50%)	2(100%)	3 (2.4%)
Viana do C	Mourela	3	1 (33.3%)	1 (100%)	0	0	0	1 (1.1%)
Total	-	-	215	41(19.1%)	154 (71.6%)	67(56.8%)[(2)]	188(87.4%)	-

[(1)] chicks breed in the wild; [(2)] 51 wild localities were analysed with intronic loci, there were 118 total detected hybrids with all markers; Ach - *A. chukar*; Hybrid^{T} - Total number of detected hybrids with all genetic markers (16); Alleles^{T} - Total number of *A. chukar* alleles; Viana do C. - Viana do Castelo.

Table 4 *A. chukar* introgressed red-legged partridge farms in Spain. Number of detected hybrids, hybrids identified per each type of marker (mtDNA, microsatellites and introns) and *A. chukar* alleles per farm.

			Hybrids identified per each type of marker				*A. chukar*
Localities	N	Hybrid^{T}	mtDNA	micros.	introns	nuclear	Alleles^{T}
			(cyt-b)	(8 loci)	(7 loci)	(15 loci)	
Albacete	241	38 (15.8%)	9 (23.7%)	37 (97.4%)	1 (2.6%)	38 (100%)	72 (1%)
Cuenca	8	3 (37.5%)	0	3 (100%)	0	3 (100%)	6 (2.4%)
Jaén	97	25 (25.8%)	5 (20%)	12 (48%)	21 (84%)	25 (100%)	109 (3.6%)
Navarra	37	29 (78.4%)	4 (13.8%)	22 (75.9%)	18 (62.1%)	26 (89.7%)	87 (7.6%)
Soria	96	25 (26%)	0	8 (32%)	18 (72%)	25 (100%)	29 (1%)
Zaragoza	93	34 (36.6%)	2 (5.9%)	14 (41.2%)	22 (64.7%)	32 (94.1%)	50 (1.7%)
Total	-	154	20 (13 %)	96 (62.3%)	80 (52 %)	149 (96.8%)	-

Ach - *A. chukar*; Hybrid^{T} - Total number of detected hybrids with all genetic markers (16); Alleles^{T} - Total number of *A. chukar* alleles.

CHAPTER 3

Phylogeography and genetic structure of the red-legged partridge (*Alectoris rufa*): more evidence for refugia within the Iberian glacial refugium

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Phylogeography and genetic structure of the red-legged partridge (*Alectoris rufa*): more evidence for refugia within the Iberian glacial refugium

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Abstract

The Pleistocene climatic oscillations promoted the diversification in avian species during the last glacial period. The red-legged partridge (*Alectoris rufa*, Family *Phasianidae*) has a large natural distribution extending from the Mediterranean to humid temperate zones. However, the genetic structure for this species is unknown. The present study investigates the phylogeography, genetic structure and demographic history of *A. rufa* across its distribution, employing both mitochondrial DNA control region sequences and nuclear microsatellite loci. Our results propose that this species was greatly affected by Pleistocene glaciations. The mismatch analyses suggest that the current populations resulted from post-glacial expansion and subsequent differentiation resulting in five diagnosable genetic clusters: Southwestern, Central-eastern, Northwestern, Balearic and French and Italian. Further, we found evidence of three glacial refugia within the currently recognized Iberian glacial refugium. The intraspecific structure revealed by both maternal and biparental phylogeographic analyses was not resolved in the phylogenetic analyses. Based on all considerations, we recommended that five management units be recognized.

Keywords: control region, Last Glacial Maximum, management unit, microsatellite, Pleistocene, refugia-within-refugia

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Introduction

Contemporary distributions and genetic structure of plant and animal populations reflect the complex interplay between historical and ongoing ecological and evolutionary processes (Avise 2000). Climate fluctuated greatly during the past 3 million years, and the distributions of most living organisms shifted markedly over that time span (Bennett 1997). During the Quaternary ice ages, substantial areas of northern Europe were covered by ice sheets, while permafrost existed in large areas of central Europe, both of which restricted the distribution of many temperate and warm-adapted species to the three southern European peninsulas of Iberia, Italy and the Balkans during the Late Pleistocene (Hewitt 2000). These southern glacial refugia subsequently acted as sources for the recolonization of Europe for different species.

The Iberian Peninsula was one of the most important unglaciated areas in Europe during the Pleistocene (Hewitt 2000; Gómez & Lunt 2007), possibly because of the latitudinal localization between arid and temperate areas and its geographical position between the Atlantic Ocean and Mediterranean Sea. Further, a complex orography and wide range of climates and microclimates existent might have promoted subrefugia within the Iberian refugium that housed multiple species during glacial maxima (Gómez & Lunt 2007). Various studies have shown the Iberian Peninsula to have been a

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refugium with 'refugia-within-refugia' revealed by phylogeographic analysis (e.g. mammals, Branco *et al.* 2002; Centeno-Cuadros *et al.* 2009; amphibians, Martínez-Solano *et al.* 2006; insects, Vila *et al.* 2005; reptiles, Paulo *et al. 2008*). However, there are few studies of bird species that have evaluated this possibility (Gómez & Lunt 2007; but see in North America Shafer *et al.* 2010), probably as consequence of their high vagility.

Complex refugial dynamics may have affected suites of species differently and at different times depending of their life history, so to truly understand their effects we must examine different taxa. The genus *Alectoris* is comprised of seven bird species that are widely distributed in the Palaearctic and are characterized by low dispersal, which would have favoured sustained geographical isolation during glacial cycles. Both Mediterranean rock (*A. graeca*) and chukar partridges (*A. chukar*) possess genetic population structure attributable to isolation during last Pleistocene cycle and subsequent population expansion during the Holocene (Randi *et al.* 2003, 2006). However, phylogeographic patterns in the red-legged partridge (*A. rufa*) have not yet been evaluated.

The red-legged partridge is endemic to the western Mediterranean where its natural distribution encompasses France, NW Italy and the Iberian Peninsula including the Mediterranean islands of Corsica, Elba and the Balearics. It occupies habitats with a wide variety of land uses, although it shows the highest densities in the centre-south of Spain, and is least abundant along the Mediterranean coast (Blanco-Aguiar *et al.* 2003). The red-legged partridge has suffered declines in abundance throughout its European range, especially during 1980s. The abandonment of traditional agricultural practices, with increasing levels of intensive agriculture and overhunting led to this diminution of red-legged partridge populations (Blanco-Aguiar *et al.* 2003). In areas where the species is actively hunted, there have been attempts to counter population decline through massive releases of captive-reared red-legged partridges, which often included hybrids with *A. chukar* (Blanco-Aguiar *et al.* 2008; Martinez-Fresno *et al.* 2008; Barbanera *et al.* 2010). Further, such releases for restocking have not included considerations of red-legged partridge subspecies and potential local adaptation; thus, farmers sell and buy partridges to and from anywhere within its broad distribution (Blanco-Aguiar *et al.* 2008; Barbanera *et al.* 2010).

There are three recognized *A. rufa* subspecies: *A. r. rufa* (Linnaeus, 1758) distributed throughout France, NW Italy, Elba and Corsica, *A. r. hispanica* (Seoane, 1894) in the N & W portions of the Iberian Peninsula, and *A. r. intercedens* (A. E. Brehm, 1857) in the E & S parts of the Iberian Peninsula and Balearic Islands (Cramp & Simmons 1980). A fourth subspecies, *A. r. laubmanni* from the Balearic Islands, was recognized by Jordans (1928). Subspecies in red-legged partridges are delimited by subtle morphological differences (Cramp & Simmons 1980) and are contentious.

A mitochondrial DNA study suggested that a population from northern Spain was genetically distinct from other populations (Martinez-Fresno *et al.* 2008), whereas more recent authors have found no such pattern (Barbanera *et al.* 2010). Thus, the relation of red-legged partridge subspecies to underlying genetic structure and to the potential effects of southern refugia remains unclear. For a more comprehensive survey, we sequenced part of the mitochondrial DNA control region (mtDNA CR) and genotyped 20 species-specific nuclear DNA microsatellites for red-legged partridges sampled throughout its range. We use these data to: (i) evaluate the intraspecific population structure in red-legged partridge species, (ii) test the hypothesis of historical isolation in subrefugia within the Iberian Peninsula refugium and (iii) infer the historical demographic processes that possibly influenced the present population structure.

Materials and methods

Sample collection and DNA extraction

Muscle tissue samples were collected from wild individuals sampled during the 2001–2004 hunting seasons in the Iberian Peninsula, Balearic Islands and France. Samples preserved at room temperature in 100% ethanol. Genomic DNA was extracted using a standard phenol–chloroform method (Sambrook *et al.* 1989). To diminish the possibility of including allochthonous DNA (*A. chukar*) in our analysis of red-legged partridge localities, we discarded samples from known locales of intensive restocking and from the surrounding areas. We also performed hybrid assignment analysis using a mitochondrial locus (Blanco-Aguiar *et al.* 2008). All locales with individuals diagnosed as possible interspecific hybrids with A. *chukar* were not used in subsequent analysis.

The sampling was more geographically intensive in the Iberian Peninsula and Balearic Islands because this area contains three of the four described subspecies, the limits of which are unclear. To our mitochondrial data, we added 23 mtDNA CR sequences of red-legged partridges from Balearic Islands, Italy and France (Table 1) deposited in GenBank by Barbanera *et al.* (2005): AJ586190-93, AJ586195, AJ586213, AJ586222, AJ586225-26; Barilani *et al.* (2007): DQ679475; Barbanera *et al.* (2009): AM850842-51. We also included sequences from Italian and French locales to augment of samples of *A. r. rufa* subspecies.

Table 1 Red-legged partridge sampling locations and sample size for each class of marker employed in this study

Map Ref.	Country	Localities	Mitochondrial study (N)	Nuclear study (N)	mtDNA sequences origin
	Spain				
1		Lugo	10	18	This study
2		León	14	20	This study
3		Valladolid	9	25	This study
4		Álava	5	33	This study
5		Madrid	5	29	This study
6		Zaragoza	6	24	This study
7		Tarragona	7	25	This study
8		Toledo	6	29	This study
9		Cuenca	7	31	This study
10		Ciudad Real	5	30	This study
11		Alicante	10	25	This study
12		Almería	6	29	This study
13		Cádiz	20	36	This study
14		Balearic Is.	13	16	This study
		Mallorca	5	–	Barbanera *et al.* (2005)
	Portugal				
15		Bragança	3	23	This study
	France				
16		Corsica	18	23	This study
		Corsica	5	–	Barbanera *et al.* (2009)
17		Porquerolles	3	–	This study
		Undetermined	1	–	Barilani *et al.* (2007)
	Italy				
18		Scarlino	3	–	Barbanera *et al.* (2005)
		Bieri	1	–	Barbanera *et al.* (2005)
		Elba	3	–	Barbanera *et al.* (2009)
		Scarlino	1	–	Barbanera *et al.* (2009)
		Undetermined	4	–	Barbanera *et al.* (2009)
	Total		170	416	

DNA amplification and sequencing

Mitochondrial DNA. The 319-base pair fragment of domain I of the mtDNA CR was amplified using primers Aru DLCR-F2 (5′-ACTCCCCTACCTAGTGTACC-3′) and Aru DLCR-R2 (5′-CCTGAAGCTAGTCATGGAGT-3′) designed from previously aligned complete nucleotide sequences of the mtDNA CR of *Alectoris* (Randi & Lucchini 1998). PCR conditions are as described by Ferrero *et al.* (2007), except for annealing temperature that was set to 59 °C. Amplified fragments were purified with Sephadex TM G-50 medium using CENTRISEP Spin Columns (Princeton Separations) and analysed in an ABI PRISM 3130xl sequencer using BigDye ® Terminator v 3.1 chemistry (Applied Biosystems).

Microsatellites. We employed 20 species-specific microsatellite loci. Sixteen loci were assayed using methods outlined in Ferrero *et al.* (2007) and the remaining four loci, Aru 1E45: F_5′-ACTGCATTGTGGACCTTCTG-3′, R_5′-TACAGCAGTGGAGGTGGTTG-3′; 1E78: F_5′-CCTTATGCCACGGAGTATGT-3′, R_5′-CTTTTGTGTCTACTAGCCCTG-3′; 1G47: F_5′-AAATGAACCTAAACCAGAGAAC-3′, R_5′-GTGTTACATTTTGATACTCACC-3′ and 1F138: F2_5′-CCTCCATTCACTCATTCACC-3′, R2_5′-GAGATCAGAAGGAAGATGGA-3′, were survey using similar methods but with annealing temperatures of PCR: 62, 62, 56 and 56 °C, respectively. Allele sizes were determined in an automatic sequencer according to Ferrero *et al.* (2007).

Phylogenetic analyses

Mitochondrial haplotypes were identified using MacClade 4.05 (Maddison & Maddison 2002), and newly diagnosed *A. rufa* haplotypes were deposited in GenBank (accession nos. HQ621760-770, 772–778, 780–801, 803–809). Phylogenetic relationships among haplotypes were inferred using neighbour-joining (NJ) and maximum parsimony (MP) methods implemented in PAUP* 4.0b10 (Swofford 2002). We also conducted maximum likelihood (ML) analyses using Garli v 0.951 (Zwickl 2006). For both the NJ and ML analyses, we selected the most appropriate model of evolution using MODELTEST 3.7 and

the Akaike information criterion (Posada & Buckley 2004). The resulting model was the Hasegawa–Kishino–Yano + Invariant sites + Gamma with the following parameter estimates: base frequencies: A = 0.3, C = 0.3, G = 0.1, T = 0.3; transition/transversion ratio of 6.4; proportion of invariant sites as 0.7016 and gamma distribution shape parameter as 0.6569. For NJ, we used a maximum likelihood distance based on this model. For the ML analysis, we did five independent runs using this model and chose the tree with the highest likelihood. For the MP analysis, we used 10 random additions, tree bisection and reconnection swapping algorithm and collapsed zero length branches. Homologous mtDNA CR sequences of chukar and rock partridges deposited in GenBank: AJ586200, 586202, 586204, 586207 (Barbanera *et al.* 2005); EF688287 (Z Huang & N Liu, unpublished); AJ222730, AJ222731 (Randi & Lucchini 1998); AJ555854 (Randi *et al.* 2003) were used for rooting the phylogenetic trees. For three methods of phylogenetic reconstruction, we derived support using 1000 nonparametric bootstraps (BP). We also inferred intraspecific genetic lineages among ingroup haplotypes using a 95% statistical parsimony network with the program TCS 1.21 (Clement *et al.* 2000). Network ambiguities (loops) were resolved using coalescence theory according to rules outlined in Pfenninger & Posada (2002).

Genetic diversity and population structure

Mitochondrial DNA. Nucleotide composition was inferred using MEGA 4 (Tamura *et al.* 2007). Estimates of mtDNA CR genetic diversity were calculated in DnaSP v5 (Librado & Rozas 2009). We did spatial analysis of molecular variance employing the program SAMOVA 1.0 to determine the number of groups of populations (K, SAMOVA-inferred clusters) within the sampled distribution. SAMOVA maximizes the total genetic variance attributable to differences among groups of populations (F_{CT}) (Dupanloup *et al.* 2002). We ran SAMOVA with K values ranging from 2 to 10 and tested the significance of fixation indices using 100 simulated annealing processes.

Microsatellites. Observed and unbiased expected heterozygosities (Nei 1978) and F_{IS} values for each sampling location were determined using the program FSTAT 2.9.3.2 (Goudet 1995). For each SAMOVA-inferred clusters, mean number of alleles per locus, gene diversity and allelic richness were calculated using FSTAT 2.9.3.2. Deviations from Hardy–Weinberg equilibrium (HWE), heterozygote deficits and heterozygote excess and linkage equilibrium per sampling location, SAMOVA-inferred clusters and overall, were tested in GENEPOP 4.0.10 on the Web with default settings (Rousset 2008). Sequential Bonferroni correction was used to test for significance in multiple comparisons (Rice 1989). The presence of null alleles was tested using MICRO-CHECKER 2.2.3. (Van Oosterhout *et al.* 2004).

We also used the Bayesian model-based clustering method deployed in STRUCTURE 2.3.1. (Pritchard *et al.* 2000) to infer K (number of genetic clusters, STRUCTURE clusters) and assign individuals to each diagnosed cluster based on multilocus genotype data. The model assumes Hardy–Weinberg and linkage equilibrium within clusters. We used the admixture model specifying sampling locations as priors (Hubisz *et al.* 2009) with correlated allele frequencies between populations (Falush *et al.* 2003). To determine the optimal value for K, we evaluated the modal value of ΔK as recommended by Evanno *et al.* (2005), with a length of the burn-in and Markov chain Monte Carlo of 10 000 each and 20 replicates per K, testing values of K from 1 to 10. Individuals were assigned to clusters for selected Ks, using 500 000 iterations following a burn-in period of 50 000 runs, if the estimated allele frequency for each individual in each cluster (Q) was ≥0.8.

Patterns and scale of mitochondrial and nuclear differentiation. We evaluated the patterns and scale of genetic differentiation F_{ST}, using two analyses in GENALEX 6 (Peakall & Smouse 2006): a Mantel nonparametric test and a spatial autocorrelation analysis. These analyses were performed with, without the islands and only within Iberian Peninsula. For Mantel test, we calculated matrix correlations between pairwise genetic and geographical distance matrices for sampling locations. Spatial autocorrelation analysis employs pairwise geographical and genetic distance matrices to calculate an autocorrelation coefficient (r) for each of a series of distance classes (Smouse & Peakall 1999). We calculated r for increasing distance class sizes. Tests for statistical significance were performed using two methods: random permutation and bootstrap estimates of r both set to 999 permutations. In addition, we also calculated the mitochondrial and nuclear differentiation F_{ST} among SAMOVA-inferred clusters, in Arlequin 3.1 (Excoffier *et al.* 2005) and GENALEX, respectively.

Bottleneck, demography and coalescence time

We tested for the signature of recent population bottlenecks in red-legged partridge localities and in SAMOVA-inferred clusters using the program BOTTLENECK 1.2.02 (Cornuet & Luikart 1996) assuming a two-phase model purported to be a better fit of microsatellite evolution (Di Rienzo *et al.* 1994), with 5% of new alleles arising via the infinite allele model and 95% from the stepwise mutation model. We used two-tailed Wilcoxon sign-rank tests to determine which sampling locations had significant heterozygote excess across loci. The M-ratio

was also used to test for reductions in population size (Garza & Williamson 2001). To determine the critical M-ratio value (M_C), we used CRITICAL_M.EXE software as recommended by Garza & Williamson (2001).

We analysed trends in historical demography of SAMOVA-inferred clusters as follows. We used mismatch distribution analysis of mtDNA data (Rogers & Harpending 1992) to evaluate different possible demographic histories of sampled red-legged partridge localities. A unimodal distribution implies that the population passed through a recent demographic expansion (Rogers & Harpending 1992). Observed distributions were compared to the expected distributions under a growth-decline model, using the program DNASP v5 (Librado & Rozas 2009). Significant difference from a model of sudden expansion was assessed using the sum of squared deviations (SSD) and raggedness index (rg) in Arlequin. As further tests for demographic expansion, we conducted Fu's F_s (Fu 1997) and Tajima's D (Tajima 1989) neutrality tests in Arlequin.

The coalescent times of red-legged partridge haplotypes were calculated according to the methods of Rooney *et al.* (2001) and Randi *et al.* (2003). For estimation of the number of nucleotide substitutions per site, we used the formula: $d = (Tv+TvR)/m$, where Tv is the number of transversions between red-legged partridges and chukar outgroups, R is the transition/transversion ratio in red-legged partridges and m is the sequence length. For the estimates, we assumed that the time since divergence between *A. rufa* and *A. chukar* species is 2 million years (Randi 1996) and that the generation time is 2 years (Randi *et al.* 2003). Coalescence time in generations is given by: $t = \tau/2u$ (Rogers & Harpending 1992). The population parameter τ (mutational timescale) and 95% quartiles were obtained from Arlequin.

For comparison to the approach outlined above, we also calculated the timing of divergence for key pairs of genetic clusters (between the Central-eastern and Northwestern clusters; Central-eastern and Southwestern clusters) using our mtDNA sequence data and the coalescence 'isolation with migration' approach in IMa2 (Hey & Nielsen 2004). Settings for the prior distributions were determined empirically via a series of initial runs with large initial parameter intervals. We used a geometric heating model with 100 chains with the first and second heating parameters assigned values of 0.99 and 0.75, respectively. We began with a burn-in period of 5 00 000 steps to ensure that the state of the chain was independent of its starting point. Following burn-in, the number of steps over which parameter values were recorded was 3 700 000 using *post hoc* evaluations of trend plots to determine whether we had adequate mixing. We used the two rates of mitochondrial control region divergence to convert the estimate of the time parameter, t, to divergence in years spanning the rates that have been reported in the literature: 5–15% per million years (see Wenink *et al.* 1994; Randi *et al.* 2003).

Results

Mitochondrial DNA sequence description, variability and phylogenetic relationships

We used 170 red-legged partridges from 18 sampling locations distributed across Spain, Portugal, France and Italy (Table 1). From the mtDNA CR sequence alignment of 319 nucleotides, we found a total of 57 haplotypes in 170 individuals of *A. rufa* (including GenBank sequences), defined by 36 polymorphic sites (11.3% of the entire sequence): 23 parsimony informative sites, 10 singleton variable sites and three insertions/deletions (see Table S1, Supporting information). For the total sampled area, haplotype diversity was high (Hd = 0.929), but average values for the number of nucleotide differences (Kt = 3.610) and nucleotide diversity (π_T = 0.011) were small. The French and Italian cluster (as defined by SAMOVA-inferred clusters, see below) showed the lowest values of mitochondrial diversity (which could be biased by the few sample sizes), while the Southwestern and Central-eastern clusters had the highest diversity values (Table 2, see below).

We found three single base insertion/deletion events (indels) for several of the red-legged partridge mtDNA CR sequences (Table S1, Supporting information): one deletion in position 192, one insertion in position 237 and one deletion in position 269. For SAMOVA-inferred clusters, the first deletion was observed in 60% of sequences from Southwestern cluster, 11% from Central-eastern, 2% from French and Italian. The insertion was observed in 2% of sequences from French and Italian SAMOVA-inferred cluster. The last deletion was detected in 10% of sequences from Southwestern and 2% from French and Italian cluster.

The ML, NJ and MP consensus trees exhibited the same topology, with three clusters apparent. The first cluster contained the outgroup haplotypes of *A. graeca* (bootstrap support for ML, NJ and MP, 90%, 92% and 95%, respectively); the second cluster had the five outgroup haplotypes of *A. chukar* (bootstrap: 95%, 98%, 97%), and the third cluster grouped all red-legged partridge haplotypes into *A. rufa* cluster (bootstrap: 79%, 79%, 97%). Within the *A. rufa* cluster, there were three haplogroups with low bootstrap values (range 51–65%; see Fig. S1, Supporting information).

In the statistical parsimony analysis of 57 *A. rufa* haplotypes, most network ambiguities (loops) were resolved but two loops remaining unresolved (Fig. 1). Haplotype 35 (Central-eastern cluster) had the highest

Table 2 Mitochondrial population diversity parameters, demographic statistics and pairwise F_{ST} values for the five SAMOVA-inferred clusters

Clusters	Northwestern	French and Italian	Southwestern	Balearic	Central-eastern
N	33	39	20	18	60
h	11	9	9	9	29
Hd	0.751	0.411	0.789	0.836	0.933
K	1.651	0.906	3.526	1.849	3.538
π	0.005	0.002	0.009	0.006	0.011
Tajima's D	**−1.8280**	**−2.0431**	0.3995	−1.3100	−0.9241
FS	**−4.9992**	**−5.1919**	−1.1954	**−4.0405**	**−20.1830**
SSD	0.0108	**0.2152**	0.0354	0.0047	0.0007
Raggedness	0.0742	0.1884	0.0977	0.0645	0.0118
τ	1.2480	n.e.	6.9003	1.6328	4.0468
τ quartile 95%	0.75–1.94	n.e.	0.41–11.24	0.82–2.61	2.04–5.71
Northwestern					
French and Italian	0.674*				
Southwestern	0.553*	0.517*			
Balearic	0.616*	0.600*	0.470*		
Central-eastern	0.236*	0.316*	0.258*	0.239*	

N, sample size; h, number of haplotypes; Hd, haplotype diversity; K, average number of differences; π, nucleotide diversity; SSD, sum of square deviation; ne., not estimated (see text); τ (tau) values and τ quartile 95% for the estimates of time since population expansion. Tajima's D, SSD and raggedness index values in bold show significant tests ($P < 0.05$), for Fu's FS test ($P < 0.02$).
*$P < 0.00001$.

relative root probability, $P = 0.10$. The network was globally consistent with our SAMOVA results although there was haplotype sharing among clusters, except for the Balearic cluster (Fig. 1).

Mitochondrial population structure

The SAMOVA results suggested five population groupings (SAMOVA-inferred clusters), as F_{CT} values approach a plateau for this value of *K* (Table S2, Supporting information): a Northwestern cluster: Lugo, León and Valladolid; a French and Italian cluster: France and Italy; a Southwestern cluster: Cádiz; a Balearic cluster: Balearic Islands; and a Central-eastern cluster: Álava, Madrid, Almería, Alicante, Tarragona, Toledo, Ciudad Real, Zaragoza, Cuenca and Bragança (Fig. 2). The AMOVA results showed that a significant component of genetic variance occurred among clusters (37.43%), among sampling locations within clusters (6.04%) and within sampling locations (56.52%) (Fixation indices: $F_{SC} = 0.097$, $F_{ST} = 0.435$, $F_{CT} = 0.374$; all $P < 0.00001$). Population diversity parameters for the five SAMOVA-inferred clusters are shown in Table 2.

Microsatellites Hardy–Weinberg and linkage equilibrium

For nuclear surveys, we employed 416 wild red-legged partridge samples from 16 sampling locations distributed across Spain, Portugal and France (Table 1). Characteristics of the microsatellite loci used in the analysis are shown in Table S3 (Supporting information). For all samples pooled, four loci, Aru 1E66, 1E97, 1B3 and 1F138, showed highly significant departures from HWE, and these were excluded in any subsequent analyses that assumed HWE.

Most of the sampling locations showed a positive F_{IS} value, except Corsica with a negative value, which reflected heterozygote deficit and heterozygote excess, respectively. The multilocus test indicated that overall heterozygote excess was not significant in Corsica ($P = 0.32$) and that none of the 20 loci had statistically significant heterozygote excess. Heterozygote deficiencies were significant ($P < 0.001$) for Madrid, Lugo, León, Bragança, Zaragoza, Alicante, Álava, Cuenca, Cádiz, Ciudad Real and Balearic Islands sampling locations. The multipopulation test showed statistical significant heterozygote deficit ($P < 0.00001$) for loci Aru 1E66, 1E97 and 1B3.

The results from MICROCHECKER suggested that locus Aru 1E66 might have null alleles in eight sampling locations, Aru 1B3 in six sampling locations, Aru 1E97 in three sampling locations, Aru 1F114 in three sampling locations, Aru 1E102 in two sampling locations, Aru 1F138 in one sampling location, Aru 1G47 in one sampling location and Aru 1E7 in one sampling location. No evidence of stuttering or small allele dominance was found in our data.

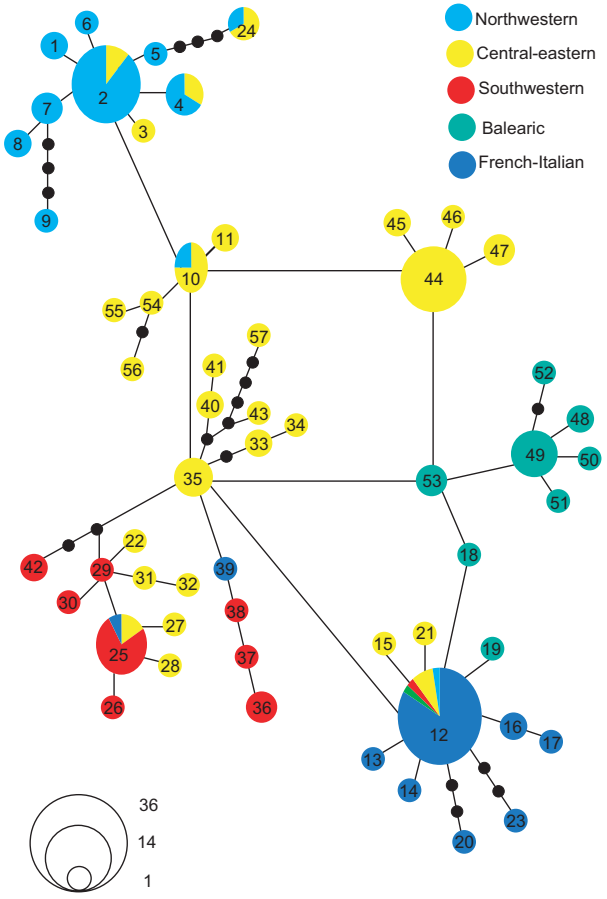

Fig. 1 Statistical parsimony network of 57 *Alectoris rufa* haplotypes (numbers). All haplotypes are separated by one mutation, and solid black circles represent intermediate haplotypes that are not present in our study. Circle sizes are proportional to haplotype frequencies. The five colours indicate SAMOVA-inferred clusters.

Nuclear genetic diversity and population structure

We surveyed 16 microsatellite loci (Aru 1E66, 1E97, 1B3 and 1F138, excluded) for 416 individuals from 16 sampling locations. From the STRUCTURE analysis, the assessment of modal value of ΔK was at $K = 2$ (high peak), the uppermost level of structure, which corresponded to the division of French cluster (Corsica assigned with 100% of assigned individuals) and no French cluster (all Spanish and Portuguese sampling locations assigned with 100% of assigned individuals, except Bragança 95.6% and Balearic Islands 50%). However, we also detected substructure within no Frech red-legged partridges with $K = 5$ (lower peak). This substructure separated the French samples into a cluster (III) with 100% of assigned individuals, and additionally split the non-French samples into four clusters (I, II, IV, V clusters, Fig. 3): all individuals from Cádiz were assigned to cluster I; samples from Madrid were assigned to cluster II with 7% of assigned individuals; León, Álava and Valladolid were assigned to cluster IV with 90%, 100%, 100%, respectively; and Cuenca and Balearic islands were assigned to cluster V with 65% and 100%. The STRUCTURE clusters for $K = 5$ were equivalent to the number and distribution of SAMOVA-inferred clusters based on mtDNA sequences (Figs 2 and 3). All subsequent discussions centre on these five clusters diagnosed using STRUCTURE (STRUCTURE clusters). There were individuals of some sampling locations unassigned to any cluster (58%, $Q < 0.8$), and 54% of these unassigned individuals belonged to Central-eastern SAMOVA inferred cluster (Fig. 3).

Nuclear genetic diversity values were calculated for each of the SAMOVA-inferred clusters (Table 3). The Corsica sampling location exhibited the smallest nuclear genetic diversity.

Patterns and scale of mitochondrial and nuclear differentiation

Mitochondrial. A pattern of isolation by distance was indicated by significant matrix correlations between genetic and geographical distances across all sampling locations (Mantel test, $r = 0.515$, $P < 0.001$, b (slope) = 0.0003), in the set of localities excluding all islands (Mantel test, $r = 0.513$, $P < 0.001$, $b = 0.0003$) and in the Iberian Peninsula only (Mantel test, $r = 0.464$, $P < 0.003$, $b = 0.0005$; Fig. S2A, Supporting information). The isolation-by-distance pattern was corroborated by spatial autocorrelation analyses. Within the Iberian Peninsula, spatial genetic structure was found with cumulative distances class sizes ranging from 100 to 800 km (Fig. 4a). Permutation tests identified significant structure from 100 km ($r = 0.337$, $P < 0.001$) up to 800 km ($r = 0.006$, $P < 0.001$). Bootstrap error bars suggested positive spatial genetic structure from 100 to 700 km. Pairwise F_{ST} values between clusters ranged from 0.236 to 0.674 (Table 2). All clusters were significantly differentiated one from each other ($P < 0.00001$). We also found that nucleotide diversity of sampling locations was negatively albeit weakly correlated with latitude ($r = 0.457$, $P = 0.049$).

Nuclear. A pattern of isolation by distance was indicated by a significant correlation of genetic and geographical distances in the total data set of 16 sampling locations (Mantel test, $r = 0.751$, $P < 0.002$, $b = 0.00009$). In the Iberian Peninsula (without islands), the correlation was not significant (Mantel test, $r = 0.186$, $P < 0.115$, $b = 0.00001$; Fig. S2B, Supporting information). However, an isolation-by-distance pattern was apparent in the Iberian Peninsula in the results of spatial autocor-

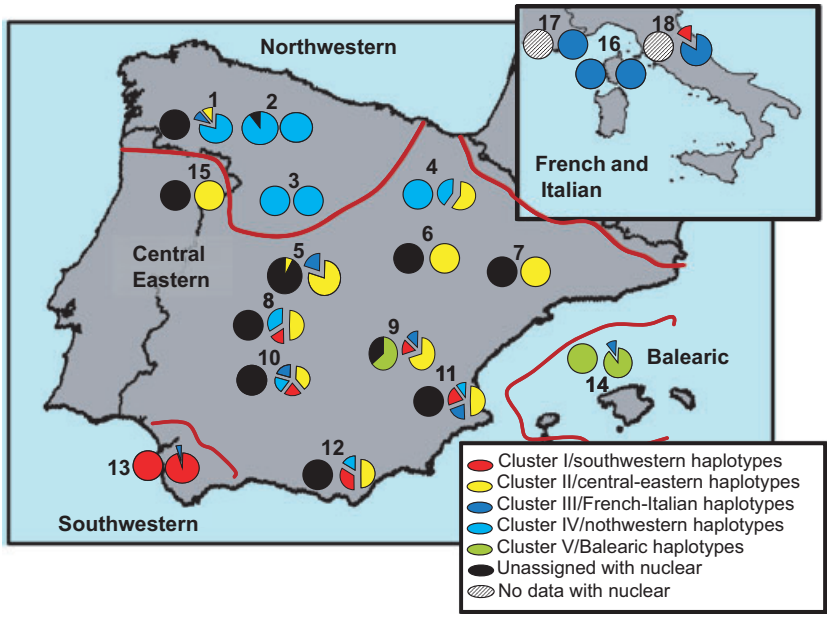

Fig. 2 Geographical haplotypes distribution of red-legged partridges based on parsimony network and SAMOVA-inferred clusters for mitochondrial results (right, exploded pie) and STRUCTURE clusters for nuclear (left, pie). Numbers show locality (Table 1). Red lines delimit SAMOVA-inferred clusters.

relation analysis. Spatial genetic structure was found with cumulative distances class sizes ranging from 100 to 800 km (Fig. 4b). Permutation tests identified significant structure from 100 km ($r = 0.061$, $P < 0.001$) up to 600 km ($r = 0.001$, $P < 0.001$). Bootstrap error bars also suggest spatial genetic structure from 100 to 600 km. Pairwise cluster F_{ST} values ranged from 0.008 to 0.164, and all clusters were significantly differentiated from each other ($P < 0.001$, Table 3).

Bottleneck, demography and coalescence time

For the bottleneck analyses, none of the Wilcoxon sign-rank tests was significant ($P > 0.05$) for either the red-legged partridge sampling locations individually or for the SAMOVA-inferred clusters. The values of M in red-legged partridge sampling locations and SAMOVA-inferred clusters always fell above 0.70. The Mc values calculated using CRITICAL_M.EXE software always were below the mean M calculated in our sampling locations. The range of M values was between 0.87 and 0.71. These results together imply that red-legged partridge sampling locations have not experienced recent demographic declines.

The mismatch distributions (Fig. 5) imply different demographic scenarios for different clusters. For the Northwestern, Balearic and Central-eastern clusters, mismatch distributions were consistent with a growth/decline model implying recent population expansion (SSD and rg tests P values >0.05; Table 2). We found

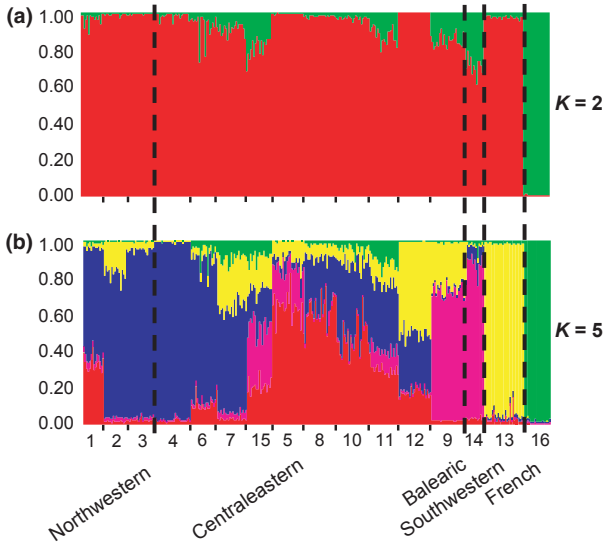

Fig. 3 Bar plots of STRUCTURE assignment tests. Each individual is represented by a vertical bar and the proportion of its genotype assigned to each of two (a) or five (b) clusters is indicated. Numbers below the plot represent the sampling locations, described in Table 1. Dashed lines separate SAMOVA-inferred clusters (see text).

negative D and F_s values, for Tajima and Fu tests of neutrality, respectively. F_s was significantly different from expectation for the Northwestern, Balearic and Central-eastern clusters, which implied population growth. We found no signal of population expansion in

Table 3 Nuclear genetic diversity and pairwise cluster F_{ST} values for five SAMOVA-inferred clusters

Clusters	Southwestern	Northwestern	Central-eastern	Balearic	French
N	36	63	278	16	23
A	7.7	9.6	14.0	6.7	4.8
A_C	6.2	6.8	7.1	6.5	4.2
P_A	5	15	60	3	1
G_D	0.68	0.73	0.71	0.72	0.54
Southwestern					
Northwestern	0.028*				
Central-eastern	0.029*	0.008*			
Balearic	0.066*	0.042*	0.034*		
French	0.164*	0.115*	0.105*	0.121*	

Number of genotyped partridges (*N*), mean number of alleles per locus (*A*), allelic richness per cluster based on a sample size of 14 complete genotypes (A_C), the total number of private alleles found in a cluster when it is compared to all the other clusters (P_A) and gene diversity (G_D). *$P < 0.001$

the French and Italian cluster (SSD *P* value = 0.002). However, this cluster showed significant deviations from neutral expectations (negative and significant Tajima's *D* and Fu's F_s). The neutrality tests for the Southwestern cluster were not significant, whereas the multimodal mismatch distribution did not deviate significantly from that expected under a model of population expansion (SSD and rg tests *P* values >0.05).The mean number of transversions between red-legged partridge and chukar haplotypes was 22.4. The overall transition/transversion bias, *R*, was 15.2 for red-legged partridge haplotypes (MEGA4). The average number of nucleotide substitutions per site, *d*, was 1.14. The rate of nucleotide substitution per site per lineage per year, λ, was 2.84×10^{-7}. We calculated the mutation rate per nucleotide site per generation, μ, as 5.68×10^{-7}, and the mutation rate per haplotype, *u*, as 1.8×10^{-4}. Using the estimates and 95% quartiles of τ (Table 2), the divergence time for the Southwestern cluster ($\tau = 6.9$) was 19 015 generations, which represents an approximate value of 38 029 years (95% CI: 2260–61 949 years). For the Central-eastern cluster, $\tau = 4.05$ with a divergence time of ≅ 22 321 years (95% CI: 11 243–31 470 years). For the Balearic cluster, $\tau = 1.63$, and the estimated divergence time was ≅ 8 984 years (95% CI: 4519–14 385) years. Finally, for the Northwestern cluster, $\tau = 1.25$, and thus divergence time was estimated to be approximately 6889 years (95% CI: 4133–10 692 years).

For our IMa2 analyses of the mtDNA, the marginal posterior probability distributions of the divergence time parameters, *t*, for both pairwise comparisons revealed well-defined relatively narrow peaks. When converted to a scale of years for the faster calibration rate of 15% per million years, the divergence times between the Central-eastern and Northwestern clusters and Central-eastern and Southwestern clusters were 27 273 years (95% HPD interval of 16 928–375 548 years) and 26 646 (95% HPD interval of 14 106–142 006 years), respectively. Using the slower calibration of 5% per million years, divergence times between the Central-eastern and Northwestern clusters and Central-eastern and Southwestern clusters were 109 091 years (95% HPD interval

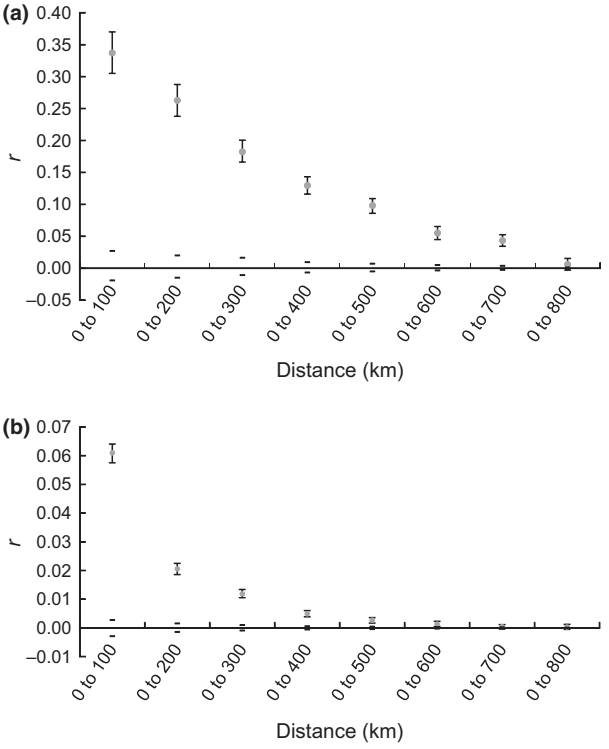

Fig. 4 Plots showing the influence of cumulative increasing distance classes from 100 to 800 km on genetic correlation (r, grey circle) of: (a) mitochondrial sequences and (b) microsatellite genotypes. Upper and lower bounds of the permuted 95% confidence interval around a random distribution of red-legged partridges, and the bootstrapped 95% confidence error bars about r are shown.

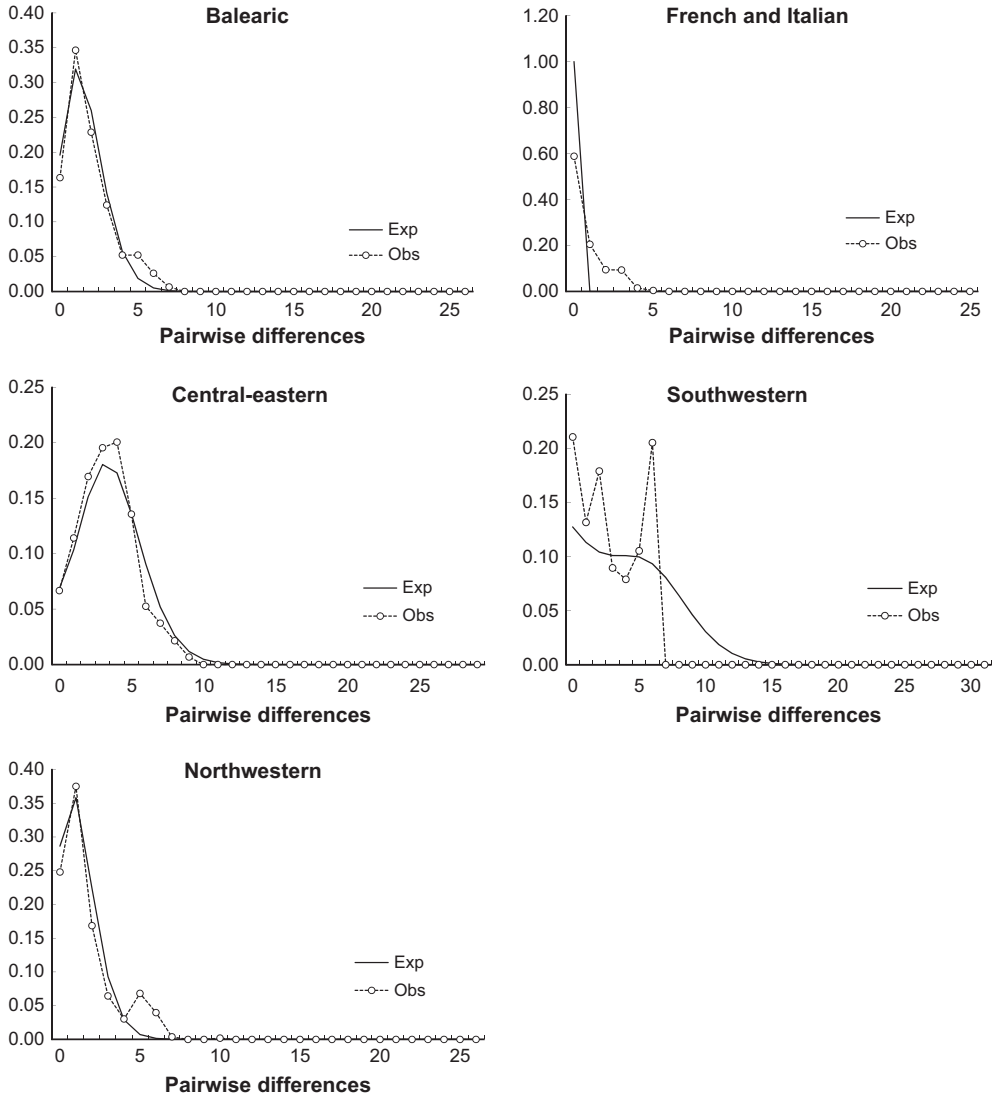

Fig. 5 Mismatch distributions for each of the SAMOVA-inferred clusters.

of 68 464–1 502 194 years) and 106 583 (95% HPD interval of 89 530–568 025 years), respectively. While these divergence times are older than those estimated above, they still place these splitting events firmly within the late Pleistocene.

Discussion

Population genetic structure

Genetic structure was revealed across the red-legged partridge distribution, comprised of five clusters: Northwestern, French and Italian, Southwestern, Balearic and Central-eastern (Fig. 2). These divisions were supported by mtDNA (SAMOVA-inferred clusters, the parsimony network, F_{ST} values) and microsatellite (STRUCTURE clusters, F_{ST} values) markers. The red-legged partridge genetic structure was compatible with isolation within refugia during Pleistocene glaciations (read below). Underlying this pattern of population differentiation was one of isolation-by-distance pattern for both maternally and biparentally inherited markers. The pattern and genetic differentiation were more striking for mtDNA CR than for nuclear markers. This is not unexpected as mtDNA markers more closely track demographic histories of populations because of smaller effective population size (Avise 2000). Concomitantly, lack of resolution in our microsatellite data set might be attributable to elevated mutation rate, the possibility of homoplasy in allele sizes (Hedrick 1999) and the potential consequences of restocking activities.

Releases of red-legged partridges for restocking activities could have diluted the contemporary genetic structure detected and might have influenced the genetic integrity of indigenous partridges because of artificial gene flow into wild populations. Perhaps, this is the case for Central-eastern cluster, which showed lower differentiation relative to other clusters (Fig. 3). This sampling area in central of Spain is where there are more intensive restocking activities (Blanco-Aguiar *et al.* 2008). Nevertheless, in this study, we discarded populations with hybrid individuals, which might contribute to detect a marked signal of genetic structure, so natural explanation may also support the lower differentiation in the Central-eastern cluster. To better circumscribe the geographical areas of the clusters, we recommend a museum phylogeographic study.

Multiple refugia within the Iberian Peninsula

Our results support the hypothesis of multiple refugia within the Iberian Peninsula refugium (Gómez & Lunt 2007), with three refugia of red-legged partridge species during Pleistocene glaciations. This study is one of the first bird evidences for 'refugia-within-refugia' pattern in the Iberian Peninsula. Based on the estimated coalescence times, high genetic diversity and structure revealed, we suggest three refugia in the Iberian Peninsula: the regions encompassed by the Central-eastern cluster, Southwestern cluster and Northwestern cluster.

The area represented by the Central-eastern cluster harbours high diversity for other taxa as well, and for these in this area, a possible Pleistocene refugium has been proposed (e.g. *Lacerta lepida*—Paulo *et al.* 2008 and *Arvicola sapidus* -Centeno-Cuadros *et al.* 2009). Interestingly, relatively low differentiation was observed from the Central-eastern to other clusters (pairwise F_{ST}, Bayesian clustering analysis; Fig. 2). This might be a consequence of both their central location within Iberia and wide distribution, which might have resulted in higher levels of gene flow upon secondary contact from all other clusters. Furthermore, this cluster has shown low assignment values for STRUCTURE analysis, which might be expected in an isolation-by-distance scenario (Pritchard *et al.* 2000).

Indeed, the area around Cádiz (Southwestern cluster) has unique ecological features and is an important transition between tropical and temperate climate zones (Reed *et al.* 2001). Moreover, Cádiz probably served as a refugium for many birds species during the Pleistocene as evidenced by abundant avian fossils, among them *Alectoris rufa* (Tyrberg 2008). Several other phylogeographic studies (*Erinaceus europeus*—Hewitt 2000; *Oryctolagus cuniculus*—Branco *et al.* 2002; *Capreolus capreolus*—Lorenzini & Lovari 2006) present evidence to support this contention. Similarly, southern subrefugium has also been described in Italy for the Calabrian region (Grill *et al.* 2009).

The northwestern portion of Iberia has been described as a subrefugium for other species (*Arvicola sapidus*—Centeno-Cuadros *et al.* 2009; *Lacerta lepida*—Paulo *et al.* 2008; *Erebia triaria*—Vila *et al.* 2005) in the Iberian Peninsula.

Divergence times and postglacial expansion

The mtDNA CR presents different evolution rates with low and hypervariable regions inside the same domain (domain I) (Randi & Lucchini 1998), and the few hypervariable sites tend to saturate quickly, which makes difficult to calibrate the substitution rates and it might be underestimated in recent species (Lucchini & Randi 1998). We employed different approaches and two rates of mtDNA CR divergence to estimate the divergence time for red-legged partridge clusters. Although we recognize the errors associated with such temporal estimates and issues with deciding which mutation rate to employ, the results imply that the diversification of red-legged partridge populations could have occurred during the last Pleistocene glaciation (Würm glaciation ≈100 000–10 000 years BP) with subsequent expansion (as suggested the correlation between nucleotide diversity and latitude, and demographic results) to its current range during the current interglacial epoch. These data are concordant with the genetic diversification in birds during Pleistocene (Avise & Walker 1998).

Analysis of mtDNA parsimony network implies that the Central-eastern cluster could have been an ancestral area for the red-legged partridge species. Coalescence time for the Central-eastern cluster indicates that this cluster could have originated during the late Pleistocene. Subsequently, partridges from this Central-eastern cluster might have expanded to their current distribution as a result of an interplay of climatic, geographical and genetic factors, ultimately giving rise to the differentiated red-legged partridge clusters evident today. Isolation with migration models suggests that both Southwestern and Northwestern clusters split off simultaneously from Central-eastern populations, and divergence time of the splits depends on the rate of mtDNA CR divergence rate used; however, the IMa estimates propose splits after the Last Interglacial Maximum (≈110 000 years) or the Last Glacial Maximum (≈30 000), which suggest the influence of climatic shifts on this differentiation. We can speculate from this that both clusters diverged as a consequence of the cold climatic conditions during the Late Pleistocene and that geographical barriers (e.g. the Guadalquivir River and Bética Mountains in the south, and the Central and

Iberian Systems in the north) may have restricted gene flow between these red-legged partridge populations and the rest of the Iberian Peninsula.

Probably isolation-by-distance pattern accentuated the differentiation among localities, but in some cases could create secondary contact zones where could admixing bordering populations. It could exist between Central-eastern and Southwestern clusters, where fluctuations in population size within refugia during Pleistocene glaciations can be detected by the multimodal mismatch distribution in Southwestern cluster. Furthermore, its parsimony network suggested two colonization events in this cluster. In the Northwestern cluster, the genetic composition of Álava sampling location is discordant between mitochondrial and microsatellite loci (Fig. 2), which could be produced as a result of contact zone (Brito 2007) between Central-eastern and Northwestern clusters. Similar mitochondrial pattern was observed in the same region between the species *Lepus granatensis* and *Lepus timidus* (Melo-Ferreira *et al.* 2005), which suggests this area as a contact zone between differentiated lineages for diverse species.

The Balearic cluster provides an interesting challenge. Natural dispersal of red-legged partridges would not seem possible if we consider that the shortest distance between the Iberian mainland and the nearest island (Eivissa) is 87 kms. We hypothesize that colonization might be because of human transport during Holocene, although the date of human settlement is uncertain (Alcover 2008). The affinities of the Balearic cluster are not clear from the parsimony network as two loops could not be resolved (Fig. 1). Based on morphological features, some authors have suggested that partridges from the Balearic Islands are intermediate between *A. r. rufa* and *A. r. intercedens* (Cramp & Simmons 1980) possibly implying multiple colonization events. The high genetic diversity in the Balearic cluster is consistent with this hypothesis, but a bimodal mismatch distribution was not observed in this cluster (Fig. 5). France might be a possible source for the original Balearic founders based on relationships inferred from the parsimony network; however, genetic and population diversity is lower in France than in the Balearic cluster. One could posit a bottleneck in France for this observed disparity, although our analyses did not detect any recent bottleneck event in France, making this hypothesis implausible. An alternate source for founders for this island population is the Central-eastern cluster (Fig. 3), which showed high diversity and greater genetic similarity to the Balearic cluster than did the French-Italian/French cluster (F_{ST} values and Bayesian clustering analysis).

Although we have few samples for the French and Italian cluster, the results imply a recent northward range expansion from a more southerly Iberian origin, a pattern similar to that observed for many other southern European species (e.g. *Oryctolagus cuniculus*—Branco *et al.* 2002; *Arvicola sapidus*—Centeno-Cuadros *et al.* 2009). While this is not consistent with the mismatch analysis, the results of the Fu's F_s (Ramos-Onsins & Rozas 2002) imply recent population growth in this region as did reduced haplotypic and nucleotide diversity estimates.

The divergence estimates suggested that the red-legged partridge species is a young species (diversification during Würm glaciation ≈100 000–10 000 years BP). The mtDNA diversity (high haplotype diversity, small nucleotide differences and nucleotide diversity) and tree topology supported this recent diversification hypothesis. The genetic structure of other recent congeneric species contrast to that of the red-legged partridge. For example, the 'sister species' *A. graeca* (Randi 1996) has more striking genetic population structure than *A. rufa*, although populations are characterized by lower genetic diversity and most populations show genetic signatures of demographic declines (Randi *et al.* 2003). The differences between *A. rufa* and *A. graeca* may be largely attributable to differences in habitat preferences. *Alectoris graeca* is an alpine species which populations are probably somewhat isolated and undergo large interannual fluctuations in population size and experience unpredictable weather conditions (Bernard-Laurent & Leonard 2000). In contrast, *A. rufa* inhabits a more contiguous landscape, which could result in greater connectivity among populations. Another congener, *Alectoris chukar*, is abundant and contiguously distributed in the mesic Mediterranean regions of Israel, and its genetic structure has also likely been determined by a combination of complex historical and demographic processes (Randi *et al.* 2006). We suggest a similar scenario for *A. rufa*, where the impact of several range expansions could be reduced by large population sizes and broad habitat tolerances in this species. Most of the clusters showed signals of population expansion (also overall populations, data not shown) and there was no signal of the decline (e.g. during 1980 decade). This could be because of the high effective population size, high reproductive rate and widely range of this species.

Taxonomic and conservation implications

The existence of the four *A. rufa* subspecies described was not apparent in our phylogenetic trees. The management unit (MU) designation is appropriate here as we did not find any consistent pattern of reciprocal monophyly for mtDNA alleles but did find significant difference in allele frequencies for both mitochondrial

and nuclear loci (Moritz 1994). The differentiation that we found among clusters (Fig. 2) was in broad agreement with the subspecies ranges described previously. The Northwestern MU covers the distribution of *A. r. hispanica* subspecies. The French and Italian MU (Corsica included) corresponds with *A. r. rufa* subspecies. The range of *A. r. intercedens* circumscribed three different MUs: Southwestern, Central-eastern and Balearic (*A. r. laubmanni*).

To aid in conservation of the present-day complement of genetic diversity of *A. rufa* species, we propose five MUs (Fig. 2) within a larger *A. rufa* evolutionary significant unit. Because restocking is commonly practiced for this species, we further recommend that individuals for restocking for each MU come from the appropriate source populations to avoid possible mixture of differentiated genotypes and potential loss of genetic diversity.

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This work is part of M.E.F.'s doctoral thesis. Her interests concern the application of molecular markers to conservation biology, evolutionary and ecological processes, currently with a special focus on partridge species. J.A.B.-A. is a post-doctoral fellow at CIBIO and has general interests in evolutionary and conservation biology applying molecular tools. S.C.L.'s research programme focuses on understanding and contrasting the mechanisms of diversification of tropical and temperate vertebrates. I.S.-B. is currently developing research works and analysis for the diagnosis of poisoning in wildlife species, in the IREC toxicology laboratory, using chromatographic and atomic spectroscopy techniques. P.J.G.N. is currently working on the development and application of molecular markers of interest in wildlife management. R.V. is interested in ecology and management of small game species. J.A.D. is a geneticist interested in conservation genetics and molecular ecology of animals, with a particular interest in managed populations.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 *A. rufa*, *A. graeca* and *A. chukar* haplotypes used in the present study.

Table S2 Fixation indices of the groups of populations inferred by SAMOVA for mitochondrial sampling locations. *P < 0.00001.

Table S3 Characteristics of microsatellite loci for SAMOVA-inferred clusters in red-legged partridge species.

Fig. S1 ML tree topology (log likelihood = −1135.6116) including MP, NJ and ML bootstrap values for 1000 replicates. Bootstrap values are above the node. Only bootstrap values >50% are shown.

Fig. S2 Mantel tests showing the relationship between pairwise genetic distances (F_{ST}) and geographical distances(in km). Calculated from mtDNA CR (A) and microsatellites (B).

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Table S1 *A. rufa*, *A. graeca* and *A. chukar* haplotypes used in the present study.

A. rufa haplotype	153	163	166	188	192	196	197	201	202	203	204	206	207	209	211	213	217	218	219	220	221	223	224	225	227	228	229	237	238	239	240	241	242	245	246	247	248	251	255	260	268	269	280	283	291	292	293
Hap 01	G	A	A	C	C	T	C	T	C	A	A	C	T	C	A	A	A	G	C	C	A	G	A	T	C	C	T	-	T	C	T	T	C	C	T	T	-	C	C	T	T	C	T	A	C	T	T
Hap 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 03	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 04	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	.	.
Hap 07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	-	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 09	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	G	C	.	.	.	-	.	.	C	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 10	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	C	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 13	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	.	-	.	.	.	.	.	.	G	.	.	.
Hap 14	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	C	C	T	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 15	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	C	.	.	C	T	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 16	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 17	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	-	.	.	.	C	T	.	C	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 18	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	C	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 20	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 21	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	-	.	T	.	.	.	.	.	.	.	.	.
Hap 22	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	T	.	.	.	-	.	.	.	C	.	.	.	.	.	.	.
Hap 23	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	C	T	.	.	.	-	.	.	.	C	-	.	.	.	.	.	.
Hap 24	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	C	-	.	.	.	.	.	.	.	.	.	.	.
Hap 25	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	.	.	C	.	.	.	.	.	.	.
Hap 26	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	A	.	C	.	.	.	.	-	.	.	.	C	.	.	.	.	.	.	.
Hap 27	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	.	.	C	.	.	.	.	C	.	.
Hap 28	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	T	.	.	-	.	.	.	C	.	.	.	.	.	.	.
Hap 29	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	.	.	C	.	.	.	.	.	.	.
Hap 30	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	-	.	.	.	C	.	.	.	.	-	.	.	.	C	.	.	.	.	.	.	.
Hap 31	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	T	.	C	.	.	.	.	.	.	.
Hap 32	.	.	.	.	-	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	T	.	.	.	.	.	.	.	.	.
Hap 33	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	A	.	.	.	.	.	C	.	.	.
Hap 34	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	A	.	.	.	.	.	C	.	.	.
Hap 35	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
Hap 36	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	C	.	.	.	-	C	T	.	C	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 37	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	C	.	.	.	-	.	T	.	C	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 38	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	C	.	.	.	-	.	.	.	C	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.
Hap 39	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	-	.	.	.	C	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.

Hap 40	. . . . C . . . . - . . . C . . . . - . . . . . C . . . .
Hap 41	. . . . C . . . . - C . . C . . . . - . . . . . C . . . .
Hap 42	. . . . C . . . . - . . . C . . . . - . . . C - . . . . .
Hap 43	. . . . C . . . . - . . . C . . . . - . . . .
Hap 44	. . . . C . . . . - . . . . . . C . - . . . .
Hap 45	. . . . . C . . . . . . C . . . . - . . . . . . C . - . . . .
Hap 46	. . . . C . . . . - . . . . . . C . - . . . .
Hap 47	. . . . C . . . . - . . . . . . C . - . . . C . . . . . .
Hap 48	. . . . C . . . . . . T . . . . - C . . C . . C . - . . . .
Hap 49	. . . . C . . . . . . T . . . . - . . . C . . C . - . . . .
Hap 50	. . . . C . . . . . . T . . . . - . . . C . . C . - . . . . . C . . . .
Hap 51	. . . . . C . . . . . . C . . . . . . T . . . . - . . . C . . C . - . . . .
Hap 52	. . . . C . . . . . . T . . . . - . . G . . . C . - . . . .
Hap 53	. . . . C . . . . - . . . C . . C . - . . . .
Hap 54	. . . . C . . . . - . . . . . T . . - . . . .
Hap 55	. . . . C . . . . - . . . . . T . . - . . . . C .
Hap 56	. . . . C . . . . C . . . - . . . . . T . . - . . . .
Hap 57	. . . . T . . . . T . - . . . C . T . . - . . . .
A. graeca	
AJ555854	T . . . - . . C A . . T . . G . . C A . . A . C T A C T G . . C T T . C - T . G . . . .
AJ222730	T . . . - . . C A . . T . . G . . C A . . A . C T A C T G . . C T T . . - . T G . . . .
AJ222731	T . . . - . T C A . G T . . G . . C A . G A . C T A C T A . . C T . C . - . . A . A . . . . .
A. chukar	
EF688287	C . . . - . . . A T . . . . G T . C A . . . G C T . C C A . . . T T C . - . . G . . C . T . C
AJ586202	C . . . - . . . A T . . . . G T . C A . . . G C T . C C A . . . T T C . T . . G . . C . T . C
AJ586200	T . . . - . . . A T . . . . G T . C A . . . G C T . C C A . . . T T C . - . . G . . C . . . C
AJ586204	T G . . - . . . A . . . . . G T G C G . . . G C T . C C A . . . . T C . - . T G . . . .
AJ586207	T G . . - . . . A . . . . . G T G C A . . . G C T . C C A . . . . T C . - . T G . . . .

Table 1S (Continued)

A. rufa haplotype	299	301	305	307	308	316	334	373	385	396	Genbank number	N		F	C	(L)
Hap 01	T	T	G	G	T	T	T	G	T	C	HQ621760	2		(0.012)	N	(a2)
Hap 02	.	.	.	.	A	.	.	.	.	.	HQ621761	1	8	(0.106)	N	(a2, b9,c5), C (h1,k1)
Hap 03	.	.	.	.	A	.	.	.	.	.	HQ621762	1		(0.006)	C	(h1)
Hap 04	.	.	.	.	A	.	.	.	.	.	HQ621763	6		(0.035)	N	(b2,c2), C (d2)
Hap 05	.	.	.	.	A	.	.	.	.	.	HQ621764	1		(0.006)	N	(c1)
Hap 06	.	.	.	.	A	.	.	.	.	.	HQ621765	1		(0.006)	N	(b1)
Hap 07	.	.	.	.	A	.	.	.	.	.	HQ621766	3		(0.018)	N	(a1,b2)
Hap 08	.	.	.	.	A	.	.	.	.	.	HQ621767	2		(0.012)	N	(a2)
Hap 09	.	.	.	.	A	.	.	.	.	.	HQ621768	1		(0.006)	N	(c1)
Hap 10	.	.	.	.	A	.	.	.	.	.	HQ621769	4		(0.024)	C	(e1,f2), N(a1)
Hap 11	.	.	.	.	A	.	.	.	.	.	HQ621770	2		(0.012)	C	(k2)
Hap 12	.	.	.	.	A	.	.	.	.	.	AJ586225	3	6	(0.212)	F	(q21,r3,s6),C(k2,i1),B(o1),S(n1),N(a1)
Hap 13	.	.	.	.	A	.	.	.	.	.	AM850850	1		(0.006)	F	(s1)
Hap 14	.	.	.	.	A	.	.	.	.	.	HQ621772	1		(0.006)	F	(q1)
Hap 15	.	.	.	.	A	.	.	.	.	.	HQ621773	1		(0.006)	C	(e1)
Hap 16	.	.	.	.	A	.	.	.	.	.	AJ586226	2		(0.012)	F	(s2)
Hap 17	.	.	.	.	A	.	.	.	.	.	AM850845	1		(0.006)	F	(q1)
Hap 18	.	.	.	.	A	.	.	.	.	.	HQ621774	1		(0.006)	B	(o1)
Hap 19	C	.	.	.	A	.	.	.	.	.	HQ621775	1		(0.006)	B	(o1)
Hap 20	.	C	A	.	A	.	.	.	C	.	AM850843	1		(0.006)	F	(s1)
Hap 21	.	.	.	.	A	.	.	.	.	.	HQ621776	1		(0.006)	C	(j1)
Hap 22	.	.	.	.	A	.	.	.	.	.	HQ621777	1		(0.006)	C	(i1)
Hap 23	.	.	.	.	A	.	.	.	.	.	DQ679475	1		(0.006)	F	(r1)
Hap 24	.	.	A	.	A	.	.	.	.	.	HQ621778	3		(0.018)	C	(j1,m1), N (a1)
Hap 25	.	.	A	.	A	.	.	.	.	.	AM850842	1	2	(0.071)	S	(n9), C (k1,h1), F (s1)
Hap 26	.	.	A	.	A	.	.	.	.	.	HQ621780	1		(0.006)	S	(n1)
Hap 27	.	.	A	.	A	.	.	.	.	.	HQ621781	1		(0.006)	C	(m1)
Hap 28	.	.	A	.	A	.	.	.	.	.	HQ621782	1		(0.006)	C	(k1)
Hap 29	.	.	.	.	A	.	.	.	.	.	HQ621783	1		(0.006)	S	(n1)
Hap 30	.	.	.	.	A	.	.	.	.	.	HQ621784	1		(0.006)	S	(n1)
Hap 31	.	.	.	.	A	.	.	.	.	.	HQ621785	1		(0.006)	C	(j1)
Hap 32	.	.	.	.	A	.	.	.	.	.	HQ621786	1		(0.006)	C	(m1)
Hap 33	.	.	.	.	A	.	.	.	.	.	HQ621787	2		(0.012)	C	(i2)
Hap 34	.	.	.	.	A	.	.	.	.	.	HQ621788	1		(0.006)	C	(i1)
Hap 35	.	.	.	.	A	.	.	.	.	.	HQ621789	5		(0.029)	C	(f1,j2,m1,p1)
Hap 36	.	.	.	.	A	.	.	.	.	.	HQ621790	3		(0.018)	S	(n3)
Hap 37	.	.	.	.	A	.	.	.	.	.	HQ621791	1		(0.006)	S	(n1)
Hap 38	.	.	.	.	A	.	.	.	.	.	HQ621792	1		(0.006)	S	(n1)

Hap 39	.	.	.	.	A	.	.	.	.	.	AM850844	1		(0.006)	F	(s1)
Hap 40	.	.	.	.	G	.	.	.	.	.	HQ621793	2		(0.012)	C	(d2)
Hap 41	.	.	.	.	G	.	.	.	.	.	HQ621794	1		(0.006)	C	(h1)
Hap 42	.	.	.	.	G	.	.	.	.	.	HQ621795	2		(0.012)	S	(n2)
Hap 43	.	.	.	.	G	.	.	.	.	.	HQ621796	1		(0.006)	C	(m1)
Hap 44	.	.	.	.	A	.	.	.	.	.	HQ621797	1	4	(0.082)	C	(e3,f2,d1,h1,i1,k2,g4)
Hap 45	.	.	.	.	A	.	.	.	.	.	HQ621798	2		(0.012)	C	(g2)
Hap 46	.	.	A	.	A	.	.	.	.	.	HQ621799	1		(0.006)	C	(i1)
Hap 47	.	.	.	.	A	.	.	.	.	.	HQ621800	3		(0.018)	C	(m1,k1,p1)
Hap 48	.	.	.	.	A	.	.	.	.	.	HQ621801	2		(0.012)	B	(o2)
Hap 49	.	.	.	.	A	.	.	.	.	.	AJ586195	7		(0.041)	B	(o7)
Hap 50	.	.	.	.	A	.	.	.	.	.	HQ621803	1		(0.006)	B	(o1)
Hap 51	.	.	.	.	A	.	.	.	.	.	AJ586192	1		(0.006)	B	(o1)
Hap 52	.	.	.	.	A	.	.	.	.	.	HQ621804	1		(0.006)	B	(o1)
Hap 53	.	.	.	.	A	.	.	.	.	.	HQ621805	3		(0.018)	B	(o3)
Hap 54	.	.	.	.	A	.	.	.	.	.	HQ621806	1		(0.006)	C	(f1)
Hap 55	.	.	.	.	A	.	.	.	.	.	HQ621807	1		(0.006)	C	(g1)
Hap 56	.	.	.	.	.	.	.	.	.	.	HQ621808	1		(0.006)	C	(p1)
Hap 57	.	.	.	.	G	C	.	.	.	.	HQ621809	1		(0.006)	C	(h1)
A. graeca																
AJ555854	.	A	A	.	.	.	C	.	.	.						
AJ222730	.	A	A	.	.	.	C	.	.	.						
AJ222731	.	C	.	C	.	.	.	.	C	T						
A. chukar																
EF688287	.	.	.	A	C	C	.	C	.	.						
AJ586202	.	.	.	A	C	C	.	C	.	.						
AJ586200	.	.	.	A	C	C	.	C	.	.						
AJ586204	.	.	.	A	C	C	.	C	.	.						
AJ586207	.	.	.	A	C	C	.	C	.	.						

Clusters diagnosed using SAMOVA (C) are indicated as follows: N, Northwestern; C, Central-eastern; F, French and Italian; S, Southwestern; B, Balearic. Sampling locations (L: a, Lugo; b, León; c, Valladolid; d, Álava; e, Madrid; f, Zaragoza; g, Tarragona; h, Toledo; i, Cuenca; j, Ciudad Real; k, Alicante; m, Almería; n, Cádiz; o, Balearic Is.; p, Bragança; q, Corsica; r, France; s, Italy). Numbers (N) and frequency (F) in the total population of the 57 mtDNA CR haplotypes found in red-legged partridge samples. Haplotype positions are aligned with nucleotide no. 1 of the complete mtDNA CR sequence of Alectoris (Randi & Lucchini 1998). New haplotype sequences are available from GenBank (Accession nos. HQ621760-770, 772-778, 780-801, 803-809).

Table S2. Fixation indices of the groups of populations inferred by SAMOVA for mitochondrial sampling locations. *P<0.00001.

Number of groups	Group composition	F_{SC}	F_{ST}	F_{CT}
2	1. León, Lugo, Valladolid. 2. Other populations.	0.320*	0.501*	0.266*
3	1. León, Lugo,Valladolid. 2. French and Italian locations. 3. Other populations.	0.244*	0.459*	0.284*
4	1. León, Lugo, Valladolid. 2. French and Italian locations. 3. Cádiz. 4. Other populations.	0.169*	0.447*	0.334*
5	1. León, Lugo, Valladolid. 2. French and Italian locations. 3. Cádiz. 4. Balearic Islands. 5. Other populations.	0.097*	0.435*	0.374*

Table S3.Characteristics of microsatellite loci for SAMOVA-inferred clusters in red-legged partridge species.

Locus	Southwestern N = 36					Northwestern N = 63					Central-eastern N = 278					Balearic N = 16					French N = 23				
	A_C	A	H_E	H_O	HWE	A_C	A	H_E	H_O	HWE	A_C	A	H_E	H_O	HWE	A_C	A	H_E	H_O	HWE	A_C	A	H_E	H_O	HWE
Aru 1A1	2.8	3	0.55	0.47	NS	4.5	5	0.72	0.70	NS	4.1	6	0.68	0.62	*	4.9	5	0.73	0.53	*	5.4	6	0.69	0.69	NS
Aru 1B3	10.2	13	0.89	0.50	**	11.0	17	0.89	0.49	**	13.2	29	0.92	0.76	**	13.9	15	0.92	0.87	NS	9.4	11	0.87	0.91	NS
Aru 1D39	4.4	5	0.58	0.55	NS	3.5	4	0.61	0.62	NS	4.2	6	0.56	0.52	*	5.9	6	0.77	0.40	*	2.6	3	0.30	0.34	NS
Aru 1E7	6.3	7	0.80	0.72	NS	8.3	9	0.85	0.80	NS	7.1	10	0.83	0.76	NS	7.6	8	0.79	0.75	NS	4.3	5	0.38	0.39	NS
Aru 1E45	7.6	10	0.84	0.77	NS	10.1	13	0.89	0.92	NS	9.4	15	0.84	0.79	NS	6.8	7	0.81	0.60	NS	4.4	5	0.60	0.56	NS
Aru 1E66	5.7	7	0.70	0.61	NS	5.2	7	0.71	0.60	NS	6.7	17	0.75	0.53	**	4.9	5	0.71	0.73	NS	1.8	2	0.08	0.08	NS
Aru 1E78	8.0	10	0.69	0.66	NS	7.7	12	0.67	0.60	NS	7.7	21	0.55	0.51	NS	4.9	5	0.64	0.73	NS	3.2	4	0.30	0.34	NS
Aru 1E93	5.0	6	0.69	0.55	NS	6.2	7	0.80	0.74	NS	6.3	8	0.80	0.79	*	5.8	6	0.72	0.68	NS	2.6	3	0.49	0.56	NS
Aru 1E97	13.9	20	0.92	0.77	*	16.2	29	0.94	0.80	*	16.4	51	0.94	0.84	**	14.4	15	0.93	0.60	**	7.1	8	0.76	0.69	NS
Aru 1E102	8.6	11	0.75	0.72	NS	7.4	11	0.78	0.74	NS	7.8	11	0.78	0.71	NS	9.0	9	0.82	0.71	NS	3.2	4	0.51	0.56	NS
Aru 1F25	6.4	8	0.76	0.75	NS	7.5	13	0.82	0.77	NS	8.4	19	0.82	0.80	NS	5.8	6	0.79	0.56	NS	5.3	6	0.70	0.78	NS
Aru 1F114	6.7	8	0.81	0.66	*	5.2	6	0.76	0.58	*	6.1	9	0.77	0.74	NS	4.9	5	0.76	0.80	NS	4.6	5	0.74	0.60	NS
Aru 1F138	2.7	4	0.50	0.13	**	3.2	4	0.56	0.41	NS	3.8	8	0.54	0.50	NS	3.9	4	0.71	0.50	NS	2.9	3	0.54	0.56	NS
Aru 1G4	6.2	7	0.80	0.83	NS	6.0	8	0.78	0.73	NS	7.0	13	0.80	0.76	*	6.8	7	0.77	0.86	NS	4.5	5	0.73	0.86	NS
Aru 1G47	3.0	3	0.66	0.63	NS	3.7	5	0.57	0.55	NS	3.3	6	0.63	0.61	NS	3.8	4	0.46	0.31	NS	3.0	3	0.60	0.69	NS
Aru 1G49	12.0	14	0.92	1.0	NS	11.4	17	0.90	0.85	NS	11.3	20	0.90	0.86	NS	12.6	13	0.92	0.86	NS	9.6	11	0.88	0.95	NS
Aru 1I121	1.9	2	0.28	0.33	NS	2.0	2	0.38	0.51	*	2.2	3	0.27	0.27	NS	1.9	2	0.17	0.18	NS	2.0	2	0.39	0.17	NS
Aru 1J76	8.2	10	0.84	0.77	NS	9.1	11	0.88	0.90	NS	10.3	15	0.89	0.85	NS	5.8	6	0.82	0.75	NS	3.9	4	0.67	0.82	NS
Aru 1K10	1.3	2	0.02	0.02	-	3.3	4	0.37	0.36	NS	3.0	4	0.38	0.33	NS	2.9	3	0.54	0.56	NS	3.8	4	0.59	0.69	NS
Aru 1M127	3.9	5	0.55	0.61	NS	4.9	8	0.62	0.41	**	4.3	10	0.61	0.59	NS	3.9	4	0.57	0.68	NS	1.6	2	0.04	0.04	-
Overall loci	6.2	7.7	0.68	0.60	**	6.8	9.6	0.73	0.65	**	7.1	14	0.71	0.66	**	6.5	6.7	0.72	0.63	**	4.2	4.8	0.54	0.56	NS

Allelic richness based on a sample size of 14 complete genotypes (A_C), number of alleles (A), unbiased expected heterozygosity (H_E), observed heterozygosity (H_O), deviations from Hardy-Weinberg Equilibrium (HWE): NS, not significant ($P > 0.01$); *, significant ($P < 0.01$); **, significant ($P < 0.001$); -, not determinated.

Fig. S1 ML tree topology (log likelihood = -1135.6116) including MP, NJ and ML bootstrap values for 1000 replicates. Bootstrap values are above the node. Only bootstrap values > 50 % are shown.

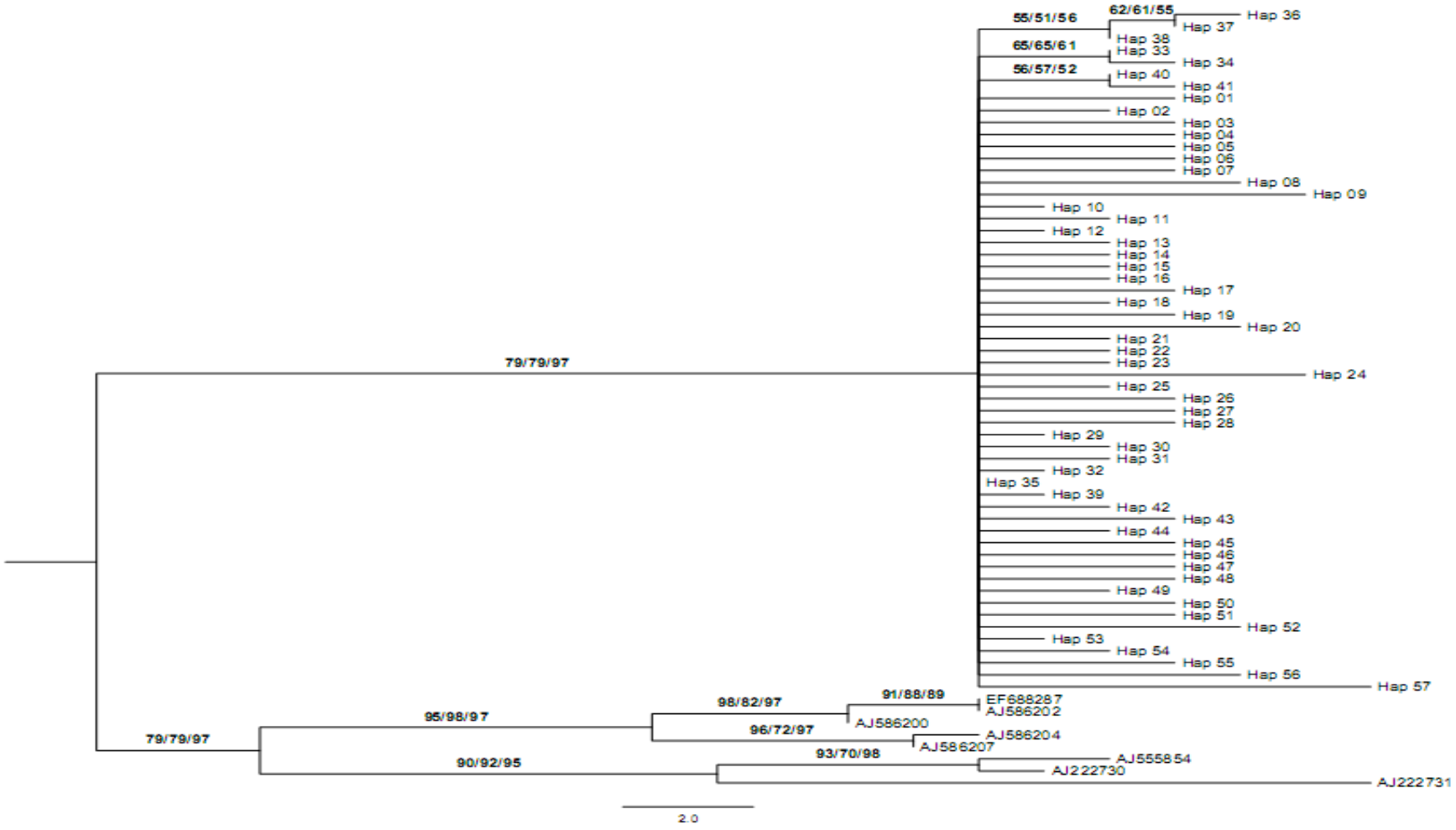
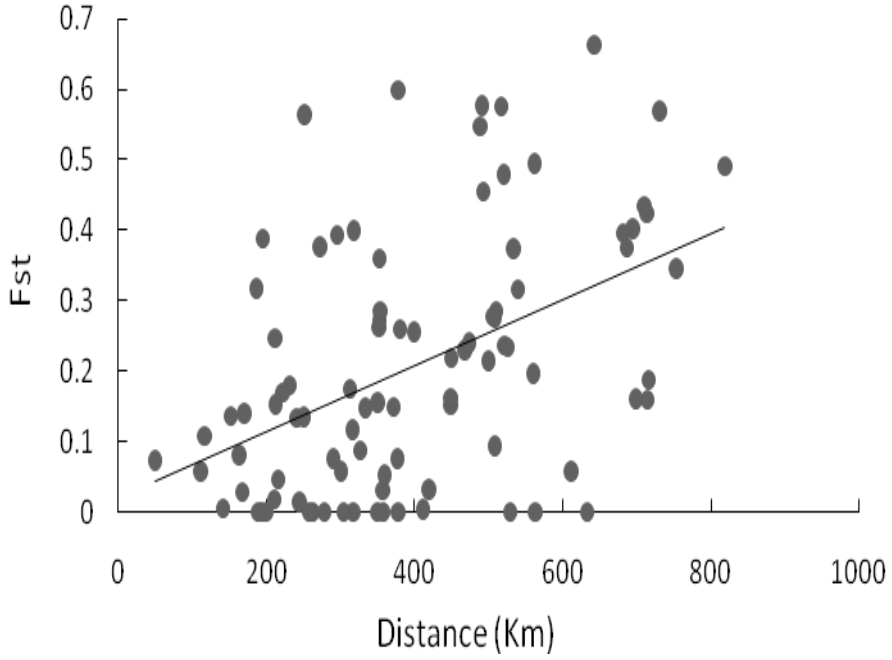
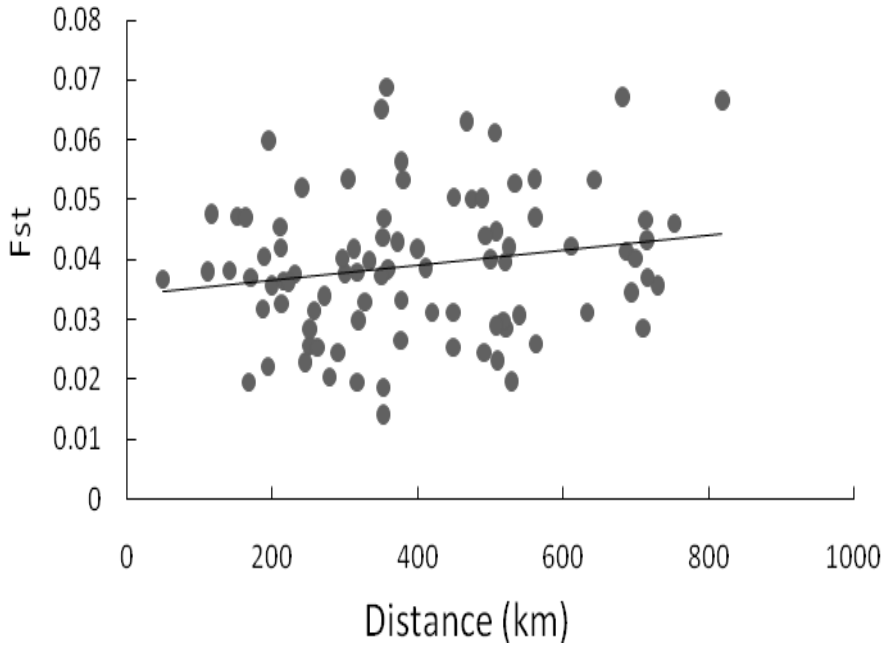


Fig. S2 Mantel tests showing the relationship between pairwise genetic distances (F_{ST}) and geographic distances(in km). Calculated from mtDNA CR (A) and microsatellites (B).

(A)



(B)



CHAPTER 4

Climatic and geographic effect on the spatial genetic pattern of a landbird species (*Alectoris rufa*) in the Iberian Peninsula

Climatic and geographic effect on the spatial genetic pattern of a landbird species (*Alectoris rufa*) in the Iberian Peninsula

Abstract

The understanding of the spatial pattern of genetic diversity may be pivotal to the adaptive conservation management of a given taxon. The red-legged partridge (*Alectoris rufa*, Galliformes) has a large natural distribution extending from the Mediterranean to humid temperate zones. The genetic structure of this species comprises five clusters, three of which are in the Iberian Peninsula, where there is evidence of glacial refugia. The partridge demographic expansion events and climatic shifts that occurred, are used as a basis to evaluate the following hypotheses regarding Iberian red-legged partridge distribution. First, we tested for the existence of relationships between genetic diversity/genetic distances and climatic factors. Second, we expected a pattern concordant with the 'abundant centre' hypothesis. Theses hypotheses were assessed using two different genetic systems, part of the mitochondrial DNA control region (n = 113) and 20 species-specific microsatellite DNA loci (n = 377). We tested our hypotheses by employing the climatic and geographic factors in 14 Iberian localities. Our results showed the influence of temperature (minimum and annual temperature) on mitochondrial haplotype and nucleotide diversity indexes. Moreover, the annual temperature distances were significantly correlated with mitochondrial and nuclear genetic distances. Furthermore, mitochondrial genetic

patterns were in accordance with the ‘abundant centre’ hypothesis. Based on all considerations, current climatic variables proved useful as regards useful describing genetic variation and differentiation in the red-legged partridge species, which may be associated with the species’ processes of local adaptation.

Keywords: census size, central-marginal, climate, genetic diversity, red-legged partridge, temperature

Introduction

Distribution, abundance of species and its diversity are greatly influenced by environmental heterogeneity (e.g., Wakeley and Aliacar 2001; Hanski and Ovaskainen 2003). Since it is possible that environmental gradients contribute to local adaptation in spatially structured populations (Doebeli and Dieckmann 2003), climatic changes may mould complex patterns in natural distributions (Jenkins and Hoffmann 2001; Carrascal and Seoane 2009).

One of the principal climatic factors is temperature. Fluctuations in temperature have had a profound impact on the distribution of organisms, which is visible in past and present times (Taberlet et al. 1998; Hewitt 2000; Jenkins and Hoffmann 2001; Walther et al. 2002; Carrascal and Seoane 2009; Provan 2013). The effects of temperature are also apparent in the intra-specific genetic patterns in an animal species' range (e.g., a high dispersal terrestrial species: *Canis lupus*, Geffen et al. 2004; avian species: *Alectoris chukar*, Randi and Alkon 1994; Huang et al. 2005; Randi et al. 2006; *Alectoris magna*, Huang et al. 2007).

Other factors may have an influence on the patterns of genetic diversity throughout a species distribution range, such as the location of populations throughout the species´ geographical range. The 'abundant centre' hypothesis suggests that central *vs*. marginal populations might correspond to high *vs*. low abundance (Vucetich and Waite 2003; Eckert et al. 2008), which predicts a high *vs*. low genetic diversity, respectively. Latitudinal and longitudinal clines may also affect the genetic diversity patterns in a species, possibly as a consequence of postglacial expansion events (Hewitt

1996; Hardie and Hutchings 2010; Ferrero et al. 2011) or environmental conditions (Geffen et al. 2004), although other more complex patterns of diversity can occur (Petit et al. 2003).

The species under study, the red-legged partridge (*Alectoris rufa* Linnaeus 1758), inhabits western Mediterranean ecosystems, whose natural distribution range encompasses Spain, Portugal, South-eastern France and North-western Italy. This species, which has reduced home ranges, is a landbird that has adapted to the warm Mediterranean climate (del Hoyo et al. 1994; Buenestado et al. 2008) and avoids extreme habitats. On the one hand, an extremely hot dry year reduces the productivity of the vegetation, nest survival (Rands 1987; Pescador and Peris 2007), juvenile survival (Doxa et al. 2012), breeding success in wild red-legged partridges (Casas et al. 2009) and partridge densities (Lucio 1990; Villanúa 2008), in addition to increasing the risk of parasitism (Calvete et al. 2003). On the other hand, heavy winters, extremely low temperatures and snowfall may decrease the survival and density in congenerous partridges (Bernard-Laurent and Leonard 2000) and in numerous European bird species (Forsman and Monkkonen 2003; Ontiveros and Pleguezuelos 2003). The present study was carried out on the Iberian Peninsula, which is located in the Eurosiberian and Mediterranean biogeographic/climatic regions (Peinado Lorca and Rivas-Martínez 1987), and has high environmental heterogeneity that favours the diversification of several endemic taxa and the evolution of its own particular biotic communities (Benayas and Scheiner 2002).

Ferrero et al. (2011) used mitochondrial and microsatellite red-legged partridge data to describe five clusters, three of which were in the Iberian Peninsula, where there is higher genetic diversity than in its northerly distribution and evidence of glacial

refugia (Ferrero et al. 2011). The Iberian Peninsula was an important glacial refugium in Europe during the Pleistocene glaciations (Hewitt 2000; Gómez and Lunt 2007), where the environmental heterogeneity may have promoted subrefugia within the Iberian refugium for numerous species (Gómez and Lunt 2007). One of these species is the red-legged partridge, with evidence of three refugia within the Iberian refugium (the regions encompassed by the Central-eastern cluster, South-western cluster and North-western cluster) during Pleistocene glaciations (Ferrero et al. 2011).

During postglacial demographic expansion events (see historical demography, Ferrero et al. 2011), partridges might have expanded to their current distribution, from Central-eastern Iberia to the northwest and southwest. The divergence time for partridge clusters (Central-eastern and North-western clusters; Central-eastern and South-western clusters) could have occurred after the Last Interglacial Maximum (≈110 000 years BP) or the Last Glacial Maximum (≈30 000 years BP), which suggests that an interplay of climatic, geographical and genetic factors shaped the genetic diversity in this species (Ferrero et al. 2011), although these relationships remain unknown.

The red-legged partridge species has undergone declines in abundance throughout its European range (Blanco-Aguiar et al. 2003). In areas in which the species is actively hunted, there have been attempts to counter population decline by means of massive releases of captive-reared red-legged partridges, which have often included hybrids with *A. chukar* (Barbanera et al. 2005; Blanco-Aguiar et al. 2008; Martínez-Fresno et al. 2008; Barbanera et al. 2009, 2010; Negri et al. 2013; Barbanera et al. 2015). However, these releases have not taken account the species' genetic structure. Indeed, farmers sell and buy partridges within its broad distribution range with no supervision (Blanco-Aguiar et al. 2008; Barbanera et al. 2010).

Releases of red-legged partridges have made the phylogeographic study in this species difficult. While some authors have not detected genetic structure in the red-legged partridge species (Barbanera et al. 2010; Rodríguez-García and Galián 2014; Barbanera et al. 2015); others such as Martínez-Fresno et al. (2008), Ferrero et al. (2011) and Barbanera et al. (2010, 2015; ancient sample), have detected some signs of intraspecific genetic structure in this species. Our awareness of the genetic alteration that partridge restocking is causing in the current genetic structure of populations has prompted us to take advantage of Ferrero et al.'s (2011) study in an attempt to unravel the relationship between genetic, geographic and climatic patterns in the Iberia Peninsula.

In the present study, we employed the14 Iberian red-legged partridge localities used by Ferrero et al. (2011). The red-legged partridge expansion and climatic shifts in the Iberian Peninsula during the Pleistocene glaciations (Ferrero et al. 2011), have been used as a basis to test three hypotheses using an ecological approach (climatic and geographic variables). First, we hypothesised an influence of climate on genetic diversity and genetic differentiation among localities. Second, following postglacial expansion from the central-eastern to the current Iberian distribution, we expected a pattern concordant with the 'abundant centre' hypothesis, predicting a decline in genetic diversity in peripheral rather than central populations. Third, the previous hypotheses were assessed using two different genetic systems, mitochondrial DNA control region and 20 species-specific microsatellite DNA loci, owing to their different effective population size, mutation rate and level of recombination. We assumed we would obtain different responses from mitochondrial *vs*. nuclear DNA, expecting a more striking pattern for mitochondrial than for nuclear DNA.

Materials and methods

Sample collection and laboratory procedures

1208 muscle tissue samples (Blanco-Aguiar et al. 2008; Ferrero et al. 2011) were collected from legally hunted wild *A. rufa* individuals in the Iberian Peninsula during the 2001-2004 hunting seasons. Because of the intensive management of this species, in addition to excluding samples from partridge farms, we also discarded those localities in which intensive restocking occurred and their surrounding areas. Furthermore, Ferrero et al. (2011) made use of a genetic conservative approach, *i.e.* all localities with any *A. chukar* mitochondrial individual (doubtful locality) were removed from the sample set.

In this study, we employed the 14 Iberian red-legged partridge localities and data set of Ferrero et al. (2011). We focused our study on the Iberian Peninsula because the barrier of the Pyrenees could have retained partridges in Iberia during periods of glaciations, thus favouring local adaptation to the environment. Island localities were excluded because evidence suggests an anthropogenic origin (*e.g.* Balearic Islands, see Ferrero et al. 2011). The muscle tissue samples collected were preserved at room temperature in absolute ethanol and at -20ºC once in the laboratory. Genomic DNA was extracted according to the methods of Sambrook et al. (1989). For the mitochondrial DNA survey, we analysed 113 samples from 14 localities (Table 1; Fig. 1). A 319 base pair (bp) fragment from the highly variable peripheral domain I of the mitochondrial

control region (mtDNA CR) was amplified using Aru DLCR-F2 and Aru DLCR-R2 primers and the PCR conditions described by Ferrero et al. (2011).

For nuclear study, we employed 377 red-legged partridges from 14 localities (Table 1; Fig. 1). We used 20 species-specific microsatellite loci: Aru 1A1, 1B3, 1D39, 1E7, 1E45, 1E66, 1E78, 1E93, 1E97, 1E102, 1F25, 1F114, 1F138, 1G4, 1G47, 1G49, 1I121, 1J76, 1K10 and 1M127; analysed as in Ferrero et al. (2007, 2011).

Genetic analyses

Mitochondrial haplotype diversity (*h*) and nucleotide diversity (π) were calculated in DnaSP v5 (Librado and Rozas 2009). Nei´s (1987) gene diversity (G_D) and allelic richness (A_C), which takes into account sample size, and were computed using FSTAT 2.9.3 (Goudet 1995). Genetic distances between pairwise localities were estimated using Φ_{PT}, an analogue of F_{ST} (Excoffier et al. 1992; mitochondrial) and F_{ST} (nuclear) as implemented in the GENALEX 6 software (Peakall and Smouse 2006). Sequential Bonferroni correction was used to test for significance in genetic distance values (Rice 1989).

Climatic variables

The average climatic parameters for each locality were characterised using a data set obtained from the Climatic Atlas of the Iberian Peninsula and employing data regarding 15 years of temperature and 20 years of rainfall (Ninyerola et al. 2005). The climatic

variables selected were: annual average temperature (AT), average minimum temperature of the coldest month (MinT), average maximum temperature of the hottest month (MaxT) and annual average rainfall (Rain). Current climate data are used to describe spatial patterns of climatic variation, and as many of the physical and geographical characteristics which are shaping climatic patterns have been stable (e.g. distance to the sea, altitude, orientation...) since the Pleistocene era, current spatial variation in climate may be useful to indirectly detect the response of an organism to climatic gradients that occurred in the past.

Geographic variables

We assessed the 'abundant centre' hypothesis by estimating the distance to edge of the localities studied. As the red-legged partridge range in Iberia is mainly limited by coastal barriers, we estimated the distance to the nearest coastal barrier for each locality.

In order to study whether partridge abundance was associated with distance to edge, we estimated the partridge index of abundance (N), using an adaptation of Vucetich et al.'s equation (4) (1997):

$N = Na/(1+CV^2)$,

where Na represents average abundance and CV is the coefficient of variation in abundance over time. As an indicator of the spatial variation of average abundance of this game bird species in Spain, we used provincial hunting bag records obtained from the Spanish Ministry of Agriculture, Fisheries and Food (Blanco-Aguiar et al. 2008). Because hunting effort can influence the number of partridges hunted, we divided the hunting bag data by the number of hunting licenses per year to obtain a provincial

average abundance (Na) (Cattadori et al. 2003; Blanco-Aguiar et al. 2008). For each locality studied, we used provincial bag records from 1973 to 1992. We excluded bag records after 1992 to avoid the influence of the massive releases of red legged partridges during middle 1990s in Spain (see Blanco-Aguiar et al. 2008). As there were no comparable hunting bag records in Portugal, we excluded the Portuguese locality in those analyses in which the partridge index of abundance was used.

Other geographic spatial patterns and estimated geographic distances were evaluated using latitude (Lat) and longitude (Lon) as geographic variables.

Statistical analyses

We conducted two different analyses using: general linear models (GLM) with genetic diversity indexes (h, π, G_D, A_C) and permutational regression with genetic distance matrices between sampled sites.

First, the GLM analyses were used to test the association between the red-legged partridges' genetic diversity indexes (mitochondrial and nuclear) *vs.* the climatic and geographic variables obtained for each locality. GLM procedures were implemented by using the MASS package (Venables and Ripley 2002) written for the R language (R Development Core Team 2012) and employing models with a normal distribution of error terms and the identity link function. The heterocedasticity of the residual was improved by transforming certain variables, e.g., arc sine (h), logarithm (N) and sample size was used as an offset when necessary. We made further biological inferences using the information theoretic approach that identifies the best set of models according to several competing hypotheses via information criteria (Burnham and Anderson 2002;

Garamszegi et al. 2009; Burnham et al. 2011; Grueber et al. 2011). The fixed effects consisted of climatic variables (MaxT, AT, MinT, Rainfall), distance to edge, partridge index of abundance (N, log-transformated) and geographic coordinates. We also considered quadratic relationships.

We used the arm and MuMIn packages in R to calculate second order Akaike Information Criterion (AICc), AICc weight (w_i), and other desired parameters (Grueber et al. 2011). As the number of localities used in the study was reduced we avoided overfitting by only assessing those models with 3 or less variables, and we employed AICc criterion in order to penalise for the addition of parameters. We present only those models that are well-supported by the data (models with a ΔAICc < 2; Burnham and Anderson 2002). Because in some cases several equally likely models were identified and no model had $w_i > 0.90$, only for report statistical parameters we model-averaged the parameter estimates and the associated variances from the set of candidate models with a ΔAICc < 2, using the model.avg function of the MuMIn package in R (Grueber et al. 2011). We considered that a variable had an important predictive value only when the 95% CI did not include zero (Garamszegi et al. 2009; Cornuau et al. 2012). For GLM, we calculated the coefficient of determination (percentage of deviance explained) for each model as follows: (deviance of the null model – deviance of the model) / deviance of the null model.

Second, the permutational regression approach was employed to assess the independence of genetic distances between localities in relation to geographical distances and climatic variables. This method, in which the permutations of distance matrices are used as a basis to perform a multivariate analysis, was originally described by Legendre et al. (1994) and is widely used in ecological and genetic approaches

(Calvete et al. 2004; Balkenhol et al. 2009). In these analyses, genetic and geographical distances represented all possible pairwise comparisons between localities, while the other variables were transformed into distance matrices by computing the Euclidean distance (i.e., the absolute value of the difference) between values for all possible pairs. Geographic distances between pairwise localities were calculated in GENALEX 6. Multiple regression analyses were performed using the PERMUTE 3.4 program (Legendre et al. 1994) on values in the matrices, and repeated after each of the 999 random permutations of the dependent variable matrix. The significance of each partial regression coefficient obtained was calculated as the probability of obtaining, in the 999 random permutations, a regression coefficient greater than or equal to that observed. Firstly, a univariate approach was used to evaluate significant variables. However, because an apparent correlation between climatic and genetic pattern may actually be driven, or at least influenced, by the geographical distance between the localities, it was necessary to filter these effects. We used a stepwise regression approach with a backward elimination procedure to assess the potential correlation between genetic and climatic distances while controlling the effect of geographical distances.

Results

Mitochondrial DNA sequence description

A total of 319 nucleotides of the mtDNA CR were sequenced in 113 samples of red-legged partridges collected from 14 localities in the Iberian Peninsula. The sequence alignment showed 42 haplotypes, defined by 30 polymorphic sites: 21 parsimony informative sites, seven singleton variable sites and two insertions/deletions (see Table S1). Haplotype sequences were deposited in GenBank by Barbanera et al. (2005): AJ586225; Barbanera et al. (2009): AM850842; Ferrero et al. (2011): HQ621760-770, 773, 776-778, 780-800, 806-809. Nucleotide diversity among the 14 localities varied from 0.0021 (León) to 0.0161 (Toledo) (Table 1). Pairwise locality Φ_{PT} values ranged from 0.000 to 0.663 and the significant pairwise values, after the application of sequential Bonferroni correction, are shown in Table S2.

Microsatellites

A total of 301 alleles were detected in the 377 wild red-legged partridges in the 20 microsatellite loci tested. The number of alleles detected per locus varied from 3 to 55, with an average of 15.05 alleles. The Nei´s gene diversity ranged from 0.657 (Tarragona) to 0.719 (Álava) (Table 1). The locality with the lowest allelic richness (5.762) was Tarragona, while the highest value (7.499) was obtained in Bragança. Pairwise locality F_{ST} values were low (from 0.014 to 0.069) and after the application of a sequential Bonferroni correction, all localities were significantly differentiated from each other (Table S3).

Patterns of genetic diversity

Molecular diversity indexes showed a correlation between different markers: Allelic richness had positive correlations with both mitochondrial diversity indexes: *h* ($F_{1,12}$ = 16.87, P = 0.0014) and π ($F_{1,12}$ = 11.66, P = 0.005), and *h* and π were correlated with each other ($F_{1,12}$ = 39.91, P < 0.0001). However, the Nei´s gene diversity index was correlated with neither any of the other mitochondrial indexes ($F_{1,12}$ < 1.58, P > 0.23) nor with allelic richness ($F_{1,12}$ = 0.089, P = 0.77).

We obtained the best set of models for each genetic index. Three models were retained to explain the mitochondrial haplotype diversity with a coefficient of determination range of 79-87 %. A polynomial relationship with the minimum temperature during the coldest month, annual average temperature and distance to range margin were included in at least one model (Table 2), thus leading to an increase in haplotype diversity with: increasing minimum temperatures, with the exception of the highest values of the minimum temperatures which underwent a decrease in genetic diversity values (Fig. 2a), thus increasing average temperature values (Fig. 2b), and the distance of the localities to the range margin (Fig. 2c). However, our results identified the polynomial correlation of minimum temperatures as the best variable with which to predict haplotype diversity (Table 3). Two models with a coefficient of determination range of 76-82 % were similarly selected for nucleotide diversity. A polynomial relationship with minimum temperatures and distance to the range margin were included in at least one model (Table 2). The relationships between nucleotide diversity and these variables (Fig. S1) were similar to those of haplotype diversity. The best variable with which to explain the nucleotide diversity was the polynomial relationship with minimum temperatures (Table 3). Two models were retained for nuclear genetic diversity, the polynomial relationship with rainfall was included in one of the models

(ΔAICc = 0.26, R^2 = 0.44), but the top model selected was the null model, suggesting that this variable had relatively little influence on nuclear genetic diversity. No model was retained for allelic richness.

The partridge index of abundance was positively correlated with the estimated distance from the margin range ($F_{1,11}$ = 5.64, P = 0.037). However, the centre-edge pattern was not evident when we assessed genetic diversity indexes (*h*: $F_{1,12}$ = 3.89, P = 0.07; π: $F_{1,12}$ = 1.52, P = 0.24; G_D: $F_{1,12}$ = 0.89, P = 0.36; A_C: $F_{1,12}$ = 0.71, P = 0.41).

Neither latitude nor longitude were correlated with haplotype diversity (Lat, $F_{1,12}$ = 0.02, P = 0.88; Lon, $F_{1,12}$ = 0.22, P = 0.64), with nucleotide diversity (Lat, $F_{1,12}$ = 1.01, P = 0.33; Lon, $F_{1,12}$ = 0.03, P = 0.87) or with allelic richness (Lat, $F_{1,12}$ = 1.37, P = 0.26; Lon, $F_{1,12}$ = 0.81, P = 0.38). Nei´s gene diversity was not correlated with longitude ($F_{1,12}$ = 1.36, P = 0.74), although it was positively correlated with latitude ($F_{1,12}$ = 12.05, P = 0.01) and negatively correlated with the interaction between latitude and longitude ($F_{1,12}$ = 9.72, P = 0.011).

Patterns of genetic differentiation

When we compared the mitochondrial and nuclear genetic distance matrices, we observed a slight correlation (Beta-value = 0.22, P = 0.039, R^2 = 0.049). The results of the regression analyses are summarised in Table 4a (mitochondrial) and Table 4b (nuclear), which includes standardised Beta values, the explained variance of R^2 and the P values. In the univariate mitochondrial models (Table 4a), increasing differences for annual temperature, rainfall, geographic and latitude distances were significantly correlated with mitochondrial genetic distances, whereas the backward stepwise

regression model retained geographic and annual temperature distances. In the univariate nuclear models, only the annual temperature distances were significantly correlated, and geographic distances were marginally significant (Table 4b). The backward stepwise regression also selected the annual temperature variable.

Discussion

The impact of climate on genetic diversity

Current climatic variables were useful to describe genetic variation and differentiation in the red-legged partridge species, probably because some variables maintain spatial patterns similar to those existing during the process of diversification.

Rainfall and temperature were related to the genetic distances between localities. When the influence of geographical distances was controlled for, our results revealed increasing genetic distances with an increase in thermic distances among localities (mitochondrial and nuclear results; Table 4a, 4b), whereas rainfall was no longer important.

Otherwise, temperature has influenced the spatial pattern of genetic diversity in the red-legged partridge species, with the highest genetic diversity values being obtained in the case of intermediate temperatures (Figs. 2a, S1a). As a species of temperate Mediterranean climate, there was a tendency for the genetic diversity to decrease in extreme habitats (i.e., the highest or lowest values of minimum temperature;

Figs. 2a, S1a). In extreme temperature habitats the species inhabits some peripheral localities (Fig. 2a), which may be more sensitive to climate warming, with changes in biodiversity and species richness (Sala et al. 2000; Barbet-Massin et al. 2012).

The red-legged partridge species was greatly affected by the last Pleistocene glaciation (Würm glaciation ≈ 100 000-10 000 years BP; Ferrero et al. 2011), when minimum values of temperature may have determined the species' survival or reproduction. According to reconstructions based on general circulation models of the Iberian Peninsula mean annual temperature throughout the course of the Last Glacial Maximum (21 000 years BP; Community Climate System Model and Model for Interdisciplinary Research On Climate), Rodríguez-Sánchez et al. (2010) describe different annual temperature zones in the Iberian Peninsula, which could fit the genetic structure found previously in this species (Ferrero et al. 2011: Fig. 2). Upon linking the information obtained by the two studies (Fig. 3), three main zones were revealed: 1. cold-temperate zone (yellow, 5-10 ºC) in the North-Northwest, which includes the North-western cluster; 2. temperate zone (orange, 10-15 ºC) in the Central region, which may agree with the Central-eastern cluster; and 3. hot zone (red, 15-20 ºC) in the Southwest and on the southeast coast of the Peninsula, which contains the Southern cluster. The second zone with high genetic variation could correspond to a possible ancestral area for this species (Ferrero et al. 2011).

The observable correspondence between the main annual temperature zones during the Last Glacial Maximum and the genetic clusters described (Fig. 3), along with temperature played an important role in both the nuclear and mitochondrial genetic dissimilarities among localities (Table 4a, 4b) and the influence of temperature on the mitochondrial genetic diversity pattern (Tables 2, 3; Figs. 2a, 2b, S1a). The overall

indicia suggest that past temperatures may be associated with the processes of local adaptation and differentiation in this species.

The polynomial correlation of minimum temperatures was identified as the best variable with which to predict mitochondrial haplotype diversity indexes, *h* and π. However, the fit was better for *h* than for π (Figs. 2a, S1a). Estimates of π could have been vulnerable to low sample numbers when the locality was particularly variable (as in the case of the red-legged partridge). On the contrary, estimates of *h* were less susceptible to the influence of under-sampling in highly variable localities (Goodall-Copestake et al. 2012). The genetic diversity pattern was more striking for mtDNA CR than for nuclear markers. This is not unexpected as mtDNA markers track demographic histories of populations more closely because of smaller effective population size (Avise 2000). Otherwise, the evolution of mtDNA has long been considered neutral, but there is evidence that it is subject to various sorts of selective pressures (Ballard and Kreitman 1995; Melo-Ferreira et al. 2014). mtDNA variation could have significant metabolic and fitness consequences, some of which are associated with temperature (Ballard and Kreitman 1995; Gerber et al. 2001; Toyomizu et al. 2002; Mozo et al. 2005). The mtDNA variation might therefore be more sensible to environmental gradients than DNA microsatellite markers. The lack of resolution in our microsatellite data set might be attributable to an elevated mutation rate, the possibility of homoplasy in allele sizes (Hedrick 1999) and the potential consequences of restocking activities.

‘Abundant centre’ hypothesis

The abundance pattern observed in this species corroborates the geographical variation in contemporary population size of the 'abundant centre' hypothesis: higher N in central populations in comparison to more peripheral populations. In general, this pattern does not appear to be associated with demographic declines in peripheral localities, as bottleneck events were not detected in our sampling localities (see Ferrero et al. 2011). Population size in this species is large, Purroy (1997) estimated between three and more than seven million breeding partridges in Spain, which could make this species less vulnerable to mild declines. The current abundance pattern could have originated from a historical process such as range expansion from the Central-eastern cluster to the limits of the Iberian distribution range (Ferrero et al. 2011). This pattern could have been preserved by decreasing effective population size and gene flow among populations towards the geographic range margins (Vucetich and Waite 2003). Furthermore, habitat quality could be associated with this current abundance pattern, since central localities could harbour optimal habitats while peripheral localities may have less favourable habitats for the species, signifying that its population size declines towards range boundaries (Brown 1984; Hengeveld 1993). In fact, the red-legged partridge shows a preference for stepparian landscapes with interspersed natural vegetation (Blanco-Aguiar et al. 2003; Buenestado et al. 2008, 2009), which are mainly located in the centre of the Iberian Peninsula.

In the red-legged partridge, the genetic diversity was not directly associated with the partridge index of abundance. However, genetic diversity increased (haplotype and nucleotide diversity) with a greater distance to edge (Figs. 2c, S1b). Moreover, the partridge index of abundance was positively correlated with the distance to edge index ($F_{1,11} = 5.64$, $P = 0.037$). Although this pattern was not very strong, these results could

support the prediction of the ‘abundant centre’ hypothesis: higher genetic diversity in central populations (high abundance) in comparison to more peripheral populations (low abundance).

Management practices

The red-legged partridge species has undergone intensive game restocking during the last few decades, which has altered the genetic structure of this species (Barbanera et al. 2010; Rodríguez-García and Galián 2014; Barbanera et al. 2015). These changes may have had effects on not only the species’ conservation (possible mixture of differentiated genotypes, loss of unique genetic traits, loss of adaptation to local environments), but also the comprehension of its historical processes. However, it was possible to find localities in which restocking events had not occurred (Martínez-Fresno et al. 2008; Ferrero et al. 2011; Negri et al. 2013; this study). We have used a conservative approach to minimise the influence of factors related to species management. Even at the risk of not being able to control all the restocking effects in the partridge species, before the loss of natural patterns as a result of current management practices, we believe that it is important not to miss the opportunity to explore the mechanisms and processes that have shaped the genetic diversity in this species.

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Table 1 Mitochondrial and nuclear genetic diversity measures for the 14 *A. rufa* localities

Country	Localities	Genetic cluster	Mitochondrial			Nuclear		
			n	*h*	π	n	G_D	A_C
Spain								
	León	NW	14	0.5824	0.0021	20	0.706	6.432
	Lugo	NW	10	0.9333	0.0092	18	0.712	6.566
	Álava	CE-NW	5	0.8000	0.0108	33	0.719	6.873
	Valladolid	NW	9	0.6944	0.0055	25	0.717	6.395
	Zaragoza	CE	6	0.8667	0.0038	24	0.679	6.827
	Tarragona	CE	7	0.6667	0.0042	25	0.657	5.762
	Madrid	CE	5	0.7000	0.0057	29	0.692	6.302
	Toledo	CE	6	1.0000	0.0161	29	0.691	6.930
	Cuenca	CE	7	0.9524	0.0109	31	0.670	6.602
	Ciudad Real	CE	5	0.9000	0.0108	30	0.706	7.253
	Alicante	CE	10	0.9333	0.0093	25	0.714	6.887
	Almería	CE	6	1.0000	0.0146	29	0.710	7.300
	Cádiz	SW	20	0.7895	0.0090	36	0.684	6.647
Portugal								
	Bragança	CE	3	1.0000	0.0127	23	0.710	7.499

Genetic clusters described in Ferrero et al. (2011): NW - North-western, CE - Central-eastern and SW - South-western. Mitochondrial: n - sample size, *h* - haplotype diversity, π - nucleotide diversity. Nuclear: n - number of genotyped partridges, G_D - mean Nei´s

gene diversity, A_C - allelic richness per locality based on a sample size of 17 complete genotypes

Table 2 Summary of GLM model selection using AICc to explain the mitochondrial genetic diversity indexes

Selected variables	AICc	ΔAICc	w_i	R^2
Haplotype diversity				
1- MinT + $MinT^2$ + AT	12.14	0	0.39	0.87
2- MinT + $MinT^2$	12.60	0.46	0.31	0.79
3- MinT + $MinT^2$ + distance to edge	12.67	0.53	0.30	0.86
Nucleotide diversity				
1- MinT + $MinT^2$	8.45	0	0.73	0.76
2- MinT + $MinT^2$ + distance to edge	10.40	1.95	0.27	0.82

Only models with ΔAICc < 2 are presented ($\Delta AICc = AICc_i - AICc_{min}$). Akaike weights ($w_i$) and coefficients of determination (R^2) are indicated. MinT - average minimum temperature of the coldest month, AT - annual average temperature

Table 3 GLM results of mitochondrial genetic indexes after model averaging

	Estimate ± SE	Confidence Interval	Relative Importance
Haplotype diversity			
(Intercept)	-1.59 ± 0.77	-3.17 / 0.002	
MinT	-0.72 ± 0.51	-1.79 / 0.354	1
MinT^2	-1.49 ± 0.25	-2.07 / -0.919	1
AT	0.11 ± 0.05	0.002 / 0.227	0.39
Distance to edge	0.29 ± 0.13	-0.011 / 0.596	0.30
Nucleotide diversity			
(Intercept)	-2.13 ± 0.21	-2.58 / -1.69	
MinT	-0.63 ± 0.23	-1.14 / -0.11	1
MinT^2	-1.09 ± 0.23	-1.61 / -0.58	1
Distance to edge	0.21 ± 0.12	-0.07 / 0.48	0.27

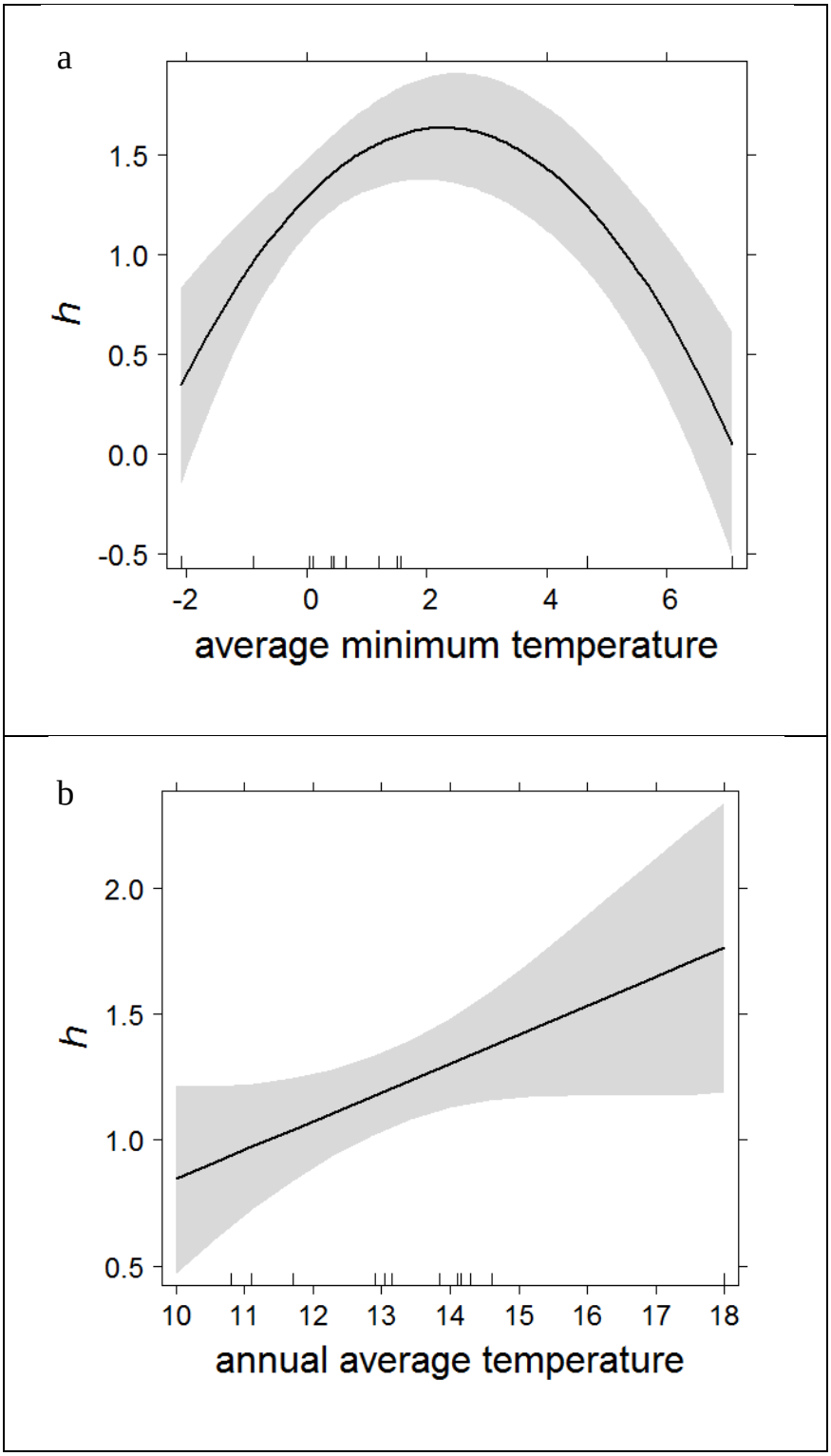
Relationships between mitochondrial diversity indexes and studied variables. Variables in bold are those for which the 95 % confidence interval did not include zero. MinT - average minimum temperature of the coldest month, AT - annual average temperature

Table 4 Correlations between genetic distances *vs.* climatic and geographic distances

Distances	Beta-values	P	R^2
a Mitochondrial			
Annual temperature (AT)	0.626	**0.001**	0.392
Minimun temperature (MinT)	0.186	0.085	0.034
Maximun temperature (MaxT)	-0.025	0.799	0.001
Rainfall (Rain)	0.405	**0.001**	0.164
Geography (Geo)	0.467	**0.001**	0.218
Longitude (Lon)	0.262	0.069	0.020
Latitude (Lat)	0.355	**0.001**	0.126
Selected model			
Geo + AT	0.18 + 0.53	**0.001**	0.417
b Nuclear			
Annual temperature (AT)	0.241	**0.021**	0.058
Minimun temperature (MinT)	-0.102	0.371	0.010
Maximun temperature (MaxT)	-0.125	0.242	0.016
Rainfall (Rain)	-0.006	0.952	0.001
Geography (Geo)	0.189	0.060	0.036
Longitude (Lon)	0.179	0.099	0.032
Latitude (Lat)	0.116	0.276	0.013

Univariate regression and selected model coefficients: standardised regression coefficients (Beta-values) and explained variance (R^2) are shown. Significant correlations are highlighted in bold

Fig. 1 Distribution of red-legged partridge localities in the Iberian Peninsula. Red lines represent Iberian genetic clusters previously described by Ferrero et al. (2011)


a
h
1.5
1.0
0.5
0.0
-0.5
-2
0
2
4
6
average minimum temperature
b
h
2.0
1.5
1.0
0.5
10
11
12
13
14
15
16
17
18
annual average temperature

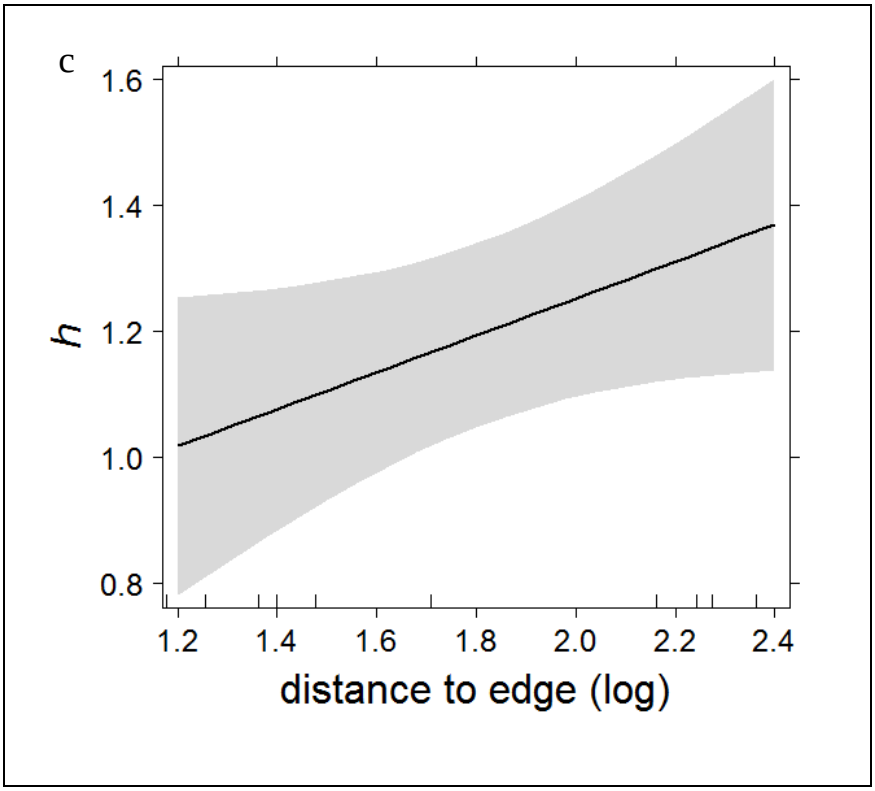


Fig. 2 Relationship between haplotype diversity (*h*, transformated) and variables selected. Shaded areas represent 95% confidence intervals of estimated marginal means.

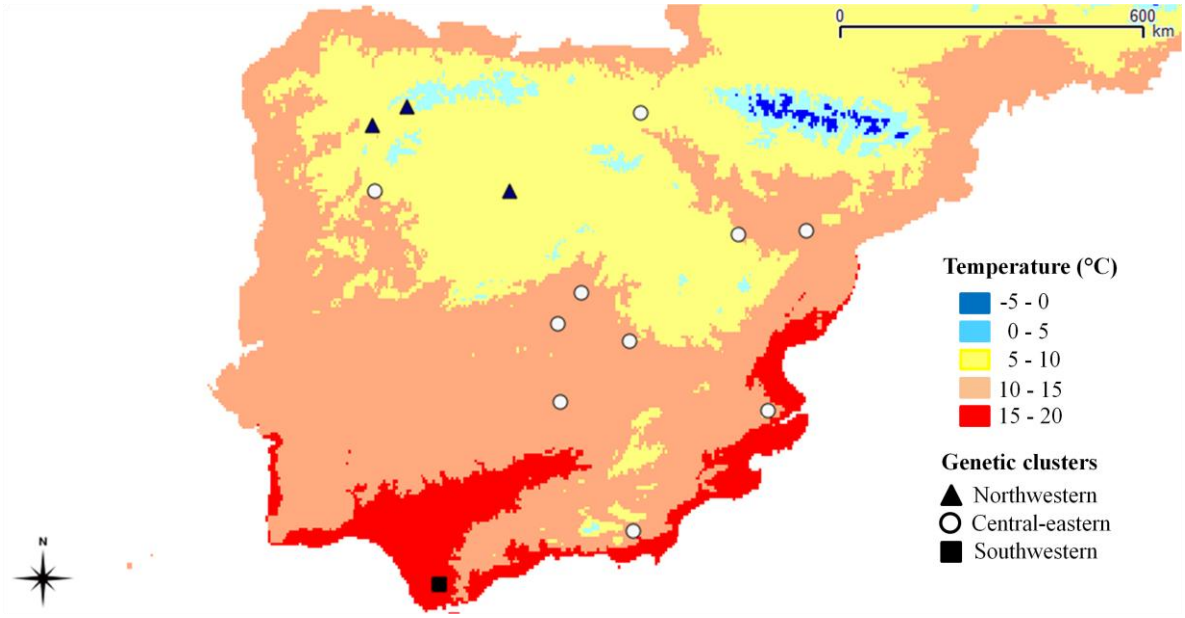


Fig. 3 Map of the Iberian mean annual temperature throughout the course of the Last Glacial Maximum (LGM, 21 000 years BP) according to reconstructions based on general circulation models (Community Climate System Model and Model for Interdisciplinary Research On Climate; based on Rodríguez-Sánchez et al. 2010). The different symbols indicate the three red-legged partridge Iberian clusters described in Ferrero et al. (2011). The LGM coastline differs from the present because the sea level was lower during the LGM.

Table S1 *A. rufa* haplotypes and variable sites

A. rufa haplotype	16 6	18 8	19 2	19 6	20 1	20 7	20 9	21 9	22 1	22 3	22 4	22 5	22 8	22 9	23 8	23 9	24 0	24 1	24 2	24 5	24 6	24 7	25 5	26 8	26 9	28 0	29 2	30 5	30 8	31 6	Genbank number	N		F	Sampling Localities (sample size)
H01	A	C	C	T	T	T	C	C	A	G	A	T	C	T	T	C	T	T	C	C	T	T	C	T	C	T	T	G	T	T	HQ621760	2		(0.018)	a2
H02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621761	1	8	(0.159)	a2,b9,c5,h1,k1
H03	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621762	1		(0.009)	h1
H04	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621763	6		(0.053)	b2,c2,d2
H05	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621764	1		(0.009)	c1
H06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	HQ621765	1		(0.009)	b1
H07	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621766	3		(0.027)	a1,b2
H08	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621767	2		(0.018)	a2
H09	.	.	.	.	.	C	.	.	G	.	G	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621768	1		(0.009)	c1
H10	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621769	4		(0.035)	e1,f2,a1
H11	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	HQ621770	2		(0.018)	k2
H12	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	A	.	AJ586225	5		(0.044)	k2,i1,n1,a1
H13	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	C	T	.	.	.	.	.	.	.	.	.	A	.	HQ621773	1		(0.009)	e1
H14	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	T	.	.	.	.	.	A	.	HQ621776	1		(0.009)	j1
H15	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	C	.	.	.	.	A	.	HQ621777	1		(0.009)	i1
H16	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	A	A	.	HQ621778	3		(0.027)	j1,m1,a1
H17	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	A	A	.	AM850842	1	1	(0.097)	n9,k1,h1
H18	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	C	.	.	.	A	A	.	HQ621780	1		(0.009)	n1
H19	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	C	A	A	.	HQ621781	1		(0.009)	m1
H20	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	C	.	.	.	A	A	.	HQ621782	1		(0.009)	k1
H21	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	A	.	HQ621783	1		(0.009)	n1
H22	.	.	-	.	.	C	.	.	.	.	.	.	A	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	A	.	HQ621784	1		(0.009)	n1
H23	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	C	.	.	.	.	A	.	HQ621785	1		(0.009)	j1
H24	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	A	.	HQ621786	1		(0.009)	m1
H25	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	C	.	A	.	HQ621787	2		(0.018)	i2
H26	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	C	.	A	.	HQ621788	1		(0.009)	i1
H27	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	HQ621789	5		(0.044)	f1,j2,m1,o1
H28	.	.	.	.	.	C	.	.	.	A	.	C	.	.	C	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	HQ621790	3		(0.027)	n3
H29	.	.	.	.	.	C	.	.	.	A	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	HQ621791	1		(0.009)	n1
H30	.	.	.	.	.	C	.	.	.	A	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	HQ621792	1		(0.009)	n1
H31	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	G	.	HQ621793	2		(0.018)	d2
H32	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	C	.	.	G	.	HQ621794	1		(0.009)	h1
H33	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	-	.	.	.	G	.	HQ621795	2		(0.018)	n2
H34	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	HQ621796	1		(0.009)	m1
H35	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	HQ621797	1	4	(0.124)	e3,f2,d1,h1,i1,k2,g4
H36	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	HQ621798	2		(0.018)	g2

H37	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	A	.	HQ621799	1	(0.009)	i1
H38	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	A	.	HQ621800	3	(0.027)	m1,k1,o1
H39	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	HQ621806	1	(0.009)	f1
H40	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	A	.	HQ621807	1	(0.009)	g1
H41	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	HQ621808	1	(0.009)	o1
H42	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	G	C	HQ621809	1	(0.009)	h1

Sampling localities (a, Lugo; b, León; c, Valladolid; d, Álava; e, Madrid; f, Zaragoza; g, Tarragona; h, Toledo; i, Cuenca; j, Ciudad Real; k, Alicante; m, Almería; n, Cádiz; o, Bragança). Numbers (N) and frequency (F) in the total population of the 42 mtDNA CR haplotypes found in *A. rufa* samples. Haplotype positions are aligned with nucleotide no. 1 of the complete mtDNA CR sequence of Alectoris (Randi and Lucchini 1998). Haplotype sequences are available from GenBank: accession nos. HQ621760-770, 773, 776-778, 780-800, 806-809 (Ferrero et al. 2011); AJ586225 (Barbanera et al 2005); AM850842 (Barbanera et al 2009).

Table S2 Mitochondrial pairwise Φ_{PT} values for the fourteen *A. rufa* localities

Cádiz	Lugo	León	Álava	Madrid	Almería	Alicante	Tarragona	Toledo	Ciudad Real	Valladolid	Zaragoza	Cuenca	Bragança	
														Cádiz
0.424														Lugo
0.569	0.073													León
0.345	0.117	0.377												Álava
0.373	0.256	**0.599**	0.133											Madrid
0.087	0.160	**0.434**	0.058	0.149										Almería
0.163	0.215	**0.454**	0.135	0.075	0.009									Alicante
0.491	**0.395**	**0.663**	0.259	0.031	0.316	0.313								Tarragona
0.218	0.053	**0.261**	0.000	0.058	0.000	0.019	0.230							Toledo
0.147	0.237	**0.575**	0.151	0.247	0.000	0.015	**0.455**	0.028						Ciudad Real
0.495	0.018	0.000	0.179	0.388	**0.285**	**0.327**	**0.480**	0.136	0.399					Valladolid
0.375	0.233	**0.577**	0.135	0.000	0.162	0.106	0.140	0.057	0.272	0.360				Zaragoza
0.241	0.277	**0.547**	0.156	0.107	0.000	0.036	0.285	0.081	0.005	**0.393**	0.152			Cuenca
0.214	0.169	**0.564**	0.004	0.000	0.000	0.000	0.160	0.000	0.075	0.317	0.000	0.032		Bragança

After sequential Bonferroni correction, statistically significant pairwise Φ_{PT} values are shown in bold.

Table S3 Nuclear pairwise F_{ST} values for the fourteen *A. rufa* localities

Cádiz	Lugo	León	Álava	Madrid	Almería	Alicante	Tarragona	Toledo	Ciudad Real	Valladolid	Zaragoza	Cuenca	Bragança	
														Cádiz
0.047														Lugo
0.036	**0.037**													León
0.046	**0.020**	**0.034**												Álava
0.053	**0.042**	**0.056**	**0.052**											Madrid
0.033	**0.043**	**0.029**	**0.042**	**0.043**										Almería
0.054	**0.037**	**0.035**	**0.045**	**0.038**	**0.023**									Alicante
0.067	**0.067**	**0.053**	**0.053**	**0.069**	**0.031**	**0.042**								Tarragona
0.050	**0.038**	**0.044**	**0.053**	**0.038**	**0.038**	**0.033**	**0.063**							Toledo
0.040	**0.029**	**0.030**	**0.031**	**0.042**	**0.022**	**0.024**	**0.044**	**0.019**						Ciudad Real
0.047	**0.045**	**0.036**	**0.038**	**0.060**	**0.023**	**0.029**	**0.040**	**0.047**	**0.030**					Valladolid
0.041	**0.042**	**0.024**	**0.026**	**0.041**	**0.025**	**0.020**	**0.037**	**0.038**	**0.014**	**0.019**				Zaragoza
0.050	**0.061**	**0.050**	**0.065**	**0.048**	**0.025**	**0.036**	**0.047**	**0.047**	**0.038**	**0.040**	**0.033**			Cuenca
0.040	**0.036**	**0.028**	**0.039**	**0.037**	**0.026**	**0.031**	**0.040**	**0.031**	**0.026**	**0.032**	**0.020**	**0.031**		Bragança

After sequential Bonferroni correction, statistically significant pairwise F_{ST} values are shown in bold.

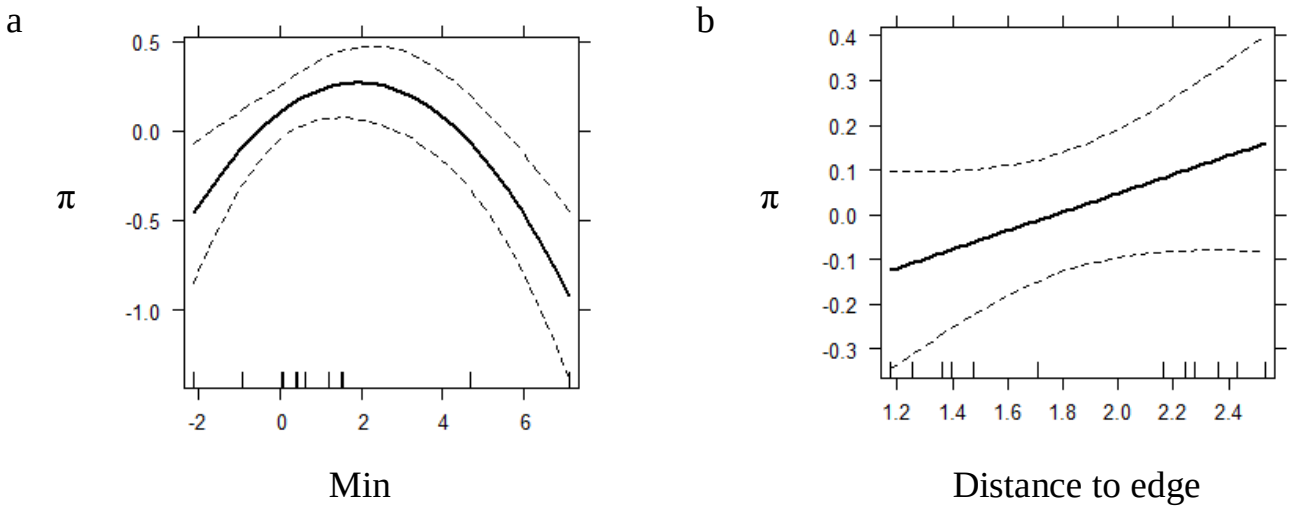


Fig. S1 Relationship between nucleotide diversity and variables selected. π - nucleotide diversity, MinT - average minimum temperature of the coldest month. Bars represent 95% confidence intervals of estimated marginal means.

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DISCUSIÓN GENERAL

Nivel molecular

En esta tesis se han desarrollado marcadores con alelos diagnóstico para tres especies de *Alectoris*: *Alectoris rufa*, *A. graeca* y *A. chukar*. A diferencia de marcadores genéticos utilizados previamente, los aquí presentados no necesitan modelos estadísticos para detectar hibridación introgresiva en poblaciones de perdiz y son más eficaces en la identificación de híbridos. Además estos marcadores carecen de las restricciones del ADN mitocondrial y de marcadores dominantes como los RADPs, y han sido testados en tres conjuntos de muestras de referencia, uno de ellos con una amplia distribución y tamaño muestral procedente de individuos de museo.

El desarrollo de estos marcadores diagnóstico de ADN creemos que es de importancia al entrar en el campo de la investigación aplicada a la conservación de especies, fin deseable para todo trabajo de investigación. Además estos marcadores podrían ser patentables y de gran utilidad en un servicio de identificación de especies cinegéticas en el género *Alectoris*.

El nivel de introgresión de perdiz chukar en perdiz roja ha sido subestimado debido a las restricciones que poseían los marcadores utilizados previamente (genoma mitocondrial, microsatélites no diagnóstico). Esta situación ha sido revelada al utilizar marcadores codominantes con alelos diagnóstico, detectando estos últimos un mayor porcentaje de introgresión y una mayor extensión de la misma en distintas localidades, siendo la incidencia mayor en localidades de granja respecto a campo. Esto nos permite afirmar que las granjas se han convertido en un enjambre de híbridos, siendo la principal fuente de híbridos para las poblaciones silvestres de perdiz.

Aunque nuestro conjunto de marcadores es una herramienta valiosa para identificar a nivel poblacional hibridación introgresiva, mediante la fórmula desarrollada por Halbert *et al*. (2005) hemos estimado el posible poder de detección de nuestros marcadores. Asumiendo que nuestros marcadores son autosómicos y que las poblaciones a analizar son enjambres de híbridos, sería necesario analizar entre 8 y 16 individuos para detectar introgresión con un 99.9%, y así poder afirmar que esa población es no tiene introgresión. Para poder decir con certeza que un individuo es puro, sería necesario analizar entre 110 y 220 marcadores. En consecuencia serían necesarios analizar más marcadores para poder afirmar con certeza la pureza de un individuo, sobretodo en poblaciones de campo, donde no tenemos enjambres de híbridos, y si encontramos poblaciones heterogéneas con toda variedad de individuos. Hace 10 años, este número elevado de marcadores sería excesivo e impracticable, por el contrario ahora con él avance de las nuevas técnicas de búsqueda de marcadores (secuenciación masiva) y de análisis de polimorfismos mediante SNPs (mediante el sistema de múltiples reacciones de SNaPshot®, microarrays), podría ser factible. De este modo, se observa un cambio de tendencia en cuanto al uso de marcadores de ADN, en contra de los microsatélites y a favor de los SNPs, debido a la rapidez de análisis en el laboratorio, evitando así el lastre de los microsatélites, es decir, alelos compartidos y homoplasia.

A pesar del manejo intenso en la perdiz roja, hemos encontrado localidades en las que no hemos detectado señales de hibridación. Estas localidades son de gran valor desde el punto de vista de conservación (ver a continuación la sección de conservación en este mismo apartado) e investigación en la especie.

Desde el punto de vista de investigación básica, en la perdiz roja hemos encontrado estructuración genética, dividida en cinco grupos de poblaciones: suroeste,

central-este, noroeste, Baleares y Francia-Italia. Esta división se obtiene con el marcador mitocondrial y marcadores nucleares. La falta de resolución en nuestros análisis filogenéticos podría ser por una baja divergencia en el ADN mitocondrial dentro de la especie (Posada y Crandall 2001).

Debido al intenso manejo de esta especie y al efecto de las sueltas de enmascarar el verdadero estatus genético de las perdices de campo y sus poblaciones, el estudio de filogeografía ha sido uno de los más complejos de diseñar. La elección de las localidades ha supuesto un importante esfuerzo de muestreo, debido a que se quería conseguir localizaciones representativas y con un número de muestras mínimo. Posteriormente, el trabajo de laboratorio ha sido no menos importante ya que al aparecer un híbrido en las poblaciones seleccionadas, la población a la que pertenecía ese híbrido era descartada para este estudio. Por todo ello ha resultado tedioso obtener unas localidades representativas del rango de distribución de la perdiz roja.

Mediante un enfoque complementario y con gran parte de las localidades utilizadas en el estudio de filogeografía, hemos realizado un estudio ecológico relacionado con los datos obtenidos de diversidad y diferenciación genética. Ha aparecido una interesante influencia de la temperatura (mínima y anual) en los patrones de diversidad genética mitocondriales, así como en ambas distancias genéticas (mitocondrial y nuclear). En base a estos resultados, la subida de la temperatura podría ocasionar una pérdida de diversidad genética en las localidades de perdiz roja, afectando negativamente.

Las sueltas de perdiz roja han afectado a la integridad genética de las perdices silvestres y han diluido la actual estructura genética de las poblaciones, a causa del flujo genético artificial que se ha producido hacia las poblaciones naturales. En trabajos de estructuración genética en perdiz roja, incluyen localidades en las que han encontrado

híbridos mitocondriales (excluyendo únicamente los híbridos en posteriores análisis), de este modo es probable que también incluyan en sus análisis localidades con perdices de suelta, no autóctonas de la zona de muestreo, y en consecuencia en sus resultados observan una dilución de la estructura genética (Barbanera *et al*. 2010; Rodríguez-García y Galián 2014).

Mientras que los microsatélites han sido ampliamente utilizados para resolver un amplio rango de cuestiones biológicas, parece que en un futuro próximo, los polimorfismos de una única base (SNPs) reemplazaran a los microsatélites, debido a las nuevas técnicas de secuenciación (Davey et al 2011) que facilitan la búsqueda de polimorfismos y su genotipado. (mapeo genético, microarrays, secuenciación masiva).

Conservación

Con los datos obtenidos en esta tesis, se aprecia claramente que el problema de la hibridación introgresiva en la perdiz roja aumenta en magnitud y tiene serias consecuencias en nuestras poblaciones silvestres. Todo esto ocurre a pesar de leyes nacionales e internacionales que prohíben la suelta de híbridos desde 1975. ¿Esto por qué sucede? Falta de implicación a nivel gubernamental puede ser una de las causas. Otra se correspondería con la carencia de marcadores eficaces, marcadores diagnóstico codominantes. Los marcadores hasta ahora disponibles han podido ser utilizados para detectar híbridos (subestima) y así tratar de cribar las granjas de perdiz, eliminando individuos híbridos. Pero creemos que las granjas son enjambres de híbridos y no sería suficiente eliminar solo los individuos híbridos, ya que en una granja con indicios de

introgresión todas las perdices son híbridas, siendo los individuos restantes también híbridos, esto se vislumbra al ser analizados con más marcadores.

La solución adecuada al problema de la hibridación sería cerrar granjas con introgresión. Esta solución quizás no sería viable desde un punto de vista económico. Una posible solución a este problema sería crear granjas de referencia a nivel regional, a partir de perdices de campo sin introgresión. Con estas perdices se renovarían por completo aquellas granjas con algún indicio de introgresión. Posteriormente, estas perdices serían utilizadas para realizar sueltas, previos estrictos controles genéticos y sanitarios. Ante cualquier irregularidad en una granja, ésta se cerraría.

La perdiz roja es un tema complejo, ya que la opción planteada podría solucionar el serio problema de las sueltas, pero éstas no han causado el declive de abundancia en esta especie. Las alteraciones en su hábitat (destrucción de hábitats, agricultura intensiva, utilización de fungicidas; Blanco-Aguiar *et al*. 2004) son otro gran problema. Por lo tanto hasta que no se solucionen los aspectos ambientales que impiden el desarrollo de esta especie, nada será de utilidad para aumentar la abundancia de perdices, ni siquiera las sueltas (Díaz-Fernández *et al*. 2013).

Planteamos una serie de estrategias para esta compleja cuestión:

-Conservación de hábitats.

- Cribar las granjas de perdices y soltar perdices sin introgresión.

- Anillar las perdices de suelta.

- Control genético y sanitario.

- Medidas de gestión.

- Monitorización.

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CONCLUSIONES

Los microsatélites diseñados en esta tesis específicamente para *A. rufa* son polimórficos y adecuados para estudiar la variabilidad y estructuración genética en esta especie. También amplifican en otras especies como *A. chukar*, *Gallus gallus* y *Perdix perdix*, siendo polimórficos en la mayoría de los loci analizados.

Los marcadores seleccionados con alelos diagnóstico para tres especies de *Alectoris*: *Alectoris rufa*, *A. graeca* y *A. chukar*, son adecuados para detectar introgresión de perdiz chukar en poblaciones de perdiz roja y de perdiz griega. De igual manera identifican introgresión de perdiz roja en perdiz griega. Este estudio tiene su aplicación en conservación y diagnóstico de especies.

Los marcadores diagnósticos no han detectado introgresión de perdiz griega en perdiz roja en las localidades de campo y granja analizadas.

El nivel de introgresión de perdiz chukar en perdiz roja, en las localidades analizadas de España y Portugal, es más elevado y ocupa una mayor extensión geográfica que lo mostrado en estudios previos.

Todas las granjas de perdiz analizadas han presentado introgresión de perdiz chukar en perdiz roja y en porcentaje mayor respecto a localidades de campo, confirmando la erosión de la calidad genética en las poblaciones silvestres como consecuencia de las sueltas de perdices.

La aplicación de estos marcadores diagnóstico ha permitido identificar localidades sin señales de introgresión, siendo de gran valor para la conservación e investigación en esta especie.

La estructura y diferenciación genética de la perdiz roja se ha visto afectada en gran medida por las glaciaciones del Pleistoceno. Encontrando evidencias de que durante el Pleistoceno la península Ibérica actuó como un refugio con numerosos sub-refugios en su interior. La posterior expansión demográfica y diferenciación genética ha producido en la especie la diferenciación de cinco grupos de poblaciones.

La temperatura es el principal factor climático asociado a los patrones de diversidad genética mitocondrial, así como a la diferenciación genética (a nivel nuclear y mitocondrial) en la perdiz roja en la península Ibérica.

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