APLICACIÓN DE LOS CONCEPTOS DE LA GENÉTICA DEL PAISAJE

A LA CONSERVACIÓN DE LAS AVES DE MONTAÑA

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1 Introducción

1 INTRODUCCIÓN

1.1 Importancia ecológica de las montañas

Las montañas son sistemas ecológicos dinámicos, complejos y de gran interés en conservación, ya que albergan una parte muy relevante de la diversidad biológica en todo el Mundo (Sala *et al.* 2000) (Körner & Spehn 2002, Rodríguez-Rodríguez *et al.* 2011). La complejidad orográfica y ambiental, junto con los usos culturales, han modelado un paisaje altamente heterogéneo y fragmentado. En este sentido, el aislamiento de las poblaciones vegetales y animales ha contribuido al desarrollo de procesos de diferenciación genética y especiación que se relacionan con la aparición de endemismos y con la existencia de una riqueza biológica excepcional (de Bello *et al.* 2010, García-Llamas *et al.* 2019a). Una característica común a todos los sistemas montañosos es su fragilidad y vulnerabilidad ante los cambios ambientales, tanto climáticos como de uso del territorio (Nogués-Bravo *et al.* 2007). Por ejemplo, en Europa, las zonas de montaña han sufrido en las últimas décadas profundos cambios en cuanto a las prácticas agroganaderas, la explotación de sus recursos naturales, el desarrollo de infraestructuras de ocio y el cambio climático. Aún así, los hábitats de montaña siguen manteniendo la mayor parte de los ecosistemas protegidos por la legislación europea (Directiva Hábitats 1992/43/CEE), a la vez que proveen a la sociedad de una notable variedad de servicios ecosistémicos (Harrison *et al.* 2010, García-Llamas *et al.* 2019b, Martín-López *et al.* 2019), tales como recursos naturales, culturales y recreativos. La demanda de servicios proporcionados por los hábitats de montaña ha sufrido profundos cambios en las últimas décadas (Morán Ordóñez *et al.* 2013). Esta situación es un reflejo de importantes cambios en las necesidades y preferencias de la sociedad que, en el caso de los países europeos, se han traducido en una disminución generalizada de la demanda de recursos relacionados con la provisión de combustible y con las prácticas agroganaderas, así como en un aumento de la demanda de valores recreativos y culturales (Wessel *et al.* 2004).

Las montañas de la península Ibérica presentan algunas particularidades más, como el hecho de situarse a caballo entre las dos principales regiones biogeográficas del sur de Europa, lo que supone que alberguen tanto especies de origen Mediterráneo como Eurosiberiano (García-Llamas *et al.* 2018). Además, estas montañas son límite de distribución actual para muchas especies animales y vegetales y han servido como refugio durante las últimas glaciaciones. Estas circunstancias las convierte en escenarios ideales para el estudio de la diversificación genética y fenotípica como origen de los procesos de especiación. El estudio de estos procesos es fundamental para el diseño de políticas de conservación que aseguren a largo plazo la diversidad genética y la resiliencia de las especies ante los cambios ambientales en el futuro.

1.2 Genética y conservación: Genética del Paisaje

La Genética surgió como disciplina en el siglo XIX, pero su aplicación en la preservación de las especies silvestres ocurrió mucho después. De hecho, no fue hasta finales del siglo XX cuando, en el marco multidisciplinar de la Biología de la Conservación (Soulé 1985), emergió como un componente imprescindible para el planteamiento de medidas de conservación en un escenario de paulatina degradación ambiental. La Genética se convirtió entonces, junto con la Ecología y la Biogeografía entre otras disciplinas, en un pilar básico para el establecimiento de las bases científicas necesarias para definir estrategias futuras de conservación en una carrera contra la pérdida de biodiversidad (Meffe & Carroll 1997).

Paralelamente al desarrollo de esta disciplina, y tras siglos de debate, el concepto de especie ha ido variando, desde el concepto tipológico de Linneo, al concepto biológico de Mayr y, más actualmente, a un concepto filogenético y evolutivo (Pigliucci & Kaplan 2006). En ese contexto, la Genética ha sido un elemento clave para la clasificación sistemática de la naturaleza, permitiendo inferir relaciones filogenéticas entre especies anteriormente impensables y descartar algunas otras relaciones asumidas. En el campo de la conservación, el concepto de especie presenta una gran relevancia social, sanitaria y legal a la hora de definir estrategias de manejo, ya que éstas normalmente se basan en las categorías taxonómicas de especie y subespecie para establecer prioridades en las políticas de conservación y, por tanto, para decidir el reparto de los recursos personales y materiales destinados a implementar esas políticas. La importancia del concepto de especie como marco de actuación se refleja en la aparición de conceptos cruciales en conservación, como las "especies indicadoras", aquellas que reflejan el estado general de conservación de los ecosistemas y paisajes, "especies bandera" aquellas especies carismáticas que actúan como referentes en campañas de conservación o "especies paraguas", aquellas especies con grandes áreas de campeo cuyo mantenimiento asegura la conservación de otras muchas especies y sus hábitats.

Tradicionalmente, la pérdida de diversidad genética ha sido un tema de debate recurrente en los estudios científicos de conservación del medio natural. Las actividades antrópicas se han considerado como aspectos determinantes de esta diversidad ya desde el comienzo de la aplicación de la Genética en Ecología (Frankel 1970, Frankel & Soulé 1981). Con el tiempo, la importancia del mantenimiento de la diversidad genética ha ido trascendiendo fuera de la comunidad científica y, actualmente, es asumida por toda la sociedad. Así, en la legislación internacional y nacional se considera claramente la necesidad de preservar la diversidad genética para conservar los valores naturales. Por ejemplo, una de las leyes básicas de conservación ambiental en España, la Ley 42/2007, de 13 de diciembre, del Patrimonio Natural y de la Biodiversidad, recoge en su preámbulo la necesidad de preservar los valores genéticos al mismo nivel que los procesos naturales, la diversidad biológica o el paisaje.

Conservar la diversidad genética significa entonces aumentar la viabilidad de las poblaciones en el paisaje, en concreto su capacidad de supervivencia y adaptación frente a los cambios (Frankham *et al.* 2002). Dado que la población es la unidad ecológica y evolutiva básica, plantear estrategias de conservación únicamente a mayores niveles (especie, ecosistema, paisaje) puede tener como resultado una pérdida efectiva de diversidad genética (Meffe & Carroll 1997). Además, el hecho de que la mayoría de las especies habitan en paisajes heterogéneos determina que, en los estudios orientados a diseñar estrategias de conservación, se deba evaluar explícitamente la variabilidad ambiental del territorio y cómo esta es modelada por las actividades humanas. En este sentido, la Ecología del Paisaje permite la realización de estudios de análisis espacial del paisaje con el objetivo de comprender de qué forma la heterogeneidad espacial es modelada por factores ambientales y antrópicos, y como afecta a los patrones de distribución y abundancia de los organismos (Forman 1995).

En la actualidad, la preservación de la variabilidad genética es uno de los principales desafíos en el campo de la conservación. A lo largo del siglo XX han ido surgiendo distintas disciplinas científicas en torno al reto de conservar la diversidad, que han ido integrando la Genética como parte fundamental en la definición de estrategias de conservación de la diversidad biológica. Algunas de ellas se han centrado en la aplicación de técnicas moleculares para la resolución de preguntas

biológicas y ecológicas, como la Ecología Molecular y la Genética de Poblaciones y otras, se han orientado a buscar soluciones a los problemas de la conservación de las especies con la ayuda de técnicas genéticas, como la Genética de la Conservación y la Genética del Paisaje.

En el año 2003 emerge la Genética del Paisaje (Manel *et al.* 2003) como resultado de la fusión entre la Genética de Poblaciones y la Ecología del Paisaje, con la finalidad de comprender la interacción entre las características del paisaje y los procesos microevolutivos específicos (flujo génico, deriva génica y selección natural). Además de este objetivo principal, a lo largo de su evolución han ido apareciendo nuevos retos, tales como la cuantificación de la heterogeneidad del paisaje (Holderegger & Wagner 2006) o la implementación de nuevas herramientas estadísticas para analizar las relaciones entre el paisaje y la genética (Cushman *et al.* 2006, Storfer *et al.* 2007). La Genética del Paisaje ha permitido reducir la brecha entre la Genética de Poblaciones y la Ecología del Paisaje, al analizar el problema de la pérdida de viabilidad genética de manera integral. De hecho, ha supuesto un salto cualitativo desde los estudios descriptivos acerca de las relaciones entre el paisaje y los datos genéticos a modelos que permiten predecir las consecuencias genéticas de los cambios ambientales (Wasserman *et al.* 2012). Las disciplinas en las que se apoya la Genética del Paisaje están bien asentadas desde hace décadas y han funcionado de manera individual o conjunta, pero era necesaria la aparición de esta disciplina para que esa integración de realizara de forma efectiva apoyada en una sólida base teórica. En una reciente reinterpretación de la concepción de la Genética del Paisaje realizada por Balkenhol *et al.* (2016), se define esta disciplina como una técnica de investigación que combina la Genética de Poblaciones y la Ecología del Paisaje con técnicas de análisis espacial para cuantificar

explícitamente los efectos de la composición del paisaje, su configuración y calidad de la matriz en procesos microevolutivos como el flujo génico, la deriva génica y la selección usando información genética neutral y adaptativa.

Desde el nacimiento de la disciplina se planteó, como uno de sus principales objetivos, la obtención de información relevante para el manejo y la conservación de las especies estudiadas (Holderegger & Wagner 2008). La aportación de la Genética del Paisaje a la conservación de los valores naturales estriba en el hecho de que permite evaluar el efecto de las barreras físicas naturales y de las actividades humanas sobre la dispersión de los individuos, así como determinar el efecto del uso del territorio como potencial fuerza evolutiva (Frankham *et al.* 2002). Además, se ha convertido en una herramienta de gestión del territorio, ya que permite evaluar la conectividad entre las poblaciones, identificar unidades evolutivamente significativas (Ryder 1986, Moritz 1994) y contribuir al diseño de políticas de conservación que fomenten el intercambio genético a través de corredores ecológicos y el mantenimiento de la diversidad genética.

En los estudios de Genética del Paisaje, tradicionalmente se han usado marcadores neutrales, no afectados por procesos de selección natural, para determinar las características genéticas, aunque cada vez es más habitual el uso de marcadores adaptativos (Wang 2013). Los marcadores neutrales están especialmente indicados para determinar el impacto de la variación de la estructura del paisaje en la conectividad genética y predecir cambios futuros. Habitualmente, se utilizan secuencias de ADN como microsatélites o SSR (*simple sequence repeat*) o STR (*short tandem repeat*), neutros y codominantes (en un 70% de los estudios), DNA mitocondrial y de cloroplastos, AFLP (*Amplified fragment length polymorphism*) y SNP (*single nucleotide polymorphism*). Los microsatélites son los más

ampliamente utilizados porque presentan varias ventajas. Además de ser marcadores neutrales, es posible aislarlos a partir de muestras diversas como pelos o plumas y por tanto, evitan técnicas intrusivas. Además pemiten caracterizar genéticamente las poblaciones, determinación la existencia de cuellos de botella, hibridación o introgresión, entre otros. También presentan algunos inconvenientes como la necesidad de caracterizar microsatélites específicos para cada especie objeto de estudio.

1.3 Paisaje, flujo génico y diferenciación fenotípica

El flujo génico es la incorporación de genes de una población en el pool genético de otra población. Se produce cuando un ejemplar nacido en una población se desplaza hasta otra, donde se reproduce, o cuando un ejemplar se reproduce en áreas diferentes. Ese flujo de genes depende entonces de los movimientos de dispersión, que están condicionados a su vez por factores tan diversos como la capacidad de desplazamiento, el comportamiento o el paisaje. La fragmentación del paisaje condiciona el desplazamiento de los individuos y, por tanto, influye en el intercambio de material genético entre las distintas áreas de reproducción. La existencia de niveles adecuados de flujo génico en las poblaciones evita la endogamia y la pérdida de eficacia biológica (*fitness*) asociada a la reducción de heterocigosidad que se produce por azar en las poblaciones pequeñas (Frankham 2005). Por tanto, el flujo génico permite mantener niveles propicios de diversidad genética y disminuye el riesgo de extinción. Además, tiene un indudable efecto sobre los procesos ecológicos y puede influir en aspectos como la capacidad de adaptación de las poblaciones a nivel local, la tasa de extinción, la diferenciación genética o la evolución en los procesos de aislamiento reproductivo, entre otros (Whitlock & McCauley 1999).

La Genética del Paisaje persigue determinar cuál es el efecto del paisaje en el movimiento de genes, teniendo en cuenta condicionantes tales como la distancia geográfica, las tasas de dispersión, las estrategias de reproducción, la existencia de barreras y la matriz de paisaje. Estos son los factores fundamentales que determinan la cantidad de ejemplares que se desplazan entre poblaciones y, por tanto, que condicionan el trasvase de genes (Storfer *et al.* 2010). De hecho, buena parte de los trabajos de Genética del Paisaje se basan en la cuantificación del flujo génico entre pares de poblaciones y la influencia que el paisaje tiene sobre ese flujo (ver Spear *et al.* 2005 y Epps *et al.* 2007). Así, una distancia de unos pocos kilómetros o una discontinuidad en el hábitat causada por la presencia de un río, una carretera o una cordillera pueden resultar infranqueables para ciertas especies de plantas, micromamíferos o reptiles, mientras que pueden no suponer ninguna barrera para el desplazamiento de grandes mamíferos y, menos aún, de aves. Precisamente, en el caso de las aves, dado que tienen una alta capacidad potencial de desplazamiento, existen ciertas características relacionadas con el comportamiento, la dispersión y el tamaño de población que pueden influir de manera notable en la dispersión reproductiva (Garant *et al.* 2005, Irwin 2005). Por otra parte, puede producirse intercambio de genes entre poblaciones entre las cuales no existe intercambio directo de ejemplares. Esto ocurre cuando existen poblaciones intermedias no muestreadas, conocidas como poblaciones fantasma, que intercambian ejemplares entre las poblaciones analizadas (Slatkin 2005). Por ejemplo, se detecta flujo génico entre las poblaciones A y B, pero realmente no existe intercambio de ejemplares entre ellas sino que existe una población fantasma C que comparte ejemplares con A y con B. A pesar de la gran utilidad que pueden presentar los estudios de Genética del Paisaje para evaluar, predecir y mitigar los

efectos del cambio antropogénico del paisaje sobre las aves (Kozakiewicz *et al*. 2018), aún existen pocos estudios centrados en este grupo.

Los movimientos dispersivos presentan entonces una importancia clave en el intercambio de genes entre poblaciones o localidades (Moore & Dolber 1989) que resultan en la reproducción entre ejemplares de poblaciones diferentes. Sin embargo, la migración, entendida como movimiento estacional, no necesariamente supone un intercambio de genes, aunque pueda suponer el encuentro de ejemplares de diferentes poblaciones en las áreas de invernada o reposo durante los viajes migratorios. El fenómeno contrario a la dispersión reproductora es la filopatría, es decir, la fidelidad a las zonas de reproducción. El grado de filopatría depende de cuestiones variadas como el sexo, la edad o el éxito en la reproducción, entre otros (Greenwood 1980, Payne & Payne 1993, Wiklund 1996).

Otro factor que puede influir en el flujo génico es el tamaño de la población. Así, a igualdad de condiciones ambientales entre diferentes áreas, las poblaciones más grandes tienden a trasmitir los genes con mayor probabilidad que las poblaciones más pequeñas. En este sentido, es necesario tener en cuenta el concepto de tamaño de población efectiva, ya que no todos los ejemplares de una población tienen la capacidad de trasmitir los genes, al variar la fecundidad en función de factores como la edad o la capacidad de reproducción. El tamaño de población efectiva es un parámetro que refleja cambios en la dinámica de poblaciones y su valor es inversamente proporcional a la pérdida de diversidad genética por deriva génica. Se considera como una herramienta clave en conservación dada su capacidad para predecir pérdidas en diversidad genética, endogamia y diferenciación genética (Weckworth *et al*. 2013).

Los fenómenos de divergencia genética promovidos por el flujo génico, el paisaje y la dispersión están estrechamente relacionados con la diversidad fenotípica. La diversidad fenotípica es una característica universal en las poblaciones naturales que afecta a caracteres de diverso tipo, como morfológicos (por ej. tamaño y morfología corporal, morfología espermática) y de comportamiento (por ej. estrategia de migración, dispersión, filopatría, canto). Estas diferencias fenotípicas pueden deberse a fenómenos de selección natural y sexual, procesos de adaptación a condiciones ambientales (Mayr 1956) o procesos de deriva génica asociados a aislamiento geográfico. La divergencia fenotípica puede promover el aislamiento reproductivo y acelerar los procesos de especiación. Habitualmente, los estudios de diversificación macroevolutiva se han centrado en características morfológicas, quedando infrarrepresentadas rasgos comportamentales o culturales (Wilkins *et al*. 2013).

La diversificación y la capacidad de adaptación a los cambios ambientales se relacionan con fenómenos como la plasticidad fenotípica y la variación genética (Ackerley 2003). En relación con el hábitat, la plasticidad fenotípica podría resultar una estrategia adecuada en ambientes cambiantes, dado que las presiones selectivas varían con frecuencia (Millien *et al*. 2006). Por el contrario, en ambientes estables, las fuerzas selectivas favorecerán características genéticas concretas que fomentarían el proceso de especiación (Rueffler *et al*. 2006). Además, estos procesos de diferenciación estarán influidos por el flujo génico entre las distintas poblaciones, de forma que el aislamiento y la fragmentación del hábitat supondrá un menor intercambio de genes entre ellas. De acuerdo con esto, la capacidad de dispersión, la filopatría y el grado de fragmentación del hábitat determinarán en gran medida el flujo génico y, por tanto, el grado de diferenciación (Slatkin 1985). El estudio de los patrones espaciales de diferenciación fenotípica puede ayudar a descubrir procesos de diferenciación intraespecífica y relacionarlos con procesos de diferenciación genética.

2 El pechiazul

2 EL PECHIAZUL COMO ESPECIE MODELO DE ESTUDIO

Para alcanzar el objetivo principal de analizar cómo la heterogeneidad del paisaje influye en el intercambio genético entre poblaciones, los estudios de Genética del Paisaje han de tomar como modelo especies que presenten un buen grado de variabilidad genética y fenotípica, así como capacidad de respuesta en función de los atributos del paisaje (Bakenhol *et al.* 2016). Por ello, en esta tesis se ha elegido como especie modelo al pechiazul *Luscinia svecica* (Linnaeus, 1758), un ave paseriforme con una extensa distribución en el Paleártico, que presenta una variabilidad genética (Johnsen *et al.* 2006) y fenotípica (Arizaga *et al.* 2006) muy acusada y nidifica en parches de matorral a lo largo de un amplio gradiente ambiental y climático (Geslin *et al.* 2002).

Otro aspecto relevante para elegir la especie objeto de estudio es su estatus de conservación. En este sentido, la preservación de la diversidad genética es uno de los retos en la conservación de los espacios naturales y de las especies amenazadas. En este sentido, el pechiazul está recogido en la legislación de la Unión Europea en el grupo de las especies que deben recibir más esfuerzos en cuanto a su conservación (Directiva Aves 2009/147/CE). Por otra parte, habita en áreas de montaña que, actualmente, representan un refugio para multitud de especies y hábitats singulares amenazados (Directiva 92/43/CEE). En la península Ibérica, su hábitat principal incluye distintas formaciones de matorral, algunas de ellas con un grado de protección elevado, como brezales, callunares y piornales, que comparten algunos de los endemismos más notables de la fauna y flora. Por ello, los resultados de los estudios realizados en esta tesis sobre el pechiazul podrán ser considerados como una contribución científica útil para el diseño de estrategias de conservación adecuadas para otras especies con las que comparte sus hábitats en las montañas ibéricas.

Hasta la fecha, los estudios realizados sobre las poblaciones reproductoras ibéricas se han limitado a aspectos biométricos (García *et al.* 2000, Campos *et al.* 2005), genéticos (Zink *et al.* 2003, Johnsen *et al.* 2006) o relacionados con cuestiones generales de su biología (Arizaga & Alonso 2015), pero no se han abordado estudios integrales desde el punto de vista de la Genética del Paisaje que permitan un mejor conocimiento de los efectos de la heterogeneidad del paisaje sobre los patrones de diferenciación genética y fenotípica de las poblaciones.

2.1 Características generales y posición taxonómica.

El pechiazul *Luscinia svecica* es un ave paseriforme migrante de pequeño tamaño (longitud: 13-15 cm; peso: 14-20 g) que, como reproductor, se distribuye por el oeste y norte del Paleártico (Gladkov 1968, Glutz von Blotzheim & Bauer 1994, Cramp 1988, Meijer & Stastny 1997, Clement & Rose 2015) con una población reproductora de 880.000-2.400.000 parejas (BirdLife International 2004). Es un ave amenazada en Europa, por lo que está incluida en el Anexo I de la Directiva Aves 2009/147/CE, que es una de las normativas de referencia en la declaración de Zonas de Especial Conservación y Zonas de Especial Protección para las Aves (ZEPA) en la Red Natura 2000, la red ecológica impulsada por la Unión Europea. Este taxón fue denominado inicialmente como *Motacilla svecica* (Linnaeus, 1758). Después, fue englobado en el género *Erithacus* y, hasta mediados del siglo XX, aparece como *Cyanosylvia svecica* (ver por ej. Corley-Smith 1959). A partir de ese momento, la denominación más ampliamente aceptada (Cramp 1988) es la de *Luscinia svecica,* que se mantiene hasta la actualidad (Clements *et al.* 2017). Sin embargo, recientemente se ha propuesto un nuevo cambio de género que supondría la denominación de la especie como

Cyanecula svecica (Collar 2017), aunque de momento esta modificación no está ampliamente aceptada.

El pechiazul una especie politípica, con un marcado dimorfismo sexual, en la que se diferencian hasta diez subespecies: *svecica*, *volgae*, *luristanica*, *tianschanica*, *abbotti*, *przevalskii*, *kobdensis*, *azuricollis*, *cyanecula*, *namnetum* y *magna* (Clements *et al.* 2017) de acuerdo a características fenotípicas del plumaje, como el patrón de colores de la garganta y el pecho de los machos (Cramp 1988). De entre ellas, las subespecies más reconocidas (AERC 2003) son: *namnetum*, *cyanecula*, *svecica*, *pallidogularis*, *magna* y *luristanica*. En todo caso, no existe un consenso unánime sobre su clasificación subespecífica, ni están determinadas con claridad sus relaciones filogenéticas (Questiau *et al.* 1998, Zink *et al.* 2003, Johnsen *et al.* 2006, Hellgreen *et al.* 2008). El alto nivel de divergencia fenotípica y genotípica existente entre subespecies y su aislamiento geográfico hacen pensar que el pechiazul se encuentra en una etapa relativamente avanzada del proceso de especiación, comparado con otros complejos subespecíficos (Johnsen *et al.* 2006). Los análisis genéticos muestran dos grupos bien diferenciados: un grupo ancestral, que incluye a las subespecies meridionales, con presencia de mancha blanca en la garganta, y otro norteño, de subespecies con mancha rojiza u ocre en la garganta (Johnsen *et al.* 2006).

En cuanto a sus caracteres fenotípicos, se ha descrito una variación cualitativa en el patrón del plumaje del pecho de los machos en función de origen, así como una variación cuantitativa significativa entre subespecies en tonalidad, color, brillo y reflexión ultravioleta (Johnsen *et al.* 2001). Estas características del plumaje de los machos se consideran determinantes como estímulos en la detección de pareja y en la diversificación intraespecífica (Peiponen 1960, Andersson & Amundsen 1997, Johnsen *et al.*

1998, Johnsen *et al.* 2001). Los pechiazules ibéricos presentan un patrón en el plumaje que les diferencia del resto de las subespecies europeas. Esta circunstancia despertó el interés en la especie por parte de exploradores, principalmente ingleses, que capturaron y clasificaron ejemplares durante sus exploraciones en la Península (Saunders 1871, Chapman & Buck 1893, Witherby 1928) (Figura 0.1).

Ibis. 1928. Pl. XX.

Almanzor, Sierra de Gredos, showing broom bushes in which nested White-spotted Bluethroats.

Figura 0.1. Expedición de Harry F. Witherby por la sierra de Gredos en busca de pechiazules (Witherby 1928).

Abel Chapman y Walter J. Buck advirtieron de estas peculiaridades en el plumaje de los machos en relación con la ausencia de mancha blanca en la medalla. A pesar de ello, las poblaciones ibéricas han sido incluidas hasta fechas recientes dentro de la subespecie de medalla blanca *Luscinia svecica cyanecula* (Clement *et al.* 2013), aunque algunos autores han ido sugiriendo que presentaban suficientes diferencias genéticas y morfológicas como para ser considerada como una subespecie diferenciada, propuesta como *Luscinia svecica azuricollis* (Mayaud 1958, Corley & Bernis 1956, Corley 1959, Zink *et al.* 2003, Johnsen *et al.* 2006, Hogner *et al.* 2013).

Figura 0.2. Macho de pechiazul ibérico.

Además de la ausencia frecuente de medalla blanca en la garganta, otra diferencia importante de los pechiazules ibéricos respecto de los de la subespecie *cyanecula* es el patrón de las bandas negra, blanca y rojiza situadas por debajo del babero azul, siendo la banda rojiza mucho más ancha que en otras subespecies (Chantler 2007, Arizaga *et al.* 2011) (Figura 0.2). La diferenciación genética de las poblaciones reproductoras ibéricas *Luscinia svecica azuricollis* (Rafinesque 1814) se ha evaluado mediante análisis de DNA nuclear (Johnsen *et al.* 2006) y mitocondrial (Hogner *et al.* 2013), que han permitido determinar que los ejemplares ibéricos constituyen un clado monofilético en el árbol de DNA mitocondrial. Por otra parte, se han descrito diferencias de morfología espermática entre las diferentes subespecies europeas, siendo la subespecie ibérica la que presenta un grado de diferenciación más acusado (Hogner *et* al. 2013). Resultados contrarios a estos fueron obtenidos por Zink *et al.* (2003), que no apreciaron diferenciación en las subespecies del sur de Europa, aunque es necesario destacar que, en su estudio, solo utilizaron una única muestra como representación de las poblaciones ibéricas. Por otra parte, en un estudio realizado por Anmarkrud *et al.* (2010) sobre el sistema MHC (complejo mayor de histocompatibilidad) class II B de histocompatibilidad en *azuricollis*, *cyanecula* y *svecica,* no se detectaron diferencias significativas entre las tres subespecies.

Se trata de una especie que realiza movimientos migratorios, aunque con notables diferencias en el patrón migratorio entre poblaciones o subespecies. Los pechiazules europeos invernan en la península Ibérica (Bernis 1963, Bueno 1990, Hernández *et al.* 2003), Italia y el Sahel (Wernham *et al.* 2002). Sin embargo, los pechiazules escandinavos presentan dos zonas de invernada: la zona central del Sahel y Pakistán y el noroeste de la India (Staav 1975, Lislevand *et al.* 2015). Se ha descrito un alto grado de fidelidad, tanto a sus cuarteles de invernada (Markovets & Yosef 2005), como a las zonas de descanso durante la migración (Cramp 1988). La filopatría ocurre también en las zonas de reproducción (Clement & Rose 2015) y así, al menos los machos, mantienen sus territorios de nidificación año tras año (Cramp 1988, Arizaga & García 2013). Esta circunstancia puede tener reflejo en la estructura genética de las poblaciones ibéricas, dado que condiciona el flujo génico entre ellas.

Figura 0.3. Área de reproducción del pechiazul. Portilla de la Reina (León).

2.2. La población reproductora de pechiazul en la península Ibérica.

En la península Ibérica, nidifica de forma exclusiva la subespecie *azuricollis*, estimándose su población reproductora en 9.000 - 12.800 parejas (BirdLife International 2004) distribuidas por los sistemas montañosos del centro y noroeste peninsular: sistema Central (Chapman & Buck 1893, Witherby 1928, Corley Smith & Bernis 1956, Blair 1957, Ern 1966), cordillera Cantábrica (Figura 0.3) y Montes de León (Ern 1966, Tellería *et al.* 1999, Domínguez & Fernández-Cordeiro 1991) y sus estribaciones (García *et al.* 2000) (Figura 0.4). Se ha citado como reproductor, de manera esporádica, en el puerto de Piqueras en La Rioja (Gómez-Manzaneque 2003) y en sierra Nevada (Merino 2010). En Portugal no existe una población nidificante estable, aunque se ha confirmado su reproducción en la serra da Estrela y en Monteshino (Moreira 2008) (Figura 0.5).

El hábitat principal de cría lo constituyen formaciones de matorral como callunares, brezales, piornales y encinares (Tellería *et al.* 1999, García *et al.* 2000, Juan & García 2008). La subespecie se localiza en un área de transición entre las dos zonas bioclimáticas más significativas de Europa, que son la región Mediterránea y la región Atlántica. El clima Mediterráneo es el que aporta mayor singularidad al área de estudio, con veranos secos e inviernos suaves, con las precipitaciones concentradas en primavera y otoño.
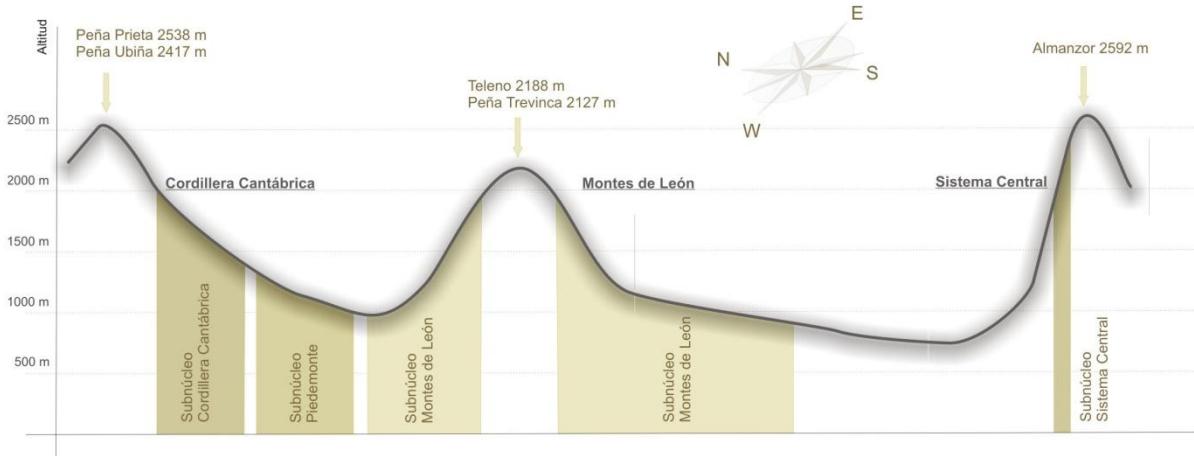
Figura 0.4. Rango de altitud ocupado por el pechiazul *Luscinia svecica azuricollis* en los sistemas montañosos en España.

En España, los datos de densidad en las áreas de cría varían entre 0,1 y 5 machos cantores/10 ha (Arizaga *et al.* 2011, Peris & Mendes 2010). En un estudio específico realizado en 2010, se obtuvo una densidad media de 1,8±0,44 parejas /10ha. Por macizo montañoso, la densidad varía entre 1,7±0,10 parejas/10ha en la cordillera Cantábrica, 2,1± 0,17 en Montes de León, 1,2±0,27 en el piedemonte de los macizos anteriores y 2,1±0,60 en el sistema Central (García 2010).

Figura 0.5. Distribución del pechiazul en España en cuadrículas UTM de 10x10 km. En azul de acuerdo con Martí & del Moral (2003) y en verde, basada en información recopilada en esta tesis.

El núcleo de población de la cordillera Cantábrica se distribuye desde la cabecera del río Sil, en Babia, entre las provincias de Asturias y León, hasta la comarca de Campoo, entre las provincias de Palencia y Cantabria. Las zonas de cría se localizan entre los 1.400 m de altitud sobre el nivel del mar, en las comarcas de Babia y Laciana (León), hasta los 2.000 m en Peña Prieta (León) (García 2010). Su hábitat prioritario lo constituyen formaciones de matorral, como brezales, piornales y callunares, siendo más común en piornales dominados por piorno serrano *Cytisus purgans* acompañado normalmente de otras especies de piornos (*Cytisus* spp, *Genista* spp), brezos (*Erica* spp) y brecinas *Calluna vulgaris*. En general, se trata de formaciones abiertas donde se alternan manchas de matorral con pastizales.

En los Montes de León ocupa la sierra del Teleno y Cabrera, en León, La Carballeda, Sanabria y la sierra de la Culebra, en Zamora, y Cabeza de Manzaneda, en Orense. También alcanza las tierras de relieve suave de las comarcas de La Valduerna y La Valdería, en León, y de Aliste y la Tierra del Pan, en Zamora. En esta zona, la subespecie nidifica en hábitats de fuerte influencia mediterránea, como encinares de escaso porte intercalados con

jarales (*Cistus* spp), en las zonas bajas (Figura 0.6), hasta piornales (*Cytisus* spp, *Genista* spp) y brezales (*Erica* spp), en las zonas de mayor altitud. La altitud en este núcleo varía entre los 733 m en Almaraz de Duero (Zamora) y los 2000 m en las sierras de La Cabrera y del Teleno. En los páramos detríticos de las provincias de León y Palencia, desde la comarca leonesa de La Cepeda hasta la comarca de La Valdavia en Palencia, aparecen pequeñas poblaciones. El pechiazul ocupa en este núcleo formaciones de matorral dominadas por brezales (*Erica australis*, *Erica tetralix*, entre otras), piornales (*Cytisus scoparius*, *Cytisus multiflorus*) acompañadas habitualmente por gayuba (*Arctostaphylos uva-ursi*).

Figura 0.6. Área de reproducción en un encinar-jaral en las estribaciones de Montes de León. Bustos (León).

En el sistema Central, la subespecie nidifica en una amplia zona, desde la sierra de Candelario, entre Salamanca y Cáceres, hasta la sierra de Guadarrama, a caballo entre las provincias de Madrid y Segovia y la sierra de Somosierra en Guadalajara, pasando por la sierra de Gredos y la sierra de Piedrahita en Ávila. Su distribución se restringe a los piornales y cambronales serranos dominados por *Cytisus purgans* (Figura 0.7). Estas poblaciones se localizan a una altitud media muy elevada, entre 1.600 m y 2.300 m en la sierra de Candelario (Santamaría *et al.* 2012).

Figura 0.7. Área de reproducción del pechiazul en un piornal en el sistema Central. La Covatilla (Salamanca).

2.3. El paisaje del pechiazul. Usos del territorio.

El paisaje que ocupa el pechiazul en la península Ibérica es un ejemplo de paisaje cultural resultante de complejas interacciones entre las fuerzas de la naturaleza y las actividades humanas. Las continuas perturbaciones asociadas a las prácticas agroganaderas tradicionales (fuego, pastoreo, desbroces, etc.) han permitido el mantenimiento a largo plazo de hábitats sucesionales (Moran-Ordóñez *et al.* 2013). Cuando las actividades agroganaderas cesan, las perturbaciones desaparecen y se inicia un proceso de sucesión ecológica. El progresivo abandono de las prácticas agrícolas y ganaderas tradicionales ha supuesto que muchos hábitats abiertos y, de forma paralela, las especies que los habitan, hayan sufrido declives acusados en las últimas décadas (Fuller *et al.* 1995). Los efectos del abandono de las prácticas tradicionales son muy complejos, dado que suponen una rápida transformación por sucesión secundaria de la vegetación, que deriva en la pérdida de pastizales a favor de matorrales y de matorrales a favor de hábitats forestales bien desarrollados (Glenn-Lewin *et al.* 1992, Gómez-Sal *et al.* 1993). El aumento de la cobertura arbórea y de matorral supone cambios en la composición de las comunidades que utilizan esos paisajes culturales (Suárez-Seoane *et al.* 2002). Por otra

parte, en las zonas donde se mantienen los usos agrícolas y ganaderos (Figura 0.8), las prácticas se vuelven más intensivas y mecanizadas, dando como resultado paisajes más simplificados que los que existían cuando el manejo del territorio se realizaba mediante técnicas tradicionales. Ante esta situación, la preservación de las especies propias de medios abiertos depende de su capacidad para colonizar los nuevos hábitats. Los elevados niveles de fragmentación de estos hábitats (Wiens *et al.* 1994, Wimberly 2006) reducen la posibilidad de dispersión de muchas especies, de forma que se incrementa la diferenciación genética y fenotípica y se erosiona su diversidad genética (Saunders *et al.* 1991).

Sin embargo, la fragmentación de los hábitats en áreas de montaña no solo es un proceso de origen antrópico. Es evidente que la altitud y la orografía conforman unas condiciones ambientales que resultan en paisajes naturalmente heterogéneos. Esta heterogeneidad se refleja en la existencia de una amplia variedad de hábitats que aparecen con una distribución parcheada en fragmentos de mayor o menor extensión y con diferentes grados de conexión estructural y funcional.

En las áreas de menor altitud, el hábitat principal de nidificación de la subespecie lo constituyen encinares degradados por décadas de intenso manejo para la obtención de leña o campos de cultivo, principalmente de cereal, y por el pastoreo de ganado menudo (ovino y caprino). En estas zonas, la mayoría de los campos de cultivo se han abandonado en las últimas tres décadas debido a la disminución de la presión agroganadera, incrementándose la presencia de matorral de encina y jaras. Asimismo, se han abandonado las actividades extractivas de leña para combustible y ha disminuido de forma notable la presencia de ganado doméstico en régimen extensivo, siendo la presencia de ungulados silvestres muy escasa. En general, la dinámica de usos del territorio en las últimas décadas ha supuesto una rápida densificación y

homogeneización de las formaciones de matorral, lo que se asocia con un mayor riesgo de incendios más severos y de mayores dimensiones.

Figura 0.8. Rebaño trashumante de ovejas en un área de reproducción de pechiazul. Ferreras (León).

En las zonas de mayor altitud, las perturbaciones ocurridas en brezales y piornales, hábitats principales para el pechiazul, se asocian con la presencia de ganado (vacuno, ovino, caballar y caprino) y con prácticas agroganaderas tales como quemas y desbroces realizadas con el objetivo de mejorar los pastos (Gómez-Sal *et al.* 1993). Estas áreas han tenido un uso intensivo durante siglos, especialmente vinculado al ganado trashumante que ha modelado el particular paisaje de los puertos de montaña, donde cada año pasaban el verano los rebaños de ganado ovino que en invierno regresaban a zonas bajas de Extremadura. Sin embargo, desde la mitad del siglo pasado, el declive de la trashumancia ha supuesto cambios muy importantes en los usos ganaderos, tales como la sustitución del ganado ovino por vacuno y caballar, la disminución en el uso del fuego para manejo de los matorrales y el aumento del desbroce mecanizado para reducir el riesgo de incendios forestales (Morán-Ordóñez *et al.* 2013). Existe también una mayor presión por parte de la comunidad de ungulados herbívoros constituida principalmente por ciervo *Cervus*

elaphus, cabra montés *Capra pyrenaica*, rebeco cantábrico *Rupicapra parva* y corzo *Capreolus capreolus* (Figura 0.9)*.*

Las principales amenazas para la especie en España están relacionadas con la destrucción y transformación de su hábitat. Las zonas de matorral son consideradas generalmente como áreas no productivas o con escaso interés para la producción de pasto, por lo que habitualmente son quemadas (Gómez-Manzaneque 2003) o desbrozadas con el objetivo de aumentar la superficie dedicada al pasto para el ganado y reducir el riesgo de propagación de los incendios. Por otra parte, en amplias zonas de su área de distribución, se han realizado plantaciones forestales de coníferas que, en pocos años, pueden suponer la desaparición definitiva de la especie. A nivel local, algunos núcleos de población se han visto afectados por el desarrollo de infraestructuras como pistas de esquí (Arizaga *et al.* 2011) y parques eólicos. En cuanto a los efectos del cambio climático, Huntley *et al.* (2007) pronostica la extinción total de la población ibérica nidificante de pechiazul, subespecie *azuricollis,* a finales del siglo XXI, así como de la subespecie *namnetum*, además de su rarefacción en buena parte de su área de distribución europea. Por otra parte, de acuerdo con Wernham *et al.* (2002), el pechiazul, al igual que otros paseriformes que invernan en el Sahel, está amenazado por las sequías que se producen en esa zona de África desde los años 60.

Figura 0.9. Garganta de Gredos (Ávila). Sistema Central.

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3 Objetivos

3 OBJETIVOS

3.1. Objetivo general

El objetivo principal de esta tesis es evaluar la influencia de la heterogeneidad del paisaje en la diversificación genética y fenotípica del pechiazul. El estudio se ha abordado a dos escalas geográficas: europea e ibérica. A escala europea, se analizan las relaciones filogenéticas entre las subespecies que constituyen el complejo subespecífico de *Luscinia svecica*, así como la correlación entre la diferenciación genética y fenotípica. A escala ibérica, se trata de comprender el papel del paisaje en la diferenciación genética y fenotípica ocurrida en las poblaciones nidificantes de la subespecie *azuricollis*. En este sentido, se analizan las relaciones existentes entre el flujo génico y el aislamiento geográfico, la resistencia del paisaje, las adaptaciones locales y los procesos microevolutivos, como la deriva génica. De forma particular, se investiga el efecto de la estabilidad de paisaje de las montañas ibéricas, así como de sus características actuales, sobre la diversidad y estructura genética del pechiazul. Finalmente, se analizan los procesos de diferenciación fenotípica de variables biométricas, morfológicas y de canto, en relación con la diferenciación genética, a lo largo de gradientes ambientales y geográficos. Estos aspectos se desarrollan en cinco capítulos, cuyo contenido se resumen a continuación.

En el capítulo 1*, Divergencia evolutiva en el complejo subespecífico del pechiazul,* se aborda la singularidad de las poblaciones ibéricas de pechiazul en el contexto europeo en cuanto a su diferenciación genética y morfológica. Se analiza la importancia de las poblaciones ibéricas, *Luscinia svecica azuricollis* y se discute la contribución de las nuevas técnicas moleculares a la clarificación de la posición taxonómica de las distintas poblaciones y de qué forma esto afecta al diseño de las políticas de conservación. En el capítulo 2, *Estructura genética local en un*

paseriforme migrante de larga distancia, se realiza una caracterización genética de las poblaciones reproductoras ibéricas más norteñas de pechiazul mediante un genotipado basado en el uso de marcadores genéticos neutrales como son los microsatélites. Se trata de conocer qué variables ambientales han podido contribuir de manera más relevante en la diferenciación y estructura genética de estas poblaciones.

En el capítulo 3, *Análisis de Genética del Paisaje en las poblaciones ibéricas de pechiazul*, se aborda, desde el punto de vista de la Genética del Paisaje, el efecto del aislamiento geográfico y la resistencia del paisaje en la diferenciación genética ocurrida en todo el área de nidificación del pechiazul Ibérico: cordillera Cantábrica, Montes de León y sus estribaciones y sistema Central. Para ello, se analizan las variaciones espaciales de la idoneidad del hábitat mediante la realización de un modelo espacialmente explícito basado en series temporales de datos de Teledetección que informan sobre características del paisaje actual, así como sobre su estabilidad en las últimas décadas, y se evalúa la conectividad funcional del paisaje mediante análisis basados en la teoría de circuitos.

En el capítulo 4, *Patrones de diferenciación morfológica en la subespecie azuricollis a lo largo de gradientes geográficos y ambientales en montañas templadas del sur de Europa*, se analiza la diferenciación de variables biométricas y morfológicas de los pechiazules nidificantes ibéricos a lo largo de gradientes geográficos (latitud y altitud) y ambientales (vegetación y clima), en relación con condicionantes como la diferenciación genética, evaluada mediante marcadores genéticos neutrales, y el aislamiento geográfico. De forma complementaria, se comprueba y discute el cumplimiento de la regla de Bergmann.

Por último, en el capítulo 5, *Factores que influyen en la diversificación del canto del*

pechiazul en la península Ibérica, se aborda el estudio de la relación entre diferenciación genética y fenotípica, pero desde el punto de vista de las señales acústicas emitidas por una especie canora con un canto tan complejo como es el pechiazul. Se analiza el efecto de la calidad del hábitat sobre las características del canto, un carácter sexual secundario relacionado con la competencia entre los machos y la atracción de las hembras y que está considerado como una señal honesta de calidad. Se analiza además el proceso de diferenciación en el canto en distintos grupos genéticos en cuanto a factores estructurales y de complejidad, abundancia de congéneres y riqueza de especies acompañantes.

3.2. Objetivos específicos

Objetivos del capítulo 1

- Evaluar la relevancia y singularidad de las poblaciones ibéricas en el contexto del complejo subespecífico del pechiazul *Luscinia svecica.*
- Determinar la diferenciación biométrica y la correlación entre caracteres genéticos y fenotípicos en algunas de las subespecies euroasiáticas de pechiazul: *azuricollis*, *cyanecula*, *namnetum* y *magna*.
- Caracterizar el plumaje de la subespecie ibérica, *azuricollis*.

Objetivos del capítulo 2

- Investigar la variación genética de las poblaciones reproductoras de pechiazul del norte de la península Ibérica (cordillera Cantábrica y Montes de León) para determinar el alcance de la diferenciación genética a escala local.
- Conocer el efecto de variables ambientales como la altitud y la vegetación en la estructura genética de las poblaciones estudiadas.

Objetivos del capítulo 3

- Evaluar el efecto del paisaje sobre el intercambio genético en el pechiazul ibérico a través de técnicas de modelado espacialmente explícito y Teledetección.
- Valorar los efectos del aislamiento por resistencia actual y estabilidad del paisaje como factores clave en la determinación de los patrones de diferenciación genética
- Determinar el efecto de los cambios en la conectividad del hábitat resultantes de las actividades antrópicas a gran escala en la estructura y la diversidad genética del pechiazul.

Objetivos del capítulo 4

- Determinar los patrones de diferenciación de caracteres biométricos y morfométricos de las poblaciones ibéricas de pechiazul a lo largo de gradientes geográficos y ambientales.
- Evaluar el cumplimiento de la Regla de Bergmann.
- Analizar el papel del aislamiento geográfico y la diferenciación genética en los patrones observados de diferenciación morfológica en las poblaciones nidificantes ibéricas de pechiazul, valorando la contribución de adaptaciones locales y procesos microevolutivos como la deriva génica.

Objetivos del capítulo 5

- Evaluar la variación espacial del canto de los pechiazules ibéricos en cuanto a su complejidad y estructura.
- Analizar el efecto de variables ambientales como la calidad del hábitat, el aislamiento geográfico, la diferenciación genética, el tipo de vegetación y el tamaño del ave en la variación espacial del canto.

3.3. Mapa conceptual de la tesis
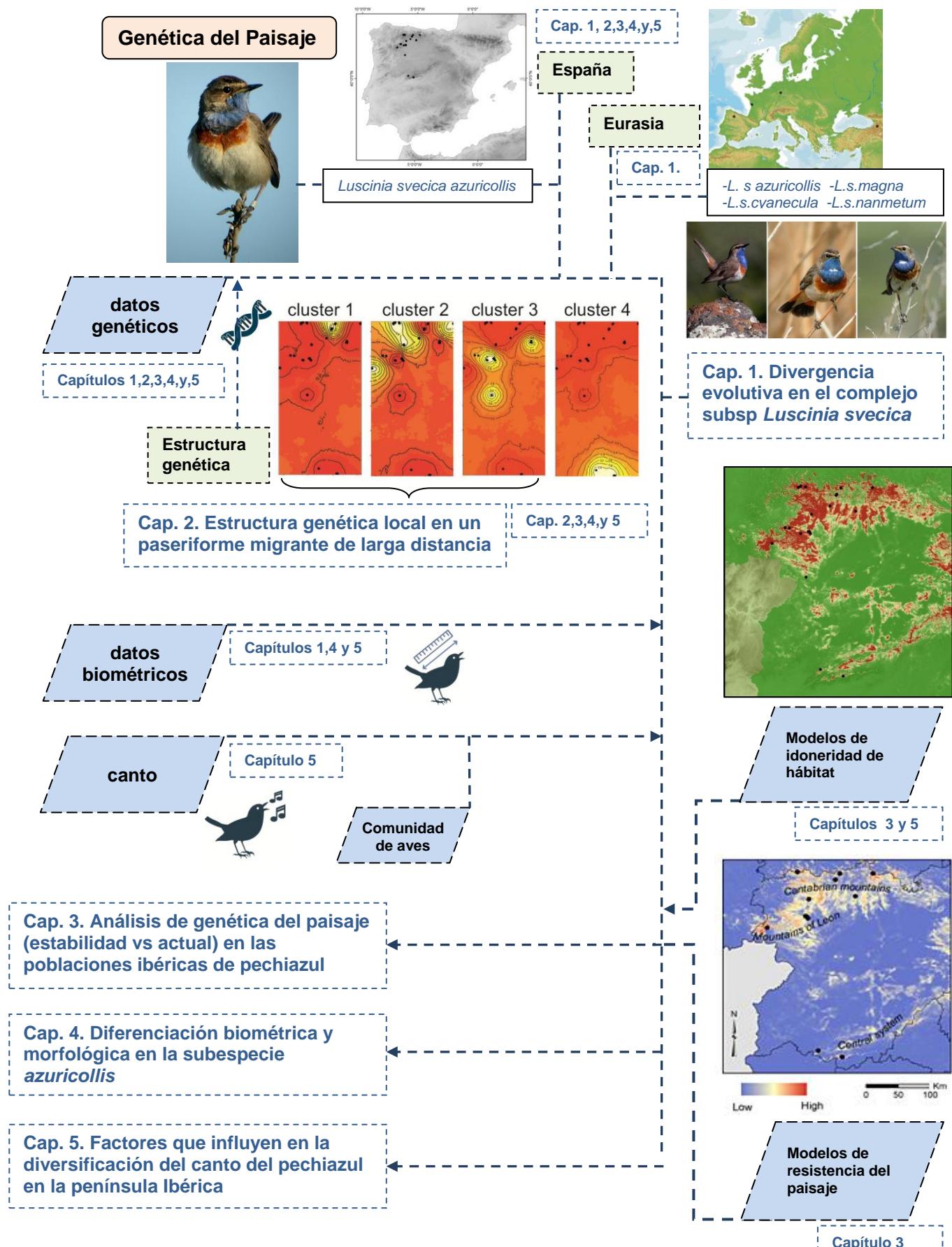
CAPÍTULO 1 Evolutionary divergences in *Luscinia svecica* subspecies complex – new evidence supporting the uniqueness of the Iberian bluethroat breeding populations

CAPÍTULO 2 Local genetic structure on breeding grounds of a long-distance migrant passerine: the bluethroat *Luscinia svecica* in Spain

CAPÍTULO 3 Current landscape attributes and landscape stability in breeding grounds explain genetic differentiation in a long-distance migratory bird

CAPÍTULOS 4 Morphological differentiation patterns of Iberian breeding bluethroat *Luscinia svecica azuricollis* across geographic gradients

CAPÍTULO 5 Environmental fit prevails over chance effects in explaining song differentiation among populations of an upland passerine

4 Capítulos

CAPÍTULO 1

Evolutionary divergences in *Luscinia svecica* subspecies complex – new evidence supporting the uniqueness of the Iberian bluethroat breeding populations.

Abstract

The assessment of evolutionary divergences within subspecies complexes provide an effective short-cut for estimating intraspecific genetic diversity, which is relevant for conservation actions. We explore new evidence supplementing the existing knowledge about the singularity of Iberian bluethroats within the *Luscinia svecica* subspecies mosaic. We compared biometric traits of Iberian males (*L. s. azuricollis*) to the closest subspecies (*L. s. cyanecula*, *L. s. namnetum* and *L. s. magna*) using general linear models and analysed the correlations between biometric and genetic differentiation (based on nuclear microsatellites) among the target subspecies with a Mantel test. Biometric differences were calculated using 63 museum skins and 63 live specimens. Genetic distances were estimated in a sample of 136 individuals. An additional characterisation of the plumage of Iberian males was shaped from 22 live specimens. We highlight the distinctiveness of Iberian birds within the subspecies mosaic since *L. s. azuricollis* had longer wings than *L. s. cyanecula* and *L. s. namnetum*, but shorter wings than *L. s. magna*. Indeed, *L. s. azuricollis* had longer tarsus and bill than *L. s. namnetum*, but shorter bill than *L. s. magna*. Biometric divergence was not significantly associated with genetic distance. Iberian males showed an all-blue plastron in 77% of specimens, a mostly non-marked black band and no white band, which distinguished them from males of *L. s. cyanecula* and *L. s. namnetum*. We conclude the importance of considering phenotypic and genotypic differences at subspecies level, which is essential for designing realistic conservation strategies addressed to preserve species genetic diversity patterns.

1. Introduction

The presence of endangered species is currently one of the main principles for establishing priorities of conservation and designing protected areas, like the Nature 2000 network in Europe. However, conservation strategies may benefit from targeting lower taxonomic levels of assessment to avoid widespread loss of genetic diversity (Meffe & Carrol 1997). Successful conservation policies should be explicitly focused on the preservation of multiple populations across the range of the species, that should be self-sustaining, healthy and genetically robust (Redford *et al.* 2011), since genetic variability determines the adaptive potential of the species and their resilience to environmental changes (Reed & Frankham 2003). In the case of bird species, the assessment of intra-specific variation has been traditionally addressed at the taxonomic category of subspecies (Winker 2010). The evaluation of bird subspecies is critical since it contributes to explain the current distribution and the biogeographic history of species (Newton 2003). Indeed, according to Phillimore & Owens (2006), it offers an effective shortcut for estimating patterns of intraspecific genetic diversity, thereby providing a useful tool

for the study of evolutionary divergence and conservation. However, it is important to have in mind that, despite the convenience of using this study level, the traditional subspecies limits, that have been based on phenotypic features, could sometimes be contradicted by the outcomes derived from modern molecular techniques (Zink *et al*. 2003, Rheindt *et al*. 2011), which makes the approach more challenging. The bluethroat (*Luscinia svecica*) is a small migratory passerine (weight 14-20 g) with breeding populations distributed through the west and north of the Western Paleartic (Cramp 1988, Dement'ev & Gladkov 1968). It has been incorporated in the Annex I of the European Union's Birds Directive (Directive 2009/147/CE) that includes the most endangered species of Europe. The species shows a high sexual dichromatism, which influences inter and intrasexual communication. In the case of males, throat and breast are dominated by a large patch of ultraviolet (UV)/blue plumage (with or without a central spot of white/chestnut coloration) and a breast band of chestnut plumage below. Females are highly variable in the extent of their throat coloration, from entirely drab to almost male-like (Cramp 1988, Amundsen *et al*. 1997). The phenotypic variation of Bluethroats constitutes a complex mosaic associated to differences in geographical morphs and life history. Ten subspecies have been described according to both male throat ornament and general plumage patterns (Cramp 1988, Del Hoyo *et al*. 2005), although a unanimous consensus does not exist about subspecies classification, neither about phylogenetic relationships (Questiau *et al*. 1998, Eybert *et al*. 2003, Zink *et al*. 2003, Johnsen *et al*. 2006). According to Zink *et al*. (2003), only two clades can be differentiated in Eurasia based on mitochondrial DNA studies. One of these groups is located in northern Eurasia and includes chestnut-spotted subspecies (*L. s. svecica*, *L. s. volgae* and *L. s. pallidogularis*). The other comprises southern subspecies with or without a white throat spot (*L. s. azuricollis*, *L. s. namnetum*, *L. s. cyanecula* and *L. s magna*). Johnsen *et al*. (2006) found evidence for low gene flow among northern and southern groups, the latter being more differentiated than the former. These authors also showed that

Bluethroat genetic structure (based on microsatellite marker analysis) was consistent with the subspecies classification (based on phenotypic features). They found significant qualitative variation in throat spot coloration and quantitative variation in hue, chroma and brightness of the UV/ blue throat coloration that possibly evolved by sexual selection through female choice, in turn leading to subspecies diversification (Peiponen 1960, Andersson & Amundsen 1997, Johnsen *et al*. 1998, 2001). Indeed, Hogner *et al*. (2013), on the basis of sperm characteristics, showed a significant differentiation between *L. s. svecica*, *L. s. namnetum*, *L. s. cyanecula* and *L. s. azuricollis* that was consistent with the findings of Johnsen *et al*. (2006) and, therefore, supported the status of these subspecies as independent taxa. The high number of differences existing between subspecies and their geographic isolation indicate that Bluethroats are currently in an advanced stage of the speciation process, when compared with other subspecies complexes (Johnsen *et al*. 2006). Iberian breeding Bluethroats have been traditionally misclassified within one of the whitespotted subspecies, *L. s. cyanecula* (Cramp 1988, Del Hoyo *et al*. 2005), although decades ago other authors as Mayaud (1958) and Corley-Smith (1959) had highlighted that they presented sufficient plumage differences to be considered as a different subspecies (*L. s. azuricollis*). Recently, Johnsen *et al*. (2006, 2007) and Hogner *et al*. (2013) have shown that Iberian populations are genetically well defined, both in nuclear microsatellites and mtDNA (but see Zink *et al*. 2003). In this sense, the accurate assessment of subspecies taxonomic position, as well as the full characterization of phenotypic and genotypic distinctiveness of subspecies, is essential for implementing adequate measures of conservation addressed to protect the Eurasian Bluethroats as a whole.

The objective of this study was to add new evidence that supplements the existing knowledge about the relevance and singularity of the Iberian breeding bluethroats in the context of the *Luscinia svecica* subspecies complex. In particular, we evaluated biometric differences
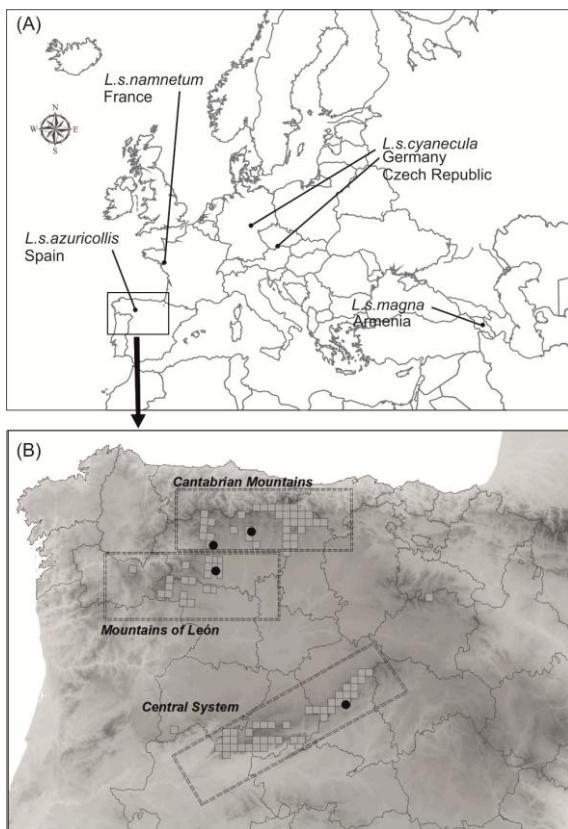
Figure 1.1. Study area. A) Map of Eurasia showing the geographic location where Bluethroat specimens from different subspecies were captured. B) Breeding distribution of Bluethroat in Spain (10 × 10 km UTM squares; Martí & Del Moral, 2003) and sampling localities.

of Iberian males (*L. s. azuricollis*) against those of the closest subspecies, according to geographic (*L. s. cyanecula* and *L. s. namnetum*) and plumage (*L. s. magna*) criteria, and tested the correlation between biometric and genetic differentiation among the target subspecies. Additionally, we aimed to carry out a specific characterisation of Iberian males according to their plumage.

Material and methods

In order to evaluate the biometric differences among subspecies, we have considered three variables: wing chord length (± 0.5mm), tarsus length, (± 0.1 mm) and bill length (± 0.1 mm) (Svensson 1992).

These data have been collected in a sample of 63 male museum skins: seven *L. s. azuricollis* (housed in the Natural History Museum [NHM] of London), 28 *L. s. cyanecula* (NHM of London, Paris and Oslo), 10 *L. s. magna* (NHM of London) and 18 *L. s. namnetum* (NHM of Paris). The sample had been used previously in Johnsen *et al.* (2006) but, whereas in that study the morphological measurements were combined into a principal component analysis (PCA), here they have been analysed separately. Additionally, we have considered the wing chord length (± 0.5 mm) measured on a sample of 63 live male specimens captured using mistnets and spring traps in their breeding areas: 24 *L. s. azuricollis*, 13 *L. s. cyanecula*, 15 *L. s. magna* and 11 *L. s. namnetum* (Figure 1.1). Biometric comparisons among subspecies were performed by applying univariate General Linear Models (GLMs) with a Tukey's Honest Significant Difference test (Tukey-HSD),which incorporates an adjustment for dealing with unbalanced sample sizes. We avoided comparisons between measurements made on museum skins and live birds, due to eventual differences in the preparation of the museum specimens that could affect results (Jenni & Winkler 1989, García *et al.* 2000).

To analyse the relationship between both biometric and genetic differentiation among the four subspecies, we applied a nonparametric Mantel test (Mantel 1967). Biometric differentiation was estimated as the Euclidean distance of the wing chord length mean values (measured on the 63 live male specimens abovementioned) among subspecies. Genetic differentiation was calculated as the pairwise genetic distance among subspecies (F_{ST} values; Weir & Cockerham 1984) using a sample of 135 individuals (36 *L. s. azuricollis*, 54 *L. s cyanecula*, 21 *L. s. namnetum* and 24 *L. s. magna*; Figure 1.1). Genotypic characterisation had been accomplished by Johnsen *et al.* (2006) on the basis of 11 heterologous microsatellite markers using standard PCR conditions. Finally, we described the plumage of the Iberian subspecies using a sample of 22 live males collected in Spain between 1999 and 2002 (Figure 1.1b). We evaluated the tone of the

breast blue plastron (light, intermediate or dark), the colour of the spot (white, chestnut or blue if it were absent) and the presence of black, white and chestnut breast bands (absent, not marked, marked or very marked). Statistical analyses were done with the packages "hierfstat" and "multcomp" of R 3.0.0 statistical software (R Development Core Team 2013).

Results

The comparison among male museum skins for the target subspecies showed that *L. s. azuricollis* had significantly longer wings than *L. s. cyanecula* whereas there was no difference in tarsus or bill length among these subspecies. In contrast, *L. s. azuricollis* had longer wings, tarsus and bill than *L. s. namnetum*, as well as shorter wings and bill than *L. s. magna*. In the case of live males, *L. s. azuricollis* had significantly longer wings than *L. s. namnetum* and shorter wings than *L. s. magna*, while there were no differences in this biometric trait with *L. s. cyanecula* (Table 1.1). Biometric divergence (based on the wing length of live specimens) was not significantly correlated with genetic differentiation among subspecies (Mantel test, r = −0.143, p = 0.661). The analysis of the plumage made on the Iberian live birds showed that the blue tone of the plastron was mostly

intermediate, while only in a few cases it was light or dark. A 77% of specimens showed an all-blue plastron (in three individuals, we found some white feathers completely covered by blue feathers) and the remaining 23% had a white spot in the blue plastron. Regarding the breast bands, the white band was not detected in any specimen, while the chestnut one was marked or very marked in all birds. The black band was absent in 22.7% of the birds, not marked in 68.2% and marked in the remaining 9.1%.

Discussion

A proper knowledge of subspecies differentiation is crucial to understand the species adaptation to different environments, the consequences of geographic isolation and the risks of population declines. In this context, subspecies classification should correctly reflect the evolutionary diversity of the target species (Zink 2004). In the case of Bluethroats, some controversy about the correct assignation of Iberian breeding birds (*L. s. azuricollis*) within the subspecies complex still exists. Currently, *L. s. azuricollis* is recognized as a separate subspecies by leading ornithologists (e.g., Clements *et al.* 2016), but some discrepancies arise (Zink *et al.* 2003, Del Hoyo *et al.* 2005).

Table 1.1. Comparison of biometric parameters among the males belonging to *L. s. azuricollis* (azu), *L. s. cyanecula* (cya), *L. s. namnetum* (nam) and *L. s. magna* (mag): mean ± SD (sample size). Table also shows the result of univariate GLMs with a Tukey-HSD post hoc analysis (*p<0.05, **p<0.01, ***p<0.001, n.s. = non-significant).

	azuricollis	cyanecula	magna	namnetum	azu-cya	azu-mag	azu-nam	cya-mag	cya-nam	nam-mag
Tarsus length (museum skins)	26.77 ± 0.84 (7)	26.23 ± 0.72 (26)	27.07 ± 0.66 (10)	24.52 ± 0.62 (18)	n.s.	n.s.	***	**	***	***
Bill length (museum skins)	8.76 ± 0.32 (7)	8.74 ± 0.44 (25)	9.53 ± 0.45 (8)	7.98 ± 0.28 (18)	n.s.	**	***	***	***	***
Wing length (museum skins)	76.86 ± 1.95 (7)	75.11 ± 1.29 (27)	80.70 ± 1.49 (10)	68.20 ± 2.62 (18)	*	***	***	***	***	***
Wing length (live specimens)	75.23 ± 1.41 (24)	75.71 ± 1.74 (14)	77.77 ± 2.93 (15)	67.55 ± 1.29 (11)	n.s.	**	***	n.s.	***	***

Our results highlighted the relevance and singularity of the Iberian subspecies within the European subspecies complex. We found that *L. s. azuricollis* is phenotypically well differentiated from the other evaluated subspecies (*L. s. cyanecula*, *L. s. namnetum* and *L. s. magna*), Showing important differences in biometry and plumage features. However, despite the microsatellite analysis made by Johnsen *et al.* (2006) revealed that *L. s. azuricollis* is genetically well differentiated from the other evaluated subspecies, we noticed that biometric differences in wing length are not significantly correlated to genetic distance among subspecies. A reason could be that phenotypic features may be the product of gradual geographic variations and clines, reflecting phenotypic plasticity in response to environmental contingencies, rather than genetic differences (Willoughby 2007). In fact, variability in biometric measurements can be related not only to genotypic features, but also to epigenomic differences. That is, birds with a similar genome may have different morphological traits depending on their habitat (Geist 1978).

Any attempt to define biologically meaningful units for conservation at intraspecific level should consider genetic, phenotypic and life history characteristics. The identification of "evolutionarily significant units" (ESUs; Ryder 1986, Moritz 1994, Crandall *et al.* 2000, Luck *et al.* 2003) can be useful for designing realistic conservation strategies. When conservation measures are based on units with evolutionary significance, areas holding high levels of genetic differentiation must be identified, giving priority to the conservation of spaces including environmental gradients that assure the viability of populations/subspecies and its adaptive potential (Meffe & Carrol 1997, Moritz 2002, Holderegger *et al.* 2006). In this sense, southern populations of Bluethroats, where the Iberian subspecies is included, are genetically well differentiated, while Northern populations (included in subspecies *L. s. svecica*, *L. s. pallidogularis* and *L. s. tianshanica*) have a relatively reduced degree of genetic differentiation (Zink *et al.* 2003, Johnsen *et al.* 2006).

Within the former group, *L. s. azuricollis* and *L. s. namnetum* may be the most ancestral forms of the species (Johnsen *et al.* 2006). Despite their ecological value, they present important conservation problems that should be specifically addressed. In particular, they show a low genetic diversity that could be caused either by genetic drift (Johnsen *et al.* 2006) or by a recent "bottleneck", resulting in an increased frequency of some rare alleles and a strong differentiation from the rest of the subspecies complex (Questiau *et al.* 1998). A low level of heterozygosity is particularly frequent in threatened species, being generally associated with low breeding success and survival rates (Frankham 1995, Haig & Avise 1996, Roques & Negro 2005).Additionally, Iberian Bluethroats are located in the southern edge of the species range in Eurasia, where available habitat is fragmented and relatively reduced due to threats related to land use (forestry, land clearing infrastructure, fires) and climate change (Martí & Del Moral 2003, Huntley *et al.* 2007).

Regarding biometry, significant differences were found among subspecies in tarsus, bill and wing length, in line with the findings of Johnsen *et al.* (2006). Dissimilarities in wing length between *L. s. azuricollis* and *L. s. cyanecula* are probably related to migration strategies. The Iberian subspecies hold birds with longer and more pointed wings that can be considered as longer distance migrants (Arizaga *et al.* 2006). This wing morphology is usually associated with a greater flight capacity to migrate further (Mönkkönen 1995) and, therefore, could imply the exploitation of different wintering areas (Arizaga *et al.* 2006).In fact, according to Arizaga *et al.* (2015), at least some birds (or populations) of *L. s. azuricollis* could spend the winter in tropical Africa (i.e. Senegal), while *L. s. cyanecula* bluethroats would have their wintering quarters within the circum-Mediterranean region specifically in southern Europe, northern Africa and the Sahel; Cramp 1988). In these areas, suitable habitat could be affected by different environmental threats (e.g. drought, land use change, human disturbances), which would mean new challenges for designing global strategies addressed to the conservation of the *L. s. azuricollis* subspecies.

The comparison of biometric data for Iberian males measured in the two evaluated samples (i.e., museum skins and live birds) showed a difference in the mean values recorded for wing length. One reason may be that the study carried out at a European scale using museum skins was only based on birds collected in central Spain, while the analysis based on live specimens included birds from the north of the country, where the level of genetic differentiation of populations is high, as described by Alda *et al.* (2013). These authors found three different genetic clusters in this area, with F_{ST} values comparable to those among several of the recognized Eurasian subspecies (Johnsen *et al.* 2006). Due to the complex genetic patterns of Iberian populations, we highlight the importance of sampling across the subspecies range for improving the reliability of ecological studies focused on this species.

The assessment of plumage characteristics showed that both the presence of a distinct white spot and the breast band patterns widely change among the four subspecies. The most distinctive plumage traits of *L. s. azuricollis*, in relation to the other subspecies, are: (i) the absence of a white spot in a large number of birds, that was present (white or chestnut) in the rest of the subspecies, with the exception of *L. s. magna* (Johnsen *et al.* 2006); (ii) the complete absence of a white breast band, which is actually a distinguishing feature that is not usually considered (Clement & Rose 2015). Breast ornamentation is strongly associated with genetic differentiation within the Bluethroat subspecies mosaic in Eurasia and, therefore, it could be useful to discriminate among subspecies in areas of secondary contact (Johnsen *et al.* 2006). Currently, Iberian populations are geographically isolated from the other subspecies.

Therefore, it would be interesting to evaluate, in a hypothetic scenario of *L. s. namnetum* expansion (Eybert *et al.* 1999), to what extent the mechanisms of reproductive isolation, based on plumage or song (Turcokova 2011), could limit the hybridization among subspecies. It has been hypothesized that the evolution of the throat spot started from a phase of an entirely blue throat

plastron, followed by an increase in the complexity of breast ornamentation (Johnsen *et al.* 2006). Nevertheless, genetic data suggest that *L. s. azuricollis* and *L. s. magna* are not as closely related as suggested by their similar phenotypic (absence of white spot) appearance (Johnsen *et al.* 2006). We conclude the importance of considering explicitly the phenotypic and genotypic differences existing at subspecies level, since this information is essential for designing realistic conservation strategies at different geographic scales (from local to continental), specifically for the case of polytypic species. Our findings reinforce the idea that the Iberian breeding subspecies should be a conservation priority to preserve the intraspecific variation in genetic diversity of the Eurasian Bluethroats. A loss of species genetic diversity would imply a reduction on its resilience against environmental changes.

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CAPÍTULO 2

Local Genetic Structure on Breeding Grounds of a Long-Distance Migrant Passerine: The Bluethroat (*Luscinia svecica*) in Spain

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Abstract

Breeding site fidelity can be determined by environmental features, which depending on their heterogeneous distribution may shape the genetic landscape of a population. We used 10 microsatellite loci to study the genetic variation of 83 bluethroats (*Luscinia svecica azuricollis*) across 14 localities within the Spanish breeding population and assess the relative influence of different habitat characteristics (physiography and vegetation) on genetic differentiation. Based on the genetic variation of this population, we identified 3 geographically consistent genetic clusters that on average showed a higher genetic differentiation than among other north European populations, even those belonging to different subspecies. The inferred genetic clusters occurred in geographic areas that significantly differed in elevation. The highest genetic differentiation was observed between sites at different mountain ranges, as well as between the highest altitude sites in the northeastern locale, whereas vegetation type did not explain a significant percentage of genetic variation. The lack of correlation between geographic and genetic distances suggests that this pattern of genetic structure cannot be explained as a consequence of isolation by distance. Finally, we discuss the importance of preserving areas encompassing high environmental and genetic variation as a means of preserving evolutionary processes and adaptive potential.

Key Words: breeding site selection, environmental factors, genetic structure, *Luscinia svecica*, microsatellites, Spain.

The interplay between gene flow and local habitat selection and its influence on species diversification constitutes a long-lasting research topic in evolutionary biology (Wright 1940; Felsenstein 1976, Hedrick 1986, Hedrick 2006). The occurrence of a species at a particular site largely depends on environmental variability, which is ultimately determined by the range of suitable habitats according to their spatial configuration and seasonal variation (Bell *et al*. 1993, Dufour *et al*. 2006). The spatial variation of ecological factors, linked both to habitat heterogeneity and quality, may also shape levels of genetic variability in wild populations (Frankham 1995, Foll & Gaggiotti 2006, Pitra *et al*. 2011). As a consequence, genetic differentiation among populations depends not only on the strength of habitat selection on each local population but also on the relative importance of dispersal. Therefore, it is expected that if habitat preferences are stronger than dispersal among local populations, local adaptation may arise in such populations even if this geographic scale is much smaller than the scale of dispersal (Wright 1940, Blondel *et al*. 2006). Strong habitat selection in heterogeneous landscapes may cause local populations to evolve traits that provide advantages under their local habitat characteristics (Kawecki & Ebert 2004). However, several factors may hamper

local adaptation. In this context, gene flow is the most important factor, since the exchange of genes between populations homogenizes allele frequencies and thus prevents genetic differentiation (Balloux & Lugon-Moulin 2002). Therefore, it is generally assumed that at small spatial scales, intraspecific variation does not occur in highly vagile organisms such as birds. This assumption would be valid if gene flow was spatially random, but evidence suggests that birds may show dispersal biases with respect to habitat (Davis & Stamps 2004, Blondel *et al.* 2006, Hull *et al.* 2008, Alda *et al.* 2011).

Birds breeding in heterogeneous landscapes may choose territories with different environmental qualities, which can affect demographic parameters and genetic diversity of populations (Penteriani *et al.* 2004, Porlier *et al.* 2009). For example, birds with migratory behavior might differ in their degrees of fidelity to their breeding and wintering sites (i.e., migratory connectivity, Esler 2000). This philopatric behavior has been associated with key features of the environment that are patchily distributed or difficult to locate, such as specialized breeding locations or food resources (Van Bekkum *et al.* 2006, Clark *et al.* 2008, Hull *et al.* 2008). Hence, migratory connectivity is directly related to gene flow, which in turn determines the geographical pattern of genetic variation within a species. Consequently, it would be expected that high levels of genetic and morphological variation among populations with strong migratory connectivity are due to low gene flow and local adaptations (Webster *et al.* 2002).

The bluethroat *Luscinia svecica* (Linnaeus 1758) is a long-distance migratory passerine that breeds throughout Europe, Asia, and Alaska. There are 10 subspecies that constitute a subspecies complex described on the basis of body size and plumage coloration of males and on differences of their breeding habitats, migration routes, and wintering areas (Cramp 1988). However, these subspecies are not recognized according to mitochondrial DNA differentiation and only a shallow divergence exists between the northern and southern subspecies, suggesting a recent divergence of these populations (Questiau *et al.* 1998; Zink *et*

al. 2003). In addition, faster evolving microsatellite markers indicate restricted gene flow among some subspecies in *L. svecica*, particularly among southern populations, which generally are more differentiated than northern populations. Furthermore, the southern group of subspecies, which includes the Spanish and French subspecies, is morphologically distinct in showing white or no throat spots, in contrast with the northern group of chestnut-spotted populations. Thus, because the Spanish subspecies *L. s. azuricollis* is clearly genetically differentiated, it and the French *L. s. namnetum* populations are proposed to be ancestral to the other European subspecies (Johnsen *et al.* 2006). In general, bluethroats show high fidelity to their migratory routes between wintering and breeding areas (Markovets & Yosef 2005; Hellgren *et al.* 2008), so the observed genetic heterogeneity among regions in Europe could be either due to isolation processes or a consequence of local adaptations of southern populations (Johnsen *et al.* 2006). Spanish bluethroats are believed to winter south of the Sahara (Arizaga *et al.* 2006) and breed in the northwestern mountains of Iberian Peninsula (Tellería 1999; Gómez-Manzaneque 2003). In the Iberian mountains, *L. s. azuricollis* occurs in a variety of habitat types greatly differing in vegetation structure and composition, altitude, and orientation. These differences can be observed at a very small spatial scale (only a few kilometers apart), providing a framework for habitat choice and some degree of local genetic divergence (Guschanski *et al.* 2008). However, there is limited knowledge of the genetic variation among bluethroat populations at such small geographic scales, with the exception of *L. s. svecica* in Scandinavia (Hellgren *et al.* 2008). Thus, the bluethroat breeding population in Spain constitutes a good model to evaluate the relationships between this site fidelity and the environmental features shaping the genetic structure at a local scale in a wide-ranging species. The main aim of this study is to examine the genetic variation of bluethroats within the Spanish breeding population, in order to determine: 1) the extent of genetic differentiation at the local scale and 2) whether landscape features have a direct influence on the genetic

structure of local populations. Different habitat characteristics (physiography and vegetation) might imply different adaptations or selection patterns for breeding individuals. Thus, we would expect to observe significant genetic differentiation among breeding sites if bluethroats are preferentially selecting certain habitat conditions. If this selection is strong, it might imply a low capability of adaptation to different environments. On the other hand, a lack of genetic differentiation could be a consequence of extensive gene flow and therefore suggest a lack of habitat selection.

Materials and Methods

Study Sites and Sampling

Breeding bluethroats were sampled across the species distribution range in northwestern Spain, from the southern slope of the Cantabrian Mountains to the Mountains of León (León province), ranging from 800 to 1900 m above sea level (Figure 2.1A). This area spans the putative limit of 2 major European biogeographic regions, the Atlantic and the Mediterranean, and features a wide diversity of habitats. Fourteen localities were sampled during the breeding season between April 2009 and August 2010

and classified on the basis of the main environmental characteristics that could directly or indirectly influence the selection of breeding sites by bluethroats (Table 2.1). Localities were assigned to the mountain range where they were sampled (Cantabrian Mountains and Mountains of León). The Cantabrian Mountains run on an east–west axis and are on average higher in altitude than the Mountains of León. They are also more influenced by the Atlantic climate and have higher precipitation than the Mountains of León. Most sampling localities were found along valley bottoms and foothills (800–1200 m) and mountain ridges (1500–1900 m) (Figure 2.1B) and were further differentiated into low- and high-altitude sites, respectively. Three main habitats were defined according to their vegetation type: brooms, mainly composed by *Cytisus* spp. and *Genista* spp.; heathlands, constituted by *Erica* spp. and *Calluna vulgaris*; and holm oak shrublands, consisting of *Quercus rotundifolia* and *Cistus* spp. (Table 2.1, Figure 2.1A, B). Bluethroats were captured with tape-lured mistnets and clap-traps baited with mealworms. Blood samples from all individuals were obtained by venipuncture of the brachial vein and stored in absolute ethanol until they were analyzed. All animals were released unharmed.

Table 2.1. Sampling localities of bluethroat (*Luscinia s. azuricollis*)

	Locality	n	Mountain range	Altit. class	Vegetation	Altitude (m)	Latitude	Longitude
1	Genicera	14	Cantabrian Mountains	High	Brooms	1777.9	42.95°	-5.49°
2	Rodillazo	2	Cantabrian Mountains	High	Brooms	1640.5	42.92°	-5.51°
3	Meroy	2	Cantabrian Mountains	High	Brooms	1592.0	42.97°	-6.22°
4	La Cueta	5	Cantabrian Mountains	High	Brooms	1566.0	43.01°	-6.18°
5	La Majúa	2	Cantabrian Mountains	High	Brooms	1895.0	42.98°	-6.02°
6	Ferreras de Cepeda	17	Mountains of León	Low	Heathlands	973.1	42.65°	-6.03°
7	La Seca	1	Cantabrian Mountains	Low	Heathlands	1122.0	42.74°	-5.60°
8	Corcos	9	Cantabrian Mountains	Low	Heathlands	1012.7	42.67°	-5.08°
9	Pobladura de la Sierra	2	Mountains of León	High	Brooms	1676.5	42.42°	-6.44°
10	Molinaferrera	1	Mountains of León	Low	Heathlands	1138.0	42.39°	-6.36°
11	Palacios de la Valduerna	13	Mountains of León	Low	Holm oak shrublands	809.4	42.33°	-5.94°
12	Villar de Golfer	3	Mountains of León	Low	Heathlands	974.3	42.35°	-6.19°
13	Bustos	8	Mountains of León	Low	Holm oak shrublands	834.0	42.38°	-6.02°
14	Toralino de la Vega	4	Mountains of León	Low	Holm oak shrublands	834.0	42.37°	-5.97°

Number of individuals sampled in each locality, classes based on physiographic and ecological characteristics, mean altitude and coordinates are indicated.
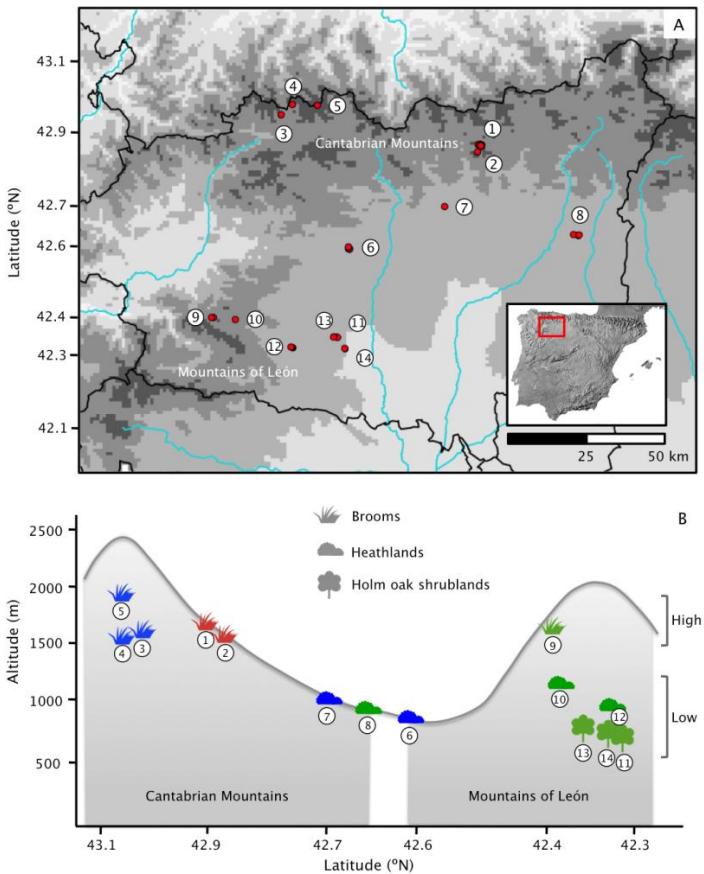
Figure 2.1. (A) Map illustrating the 14 bluethroat localities sampled in northwestern Spain. Gray layers, from light to dark, correspond to elevations 400–800 m, 800–1200 m, 1200–1600 m, and 1600–2600 m. Black lines represent province limits and blue lines are main rivers in the area. Numbers refer to localities in Table 2.1. (B) Schematic representation of the relief profile of the study region. Mountain range, altitude classes, and vegetation type for each locality is indicated. Colors represent genetic clusters to which localities were assigned; black (red): cluster K-NE, northeastern localities; medium gray (blue): cluster K-NW, northwestern and central areas; and light gray (green): K-S, southern sites. Colors between parentheses refer to the color version of the figure.

DNA Extraction and Microsatellite Genotyping

Total genomic DNA was extracted from blood using a standard ammonium acetate precipitation protocol (Perbal 1988) following Proteinase K digestion. All samples were genotyped for 12 microsatellite loci: Aar8, Ase19, Cuμ4, Cuμ10, Fhu2, Hru7, Mcy4, PAT MP 2-43, Pdo5, Phtr2, PmaC25, and Ppi2 (Ellegren 1992, Primmer *et al.* 1996, Double *et al.* 1997, Fridolfsson *et al.* 1997, Otter *et al.* 1998, Gibbs *et al.* 1999, Martínez *et al.* 1999, MacColl *et al.* 2000, Richardson *et al.* 2000, Saladin *et al.* 2003). The microsatellites were co-amplified in 4 multiplex polymerase chain reactions (PCRs; Mix1: Fhu2, PmaC25, Ptc2; Mix2: Ase19, Cuμ4, PAT MP 2-43; Mix3: Cuμ10, Hru7, Mcy4; Mix4:

Aar8, Pdo5, Phtr2), following the QIAGEN Multiplex PCR kit protocol for 30 cycles and 3 different annealing temperatures (60 °C for Mix1, 57 °C for Mix2 and 48 °C for Mix3 and 4). Reactions were prepared in a final volume of 7 μL including: 3.5 μL of Qiagen 2X PCR Master Mix, 0.7 μL of 10X primer mix (2 μM each), 1 μL DNA (ca. 25 ng/μL) and 1.8 μL of RNase-free H2O. Fluorescently labeled PCR products were analyzed on an ABI3130xl DNA Analyzer Applied Biosystems) and allele sizes were determined using GeneMapper 3.7 software (Applied Biosystems).

Data Analysis

Data were checked for null alleles and genotyping errors using MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004). We estimated the

following genetic diversity parameters: number of alleles (N_A), allelic richness permuted by the lowest number of individuals genotyped in a locality (A_R), observed and expected heterozygosity (H_o and H_e) and inbreeding coefficient (F_{IS}) using FSTAT 2.9.3 (Goudet 1995). Departures from Hardy–Weinberg equilibrium were assessed in GenoDive 2.0b20 (Meirmans & Van Tienderen 2004). To investigate the genetic structure and spatial location of genetic discontinuities within the breeding population, we first employed a Bayesian clustering method without prior assignment to their locations of origin. For that purpose, we used GENELAND 3.2.2 (Guillot *et al.* 2005; Guillot *et al.* 2008), which utilizes both genetic information and geographic coordinates from each individual to infer population structure. We initially ran 10 independent Markov Chain

Monte Carlo (MCMC) simulations using the following parameters: 5×10^5 iterations, maximum rate of Poisson process fixed at 50, maximum number of nuclei in the Poisson–Voronoi tessellation fixed at 150, and the Dirichlet model for allele frequencies. Since the number of genetic populations was unknown, we allowed the number of clusters (K) to vary on a wide range from K = 1 to K = 10. Next, we determined the best number of clusters from the highest likelihood number of K obtained from these runs and ran the MCMC 20 times with K fixed to the value identified in the first step. We then computed the posterior probability of population membership for each pixel of the spatial domain (150 × 150 pixels) and for each individual for each of the 20 runs (with a burn-in of 5×10^4 iterations).

Table 2.2. Genetic diversity of bluethroat based on microsatellite loci for the whole population and for each of the three genetic clusters (K-NE, K-NW and K-S) inferred in GENELAND.

| | Locus | | | | | | | | | | | | |
	Ase19	Cuµ4	Cuµ10	Hru7	Mcy4	PAT MP 2-43	PmaC25	Ppi2	Ptc2	Phtr2	Pdo5*	Aar8*	Mean (SD)
K-NE (*n*=16)													
N_A	4	5	3	7	5	4	3	5	2	8	5	1	4.636 (1.747)
A_R	3.597	4.818	2.988	6.613	4.812	3.682	2.786	5.000	2.000	7.316	4.734	1	4.395 (1.604)
H_o	0.500	0.938	0.250	0.750	0.688	0.750	0.286	0.545	0.286	0.857	0.214	0	0.551 (0.079)
H_e	0.606	0.729	0.425	0.706	0.644	0.631	0.508	0.773	0.516	0.835	0.541	0	0.634 (0.039)
F_{IS}	0.212	-0.183	**0.6**	-0.027	-0.123	-0.122	0.19	0.231	0.323	-0.007	**0.508**	na	0.090 (0.081)
K-NW (*n*=27)													
N_A	5	4	3	7	6	4	3	5	3	11	7	1	5.273 (2.412)
A_R	4.39	3.963	2.394	6.496	5.227	3.344	2.984	4.963	2.653	8.729	5.729	1	4.625 (1.890)
H_o	0.556	0.593	0.115	0.923	0.852	0.593	0.500	0.731	0.519	0.889	0.200	0	0.588 (0.078)
H_e	0.652	0.706	0.245	0.814	0.748	0.607	0.520	0.795	0.52	0.875	0.562	0	0.64 (0.054)
F_{IS}	0.050	0.097	0.053	-0.06	-0.092	-0.091	0.021	0.192	0.046	0.144	**0.695**	na	0.033 (0.042)
K-S (*n*=40)													
N_A	5	6	3	10	8	6	3	7	4	11	6	1	6.273 (2.611)
A_R	4.074	5.274	2.579	7.307	6.582	3.952	2.983	5.228	2.769	8.504	4.622	1	4.897 (1.927)
H_o	0.650	0.625	0.25	0.895	0.850	0.450	0.579	0.605	0.462	0.775	0.176	0	0.574 (0.069)
H_e	0.637	0.751	0.267	0.850	0.793	0.421	0.596	0.763	0.535	0.864	0.491	0	0.634 (0.058)
F_{IS}	-0.025	**0.356**	-0.04	-0.111	-0.081	0.180	0.149	0.111	0.117	-0.037	**0.683**	na	0.052 (0.036)
ALL (*n*=83)													
N_A	6	6	3	10	8	6	3	7	4	13	8	1	6.727 (3.003)
A_R	4.185	5.025	2.638	6.911	5.996	3.692	2.977	5.173	2.587	8.577	5.179	1	4.813 (1.870)
H_o	0.590	0.675	0.207	0.875	0.819	0.554	0.500	0.640	0.450	0.827	0.192	0	0.571 (0.070)
H_e	0.635	0.740	0.289	0.833	0.755	0.563	0.561	0.783	0.527	0.876	0.517	0	0.636 (0.049)
F_{IS}	0.07	0.089	**0.282**	-0.051	-0.085	0.016	0.109	**0.182**	0.146	0.056	**0.629**	na	0.058 (0.039)

n: number of samples, N_A: number of alleles, A_R: allelic richness standardized to the minimum sample size, H_o: observed heterozygosity, H_e: expected heterozygosity, F_{IS}: inbreeding index. Bold values indicate significant departures from Hardy–Weinberg equilibrium ($P < 0.05$). *indicates loci that were not included in the analyses.

Spatial patterns of genetic differentiation across the full landscape were visualized using the "Genetic Landscape Shape interpolation" analysis implemented in Alleles in Space 1.0 (Miller 2005). This analysis infers a genetic surface based on interindividual distances of sampled individuals and on interpolated distances in areas where individuals were not sampled. Across the genetic landscape, the peaks and troughs indicate high and low genetic distances between individuals, respectively. To test genetic differentiation among all sampling localities and to assess whether the inferred genetic clusters, the physiographic or habitat characteristics (i.e., mountain range, altitude, and vegetation) explained a higher percentage of the genetic variance, we performed an analysis of molecular variance (AMOVA) in GenoDive 2.0b20. Moreover, we calculated the genetic diversity parameters previously explained for each group of localities obtained from the best partition in AMOVA. In addition, we tested the effect of geographic distance on the observed genetic differentiation of the bluethroat. We calculated Euclidean and altitudinal distances between localities and individuals, and tested their correlation with their genetic distance (pairwise $F_{ST}/1-F_{ST}$ between localities and Smouse & Peakall distances between individuals; Smouse & Peakall 1999, using Mantel tests, Mantel 1967). We used partial Mantel tests (Smouse *et al*. 1986) to assess the association between altitudinal and genetic distances while controlling for the influence of Euclidean geographic distances and vice versa (i.e., the association between geographic and genetic distances controlled by altitudinal distances). These analyses were performed in GenoDive 2.0b20 and their statistical significance was assessed by 10 000 randomizations. Further relationships of altitude of sampling localities with genetic diversity parameters (N_A, A_R, H_o, H_e) were tested by Pearson correlations. Statistical support for the hypothesis that localities with different habitat features differ in genetic diversity was tested by Pearson correlations. Statistical support for the hypothesis that localities with different habitat features differ in genetic diversity was tested

using a type-III analysis of variance (ANOVA), with altitudinal block (high or low) and mountain range (Cantabrian Mountains or Mountains of León) as factors and each of the genetic diversity parameters as response variables. Finally, to address if the assignment of birds to each of the inferred genetic clusters was independent of altitude, vegetation, and mountain range of their sampling localities, a log-linear analysis of frequencies was performed. The log-linear analysis is considered an ANOVA-like design of frequency data. Specifically, it is used to test the different factors that are used in a cross-tabulation with categorical factors and their interactions for statistical significance (StatSoft-Inc. 2007). All these analyses were performed in STATISTICA 8.0 (StatSoft-Inc. 2007).
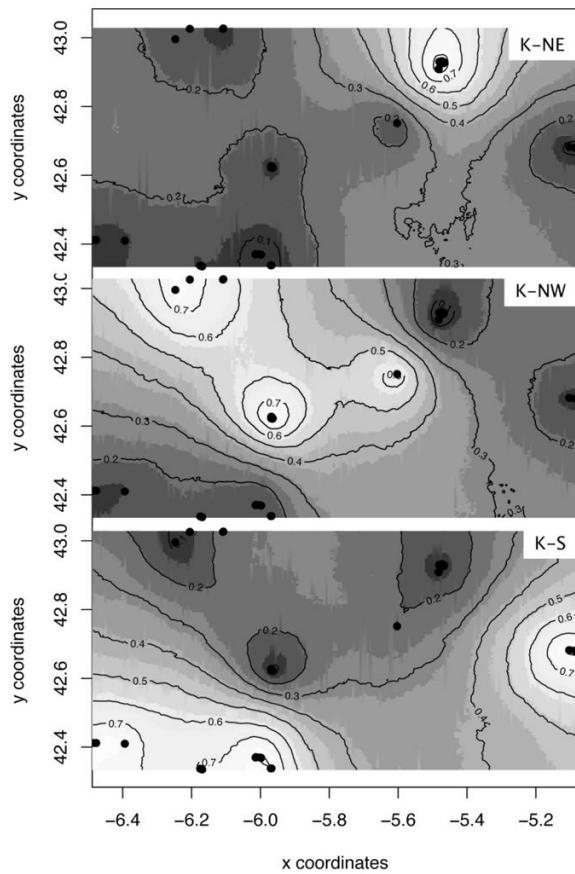
Figure 2.2. Maps of the posterior probabilities to belong to each genetic cluster inferred in GENELAND. Color gradient represents high (white) to low (gray) posterior probabilities.

Results

Eighty-three bluethroats were captured and genotyped for 12 microsatellite loci. Evidence of null alleles was found for locus Pdo5 and consequently it was not included in further analyses. Also, Aar8 turned out to be monomorphic and was removed. Overall, the number of alleles ranged from 3 for loci PmaC25 and Cuμ10 to 13 for locus Phtr2 (average NA = 6.727 ± 3.003 standard deviation [SD]). Observed heterozygosity per locus ranged from 0.207 to 0.875 with an average value of Ho = 0.571 ± 0.070 SD (Table 2.2). The Bayesian clustering analysis performed with GENELAND suggested an optimum structure of three genetic clusters in over 85% of the MCMC iterations. One cluster (K-NE) consisted of the individuals from northeastern localities of Genicera and Rodillazo. The second cluster (K-NW) was formed by the northwestern and central localities: Meroy, La Cueta, La Majúa, Ferreras de Cepeda, and La Seca. The third cluster (K-S) included the southernmost localities (Pobladura de la Sierra, Molinaferrera, Villar de Golfer, Bustos, Toralino, and Palacios de la Valduerna) but also the most eastern one (Corcos) (Figure 1B and Figure 2.2). The three clusters showed similar and significant pairwise F_{ST} values, such as $F_{ST} = 0.025$ (P = 0.007) between K-NE and K-NW, $F_{ST} = 0.024$ (P = 0.004) between K-NE and K-S, and $F_{ST} = 0.020$ (P = 0.000) between K-NW and K-S. All individuals were assigned with high probabilities (>80%) and none of the sampled localities contained individuals assigned to more than one genetic cluster. The genetic surface obtained in the Genetic Landscape Shape interpolation analysis showed sharper "ridges" in the southwestern part of the range, indicating the greatest genetic distances between localities from Mountains of León and western Cantabrian Mountains (Figure 2.3). Furthermore, this analysis indicated that genetic distances decreased in areas to the east of the main genetic discontinuity, with the exception of the localities in the northeastern Cantabrian Mountains, which also indicated high genetic differentiation. Qualitatively similar results were obtained regardless of the grid size or distance weighting parameters chosen. Likewise, use of raw genetic distances or residual genetic distances had no effect on the relative shape of the landscape surface. The AMOVA analyses indicated that most of the molecular variation resided among individuals within the breeding population ($F_{IT} = 0.919$). The remaining genetic variation was best explained by differences among the three genetic clusters inferred in GENELAND ($F_{CT} = 0.026$, P < 0.001), and no significant differences were found among localities within clusters (Table 3). Partitions according to altitude classes and mountain ranges explained significant although lower percentages of genetic variation, but vegetation was nonsignificant (Table 3). Genetic diversity parameters were very similar among the 3 inferred clusters (ANOVA, all P > 0.104) and compared with the whole population, although lower genetic variability was found in cluster K-NE (Table 2.2). Furthermore, none of the genetic diversity parameters were significantly correlated with the altitude of the sampling localities (all P values > 0.148) or were significantly different between mountain ranges (all P values > 0.157). On the other hand, Ho values were almost significantly different between altitude classes (ANOVA $F^{1,11} = 3.488$, P = 0.088), suggesting a tendency for lower genetic diversity in localities at a higher altitude. Furthermore, the altitude at which individuals were sampled was significantly different among the 3 genetic clusters, after controlling for their geographic position (i.e., latitude and longitude; ANOVA $F^{2,78} = 116.252$, P < 0.001), with K-NE at the highest altitude (post-hoc Tukey Test: P = 0.0002 for K-NE vs. K-NW and P = 0.0002 for K-NE vs. K-S) and K-S at the lowest (post-hoc Tukey Test: P = 0.0002 for K-S vs. K-NW). The log-linear analysis indicated that the best model for sample distribution did not include any interaction involving the variable genetic cluster (all P values > 0.501). Only the interaction between the variables genetic cluster and mountain range was close to significance ($\chi^2_2 = 5.457$, P = 0.065), indicating a trend for samples from cluster K-S to be more frequent in the Mountains of León than in the Cantabrian Mountains. As expected for these highly correlated variables, the interaction between vegetation and altitude was significant in the model ($\chi^2_2 = 6.306$, P = 0.043), indicating that

samples belonging to broom-type vegetation were more frequent at high altitudes and samples in shrub lands were more frequent at low altitudes. The Mantel test found a nonsignificant correlation between geographic or altitudinal distances and genetic distances between bluethroat localities (Mantel's r = 0.061, P = 0.319 and r = 0.007, P = 0.456, respectively), indicating that geographic distance between localities has no effect on their genetic differentiation. On the other hand, correlations were significant when individuals instead of localities were considered (Mantel's r = 0.051, P = 0.017 for the geographic distances and r = 0.060, P = 0.025 for the altitudinal distances). However, when the effect of altitude was controlled by Euclidean geographic distances and vice versa, correlations were not significant (Partial Mantel's r = 0.014, P = 0.317 and r = 0.023, P = 0.239).
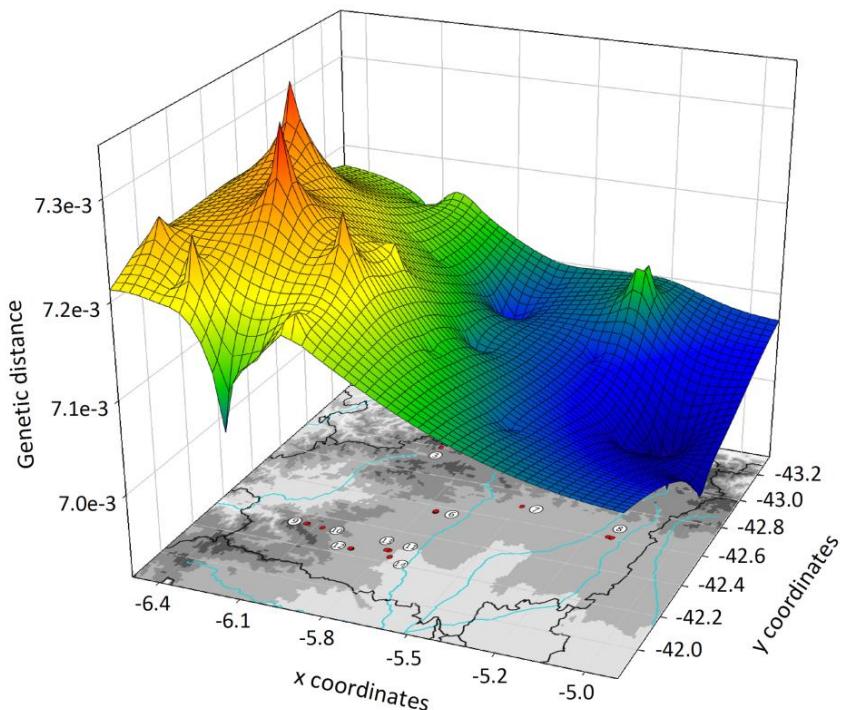
Figure 2.3. Genetic Landscape Shape interpolation based on a 50 × 50 grid and a distance weighting value (a) of 0.2. Surface plot heights are proportionate to genetic distances.

Discussion
Higher Genetic Structure but Lower Diversity in Spanish Than in European Bluethroat Populations.

Three genetic clusters were identified within the Spanish breeding range of *L. s. azuricollis* (Figure 1B and Figure 2.2), which were almost equally divergent from each other, indicating the existence of well-delimited genetic groups at a local spatial scale and restricted effective dispersal (gene flow) (Clark *et al.* 2008). Our work provides additional evidence for a significant and much stronger genetic structure in Spain than in northern Europe, considering that the observed values were one order of magnitude greater than those found among all bluethroat populations in Scandinavia (F_{ST} = 0.002; Hellgren *et al.* 2008).

Table 2.3. Analysis of molecular variance performed between the bluethroat localities analyzed

Partition tested	% var. among groups	F_{CT}	F_{SC}	F_{ST}	F_{IS}
Among Localities (All) (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14)	2.2			0.022**	0.054*
Between Mountain ranges (Cantabrian Mt.) *vs* (Mt. León)					
(1, 2, 3, 4, 5, 7, 8) *vs* (6, 9, 10, 11, 12, 13, 14)	0.1	0.001*	0.025**		0.089**
Between Altitude classes (High) *vs* (Low)					
(1, 2, 3, 4, 5, 9) *vs* (6, 7, 8, 10, 11, 12, 13, 14)	1	0.010*	0.020*		0.089**
Among vegetation types (Brooms) *vs* (Heathlands) *vs* (Shrublands)					
(1, 2, 3, 4, 5, 9) *vs* (6, 7, 8, 10, 12) *vs* (11, 13, 14)	0	0	0.025*		0.089**
Among genetic clusters (K-NE) *vs* (K-NW) *vs* (K-S)					
(1, 2) *vs* (3, 4, 5, 6, 7) *vs* (8, 9, 10, 11, 12, 13, 14)	2.6	0.026**	0.004		0.054*

F_{IS}: variation among individuals within localities, F_{ST}: variation among localities within the population, F_{SC}: variation of localities within groups, F_{CT}: variation among groups within the population.*values indicate significant probabilities at $P < 0.05$ and **values indicate significant probabilities at $P < 0.01$. Numbers correspond to locality codes in Table 2.1.

Furthermore, the levels of genetic differentiation within the Spanish subspecies were in the range of those obtained among distinct bluethroat subspecies across Europe (significant pairwise F_{ST} = −0.004 to 0.174, average pairwise F_{ST} = 0.044 ± 0.043 SD). Indeed, at the continental scale, the highest values of genetic differentiation between bluethroat subspecies were those involving comparisons with *L. s. azuricollis*, whereas the lowest were those comparing the subspecies with a northern distribution (Johnsen *et al.* 2006, Hellgren *et al.* 2008). Our data were congruent with previous studies, with 9 out of 10 microsatellite loci in common but lower sampling size, indicating that *L. s. azuricollis* is the subspecies with the lowest genetic variability. On average, the Spanish population holds 38.6% ± 21.6 SD of all the species alleles, although ranging from 76.9% to 16.6% depending on the locus considered (Johnsen *et al.* 2006). One possibility is that the low genetic diversity of bluethroats breeding in Spain is a consequence of their geographic and genetic isolations, because the associated effects of genetic drift may both decrease genetic diversity and increase differentiation (Frankham *et al.* 2002). In addition, the apparently high philopatry and low gene flow at local scales compared with northern European populations (Hellgren *et al.* 2008), and the fact that *L. s. azuricollis* is basal to the remaining European subspecies (Johnsen *et al.* 2006), might also support an isolation of Spanish breeding bluethroats and suggest a relatively independent evolution for this subspecies. This might explain their pattern of greater genetic differentiation, because besides the effect of geographic distance, the isolation of local populations would promote more rapid evolutionary change within the breeding population, and thus more rapid differentiation from the European populations from which it is isolated (Wright 1940). Furthermore, this pattern of genetic variation agrees with a nonmutually exclusive hypothesis proposing an inverse relationship between population differentiation and latitude (Martin & McKay 2004). Our results support the arguments of several authors that increased seasonal variation in climatic conditions at higher latitudes may result in broader tolerance of northern organisms to environmentally changing conditions. Thus, a greater adaptation capability could reduce costs of dispersing between populations, resulting in relaxed philopatric behavior and also in higher levels of gene flow and reduced genetic differentiation among high latitude populations (Martin & McKay 2004, Croteau *et al.* 2007, Berg *et al.* 2010). In contrast, strong fidelity to breeding sites at lower latitudes would prevent gene flow among different populations and might reduce genetic variation for dispersal behavior (Both & Visser 2001).

Environmental Factors Shaping Genetic Structure and Diversity

Our study helps identify some of the key factors conditioning species dispersal and distribution,

and contributes to a growing body of work that suggests that landscape features influence dispersal and gene flow among bird populations (Bruggeman *et al.* 2010, Coulon *et al.* 2010, Milá *et al.* 2010, Thomassen *et al.* 2010, Alda *et al.* 2011). As has been described in previous studies, we found that geographic distance by itself is not a factor determining genetic differentiation in the bluethroat, neither at a local nor at a continental scale (Johnsen *et al.* 2007). In this case, altitude and mountain range of the localities explained significant percentages of genetic variance (Table 3) and were likely responsible for the observed genetic differentiation, as revealed by the significant differences in altitude among clusters, as well as the almost significant association observed between mountain ranges and the inferred genetic clusters. Indeed, these factors were clearly reflected in the landscape analyses of genetic structure, which showed genetic differentiation of the localities in Mountains of León, as well as those in the highest northeastern localities (Figures 2 and 3). Moreover, these areas that encompass high environmental and genetic variations are particularly important for maximizing adaptive diversity and consequently should be prioritized for conservation (Thomassen *et al.* 2010). In the end, we must be aware that the variables defined for this study are correlated with ultimate factors, such as climate, which will condition phenology and habitat availability. Therefore, we must keep in mind the combined effect of multiple factors on avian habitat selection that consequently gives rise to the observed genetic structure (Milá *et al.* 2010). Limited or differential availability of those features selected by a species across its range distribution may not only explain genetic structure but also differences in population sizes and consequently in genetic diversity (Salvi *et al.* 2009). We observed a general, although nonsignificant, tendency for lower genetic diversity at high altitude localities. Such patterns of differentiation in altitude are expected in organisms with low dispersal abilities but are remarkable in species with high potential for dispersal, especially given the small geographic scale of our study (Martínez-Solano & González 2008, Milá *et al.* 2010). Although our limited sampling size precludes drawing definite conclusions regarding this issue, we might deduce, based on this trend and the genetic differentiation of some high-altitude sites (e.g., cluster K-NE), that a limited number of individuals reach these regions. We further hypothesize that climate variables, such as time differences in the melting of snow at increasing altitudes, might limit habitat availability and thus hinder colonization of breeders and eventually gene flow (Santos González *et al.* 2010). Our results suggest that the environmental differences across the range explain the putatively neutral genetic variation, rather than by isolation by distance, which further indicates that this pattern of genetic structure might likely be shaped by adaptive differentiation (Salvi *et al.* 2009, Thomassen *et al.* 2010). However, the mechanisms underlying the observed genetic structure remain unknown. In our case, genetic differentiation between low- and high-altitude sites could be associated with differences in life-history traits. These differences could be the result of divergent selection pressures, which could have a role in restricting gene flow and leading to local adaptations and differentiation (Milá *et al.* 2010). On the other hand, under a high migration connectivity scenario, birds arriving from different wintering areas or at different times could select different breeding sites depending on their ecological characteristics. In other species, this pattern has been detected on the basis of genetic differences in birds arriving or breeding at different times in the same place (Moore *et al.* 2005, Casagrande *et al.* 2006, Porlier *et al.* 2009). Nevertheless, for the bluethroat, it is still unknown whether Spanish breeding birds show a pattern of temporal genetic differentiation or originate from different wintering areas (Arizaga *et al.* 2006). Further research with broader geographical sampling and additional genetic and morphological markers would be necessary to test these hypotheses, as adaptive changes in morphology often evolve at a faster rate than neutral genetic markers and may reflect noncongruent patterns of differentiation (Marthinsen *et al.* 2007, Milá *et al.* 2009).

Implications for Conservation

The strength of local selection informs how a species might react in diverse and dynamic environments and influences its potential for adaptation in the face of future climate change (Walther *et al.* 2002, Thomassen *et al.* 2010). In this respect, it is necessary to bear in mind that in the Iberian Peninsula, there is no suitable habitat for the bluethroat further north of the Cantabrian Mountains. Consequently, under a global warming scenario, the northward expansion of the Spanish subspecies would be limited (Walther *et al.* 2002, Förschler *et al.* 2011). It remains unclear if the proposed site selection and philopatry is strong enough to hamper the adaptation of individuals from clusters K-NE and K-NW to a southern and more Mediterranean habitat under a global warming scenario. On the contrary, if lowland Mediterranean habitats were to expand under such climatic scenario, bluethroats might expand their populations from those already extant in those regions (K-S). Ultimately, all of the above strengthen the importance of preserving the evolutionary potential held in these areas encompassing both high environmental and genetic variations.

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CAPÍTULO 3

Current landscape attributes and landscape stability in breeding grounds explain genetic differentiation in a long-distance migratory bird

Abstract

Numerous studies have highlighted a major role of isolation by both geographic distance and current landscape resistance in controlling bird population genetic differentiation. However, the importance of past landscape features or landscape temporal stability in shaping population genetic structure remains undervalued, particularly in birds.

We assessed the role of isolation by landscape resistance - derived from current landscape attributes and measures of landscape stability -, in comparison to geographic isolation, as drivers of genetic differentiation of the Iberian bluethroat (*Luscinia svecica azuricollis*), a migratory bird whose populations breed in fragmented and dynamic landscapes affected by land use change. First, we characterized bluethroat genetic structure using microsatellite genotypes and evaluated genetic distances. Then, we built species distribution models using as a predictor a time-series (two decades) of values of the Normalized Difference Vegetation Index as proxies of recent past landscape stability and current landscape features, to ultimately generate landscape resistance values through different functions. Finally, we used maximum-likelihood population effects models to evaluate the relationships between genetic distances and both geographic and landscape resistance distances. We found a genetic structure of four clusters in the Iberian bluethroats populations, as well as a high level of genetic differentiation. Genetic structure was better associated with landscape resistance, rather than with geographic distance. The highest values of habitat suitability corresponded to areas where vegetation remained mostly stable during the two decades prior to bird surveys, with low annual precipitation and spring temperature, being the relationship between gene flow and presence of intervenient habitat among populations linear or quasi-linear. Our results suggest that conservation policies and land management practices that promote the maintenance of semi-open pasture-shrub mosaics (e.g. through extensive livestock grazing) can strongly benefit Iberian bluethroat populations, improving gene flow and population connectivity.

Keywords: gene flow, landscape genetics, landscape resistance, Normalized Difference Vegetation Index (NDVI), *Luscinia svecica azuricollis***.**

Introduction

In the Mediterranean region, centuries of complex interactions between nature and human activities, such as agriculture, forestry and livestock traditional farming practices, have shaped heterogeneous and dynamic landscape mosaics of semi-natural open habitats with high conservation value (Fowler, 2003). Nowadays, the persistence of these human-shaped landscapes is compromised by two opposite trends of land use change: abandonment of traditional activities and agricultural intensification (Álvarez-Martínez *et al.* 2014, Cervera *et al.* 2016). Typically, secondary succession processes after land abandonment are leading to landscape homogenisation in marginal lands, while land intensification is occurring in the most productive areas, like the fertile valley bottoms (Kuemmerle *et al.* 2016). As a consequence of these changes, species living in semi-natural open habitats are suffering progressive isolation due to fragmentation and loss of suitability of their habitats (Suárez-Seoane & Baudry 2002, Caplat & Fonderflick, 2009, Herrando *et al.* 2014).

In heterogeneous landscapes, current landscape composition (i.e., availability of suitable habitat) and configuration (i.e. spatial connectedness) determine species dispersal and gene flow, with habitat loss and fragmentation potentially leading to population size reduction and genetic differentiation, either through genetic drift or natural selection (Johnson *et al.* 1992, Barton 2001, Wang & Summers 2010, Jackson & Fahrig 2016). When landscapes are highly dynamic due to quick changes in land use/cover, the explicit consideration of landscape stability associated to habitat management actions is particularly valuable to understand population genetic patterns (Fuller *et al.* 1995; Frankham 1995, 2005). Recent past landscape changes (landscape legacies) can be related to extinction-colonization processes, including sequential founder events (Wade & McCauley 1988), acting as potentially important evolutionary forces (Barr *et al.* 2008, Amos *et al.* 2012, 2014, Athrey *et al.* 2012).

Numerous studies have highlighted a major role of isolation by geographic distance (IBD) in controlling gene flow and, consequently, population genetic structure across the landscape (Wright 1946, Spear *et al.* 2010). However, thanks to recent technical advances, it has been demonstrated that, in heterogeneous land mosaics where the landscape matrix might limit species dispersal (McRae, 2006; Peterman *et al.* 2014), isolation by current landscape resistance is most correlated to gene flow (Dudaniec *et al.* 2016; Milanesi *et al.* 2017). So far, relative fewer studies have used past landscape patterns (landscape stability) to evaluate the role of isolation by landscape resistance (but see Zeller *et al.* 2012, Fant *et al.* 2014, Bishop-Taylor *et al.* 2017). Among the data sources available for evaluating the role of past landscape patterns in landscape genetics, multi-temporal series of remote sensing data show the greatest potential (Manel *et al.* 2003). Such data can be easily integrated with other ecoclimatic datasets, affording opportunities to test hypothesis at different temporal scales and develop new research paradigms (Porter *et al.* 2009). Despite their usefulness, remote sensing data have been rarely used in landscape genetic studies, probably due to a gap of interdisciplinary integration between landscape ecologists and population geneticists (Storfer *et al.* 2007; Balkenhol *et al.* 2015, Epps & Keyghobadi 2015).

In this study, we develop a species distribution modelling approach based on remote sensing data to evaluate the role of isolation by current landscape resistance and landscape stability, in comparison to isolation by geographic distance, as drivers of current patterns of genetic differentiation in Iberian bluethroats (*Luscinia svecica azuricollis)*. The Iberian bluethroat is a passerine breeding in fragmented and shifting semi-natural mosaics associated to young stages of the ecological succession (i.e., degraded holm oak shrublands, heathlands and broom formations; Alda *et al.* 2013), where the species benefits from abundant nesting and foraging opportunities (García-Tejero *et al.* 2013; Arizaga *et al.* 2017). The persistence of these mountain habitats in the species breeding range is threatened by land abandonment, which leads

to a loss of open spaces and, subsequently, to a landscape homogenization towards shrublands and woodlands (Morán-Ordóñez *et al.* 2012) and a decrease in breeding habitat availability. Bluethroat habitat availability changes across the landscape rapidly due to disturbance events (i.e. fires and grazing) followed by secondary succession (i.e. shrub encroachment), which results in a framework for habitat choice and local genetic divergence at relatively *small scale* (Guschanski *et al.* 2008). In a previous study, Alda *et al.* (2013) investigated the genetic structure of *L. s. azuricollis* in its northernmost breeding grounds. Despite bluethroats are long-distance migrants with great mobility, Alda *et al.* (2013) inferred three highly divergent genetic groups in the Iberian subspecies. However, their results did not support the hypothesis that geographic distance was the driver of this differentiation. Here, we expand the scope of that study by re-testing the isolation by distance hypothesis across the whole distribution range of the Iberian subspecies, considering, as alternative hypotheses, that landscape stability (recent past landscape resistance) and/or current landscape resistance are the drivers of genetic differentiation. This work would provide insights into the effect of habitat connectedness changes resulting from large-scale habitat management activities on the genetic structure and diversity of a highly vagile species.

Material and methods

Study model

The bluethroat *Luscinia svecica* is a long-distance migratory passerine that breeds from Iberia to Eastern Siberia and Alaska, overwintering in the North of Africa and the South of the Iberian peninsula (Cramp, 1988). Bluethroats show great site fidelity to their breeding grounds (Arizaga & García, 2013), so the genetic diversity observed in Europe could be due to geographical isolation and local adaptation processes (Johnsen *et al.* 2006). Ten subspecies have been described according to both male throat ornament and plumage patterns, although the taxonomic classification of this subspecies complex is not exempt from

controversy (García *et al.* 2017). The subspecies complex is associated with phenotypic variations and life history. Bluethroats breed in a wide variety of habitats across their distribution range, from the sea level up to 4000 m a.s.l.: (i) In North and Central Europe, the species is mostly associated with alpine and subalpine low scrublands and wetlands; (ii) in France, it breeds in salt meadows; and, (iii) in the Iberian peninsula, bluethroats nest in broom and holm oak shrublands (Cramp, 1988). The Iberian subspecies *L. s. azuricollis,* located at the southernmost edge of the species' range in Eurasia, is genetically and phenotypically well differentiated from the other subspecies (Johnsen *et al.* 2006, Hogner *et al.* 2013, García *et al.* 2017) and shows higher genetic differentiation than their north-European counterparts (Alda *et al.* 2013).

Bird sampling, DNA extraction and microsatellite genotyping

Between 2010 and 2011, we sampled 266 bluethroats in 20 breeding localities distributed all across the whole *L. s. azuricollis* range in the Iberian Peninsula: nine in the Cantabrian Mountains, nine in the Mountains of León and two in the Central System (Figure 3.1; Tables Chap.3.1S1 and Chap.3.2S1 from Appendix Chap.3.S1). From this set of samples, 83 had been included in an earlier study (Alda *et al.* 2013). Bluethroats were captured using tape-lured mistnets and clap-traps baited with mealworms (Johnsen *et al.* 2006). We extracted blood samples by venepuncture of the brachial or jugular vein, preserved them in absolute ethanol and stored at -20°C. Genomic DNA from blood was extracted and samples were genotyped for 12 microsatellite loci: Aar8, Ase19, Cuµ4, Cuµ10, Fhu2, Hru7, Mcy4, PAT MP 2-43, Pdo5, Phtr2, PmaC25, and Ppi2. Two of the 12 loci screened were removed from further analyses because Pdo5 showed strong evidence of null alleles and Aar8 was monomorphic. A more detailed description of DNA extraction and microsatellite amplification can be found in Alda *et al.* (2013).

Genetic patterns and processes

We investigated the genetic structure of Iberian bluethroat breeding populations using GENELAND v. 4.0.4 (Guillot *et al.* 2005) (See Appendix Chap.3.S2 for further information on GENELAND settings). The differentiation was evaluated using F_{ST} values, as a measure of pairwise genetic distance (Weir & Cockerham, 1984), with the "hierfstat" R-package (Goudet 2005). Bonferroni correction was applied on p-values for F_{ST}. We used ANOVA to test differences in genetic diversity among clusters and localities on the basis of the number of different alleles (N_a), the observed heterozygosity (H_{obs}) and the expected heterozygosity (H_{exp}), that were calculated in GenAIEx v.6.5 (Peakall & Smouse 2012) and FSTAT v.2.9.3 (Goudet 1995).

The Inbreeding Fixation Index (F_{IS}) was quantified for each cluster and locality using GenAIEx v.6.5. Additionally, the effective population size (Ne) was estimated for each cluster to explore both current population size and population demographic history using LDNe v.1.31 software (Waples & Do 2008). This analysis is based on the Burrow's measure of linkage disequilibrium and assumes that genetic drift is the only process responsible for the signal in the data, instead of selection, mutation or migration processes. We excluded those alleles with a frequency lower than 0.02 and calculated 95% confidence intervals using a jack-knife approach.

Finally, we tested for genetic signals of a rapid reduction in population size at both cluster and locality levels using the program BOTTLENECK 1.2.02 (Piry *et al*. 1999). We assumed a two-phase model of multi-step mutation, accounting for 5%, 10% and 20% of all mutations. Significance of heterozygosity excess was determined using the Wilcoxon signed-rank test, which provides the most powerful statistical results for tests of bottlenecks in datasets with small samples sizes and less than 20 loci. Following statistical recommendations for the locality level analyses (Kalinowski 2005, Peery *et al.* 2012), we only included those sites where 12 or more individuals were genotyped (12 sites; Table Chap.3.1S2).

Current and recent past landscape patterns: Habitat suitability modelling analysis

We modelled habitat suitability for Iberian breeding bluethroats using MaxEnt 3.3.3k (Phillips *et al.* 2006), which is a machine learning method designed for evaluating the species-environment relationship on the basis of species presence-only data (Elith *et al.* 2006, 2011). Since our focus was on identifying areas holding suitable habitat between the existing populations, rather than predicting the potential distribution patterns over the entire Iberian Peninsula, the extent of the study area was fixed as a squared window including the current breeding range of the subspecies in the Iberian Peninsula (120.000 km^2; Figure 3.1).

Bluethroat occurrence data used for model calibration included locations covering the whole breeding range of the subspecies that were sourced from own fieldwork during the period 2010-2011, local publications, museum collections and local ornithologists. A total number of 796 field surveys were conducted following a point transect sampling method (Bibby *et al.* 2000) during the breeding season (from 1st May to 15th June, 2010 and 2011), three hours after sunrise and under good weather conditions. At each sampling point, species presence was recorded during five minutes by both visual and hearing contacts. After removing duplicates (we kept only one observation per 200 m grid cell, the spatial resolution of the analysis), the number of records available to fit the models was of 262 (Figure 3.2a). The background dataset was a sample of 10,000 random points collected across the study area, encompassing the prediction locations and informing on the distribution of the covariates in the landscape.

We selected a set of environmental variables informing on climate, topography and vegetation that are likely to influence breeding habitat suitability for bluethroats (Table 3.1): (i) Climatic variables included the average mean and minimum temperatures of spring (March, April and May; corresponding to species arrival to

breeding sites, mating and nesting) and summer (June, July and August; corresponding to fledging and post breeding months prior to migration), the annual seasonality (standard deviation of monthly temperature as a measure of continentality) and the total annual rainfall. These predictors were derived from the Climatic Digital Atlas of the Iberian Peninsula (Ninyerola *et al.* 2005) that was built at 200 m of spatial resolution based on climatic data for the period 1951-1999. (ii) Topographic factors included slope and southness (as a measure of aspect), both extracted from a 200m digital elevation model (Spanish Geographic Institute), as well as annual total radiation. (iii) Vegetation was estimated using a temporal series of Normalised Difference Vegetation Index (NDVI; Rouse *et al.* 1973) values derived from NOAA-AVHRR satellite images at 1 km resolution for the period 1987-2010. NDVI is a simple and easily interpretable index that combines visible and near-infrared reflectance measurements. It is considered as a proxy of net primary production and vegetation vigour, emerging as a powerful indicator to explore the link between animal distribution and available resources (Pettorelli *et al.* 2011).

Satellite images were daily collected and geometrically and atmospherically corrected using standard calibration coefficients (Rao & Chen 1999) at the Spanish Remote Sensing Laboratory of Valladolid University. After eliminating the clouds, radiometric errors were reduced using the monthly maximum value composite method (Holben 1986). The annual mean of monthly NDVI composites for 2010 (contemporary to bluethroat sampling; $NDVI_{2010}$) for each pixel was then used to estimate spatial variation on current vegetation condition. We also calculated the coefficient of variation of annual series of NDVI values collected across the period 1987-2009 (more than two decades before the year of species sampling; $NDVI_{CV}$), and used it as an estimate of the recent past landscape stability, vegetation composition and vigour.

We fitted three models accounting for breeding habitat suitability across the landscape: (i) A CLP-Current Landscape Patterns - model based on climate, topography and current NDVI values ($NDVI_{2010}$); (ii) a LS- Landscape Stability - model based on climate, topography and the $NDVI_{CV}$ as a measure of recent past landscape stability; and, (iii) a CLP&LS-Current Landscape Patterns and Landscape Stability - model accounting for the additive effect of current landscape and recent past landscape stability ($NDVI_{2010}$ and $NDVI_{CV}$, respectively), along with climatic and topographic variables. To minimize multi-collinearity problems, we evaluated the Pearson's pairwise correlations between all candidate predictors on a sample of 50000 points randomly distributed across the study area. When two or more variables were strongly correlated ($r_{Pearson} > 0.7$; Tabachnick & Fidell 1996) we kept the variable with a more direct interpretation from the point of view of the ecology of the species. Variables retained for fitting the models are detailed in Table 3.1 and Figure 1S3. The logistic output of the models can be interpreted as a habitat suitability index ranging from 0 to 1 (García *et al.* 2007). A value near 1 represents sites where the habitat is optimal for the bird; a value near 0 corresponds to unsuitable sites.

We controlled the complexity of the response shapes by allowing only for linear, quadratic and product features. Models with these restricted feature types are smoother than those fitted with the default settings, less prone of being fitted to data idiosyncrasies and potentially better at predicting new places (Merow *et al.* 2014). Model predictive performance (discrimination ability; Guillera-Arroita *et al.* 2015) was assessed using the area under the receiver-operator characteristic curve (AUC; Hanley & McNeil, 1982), adapted for its use with presence-background samples (Phillips *et al.* 2006). AUC values range from 0 to 1, where 1 indicates perfect model discrimination and 0.5 a model no better than random (Elith *et al.* 2006). To reduce bias in the assessment of model performance, we performed statistical resampling, where AUC values were calculated by 10-fold cross-validation. This procedure is used to avoid overfitting by testing predictive accuracy on withheld portions of the data (Phillips *et al.* 2009). We also calculated the Akaike Information

Criterion (AIC; Burnham & Anderson, 2002) value for each Maxent model using the package ENMeval (Muscarella *et al.* 2014), in order to evaluate which model best fit the observed data.
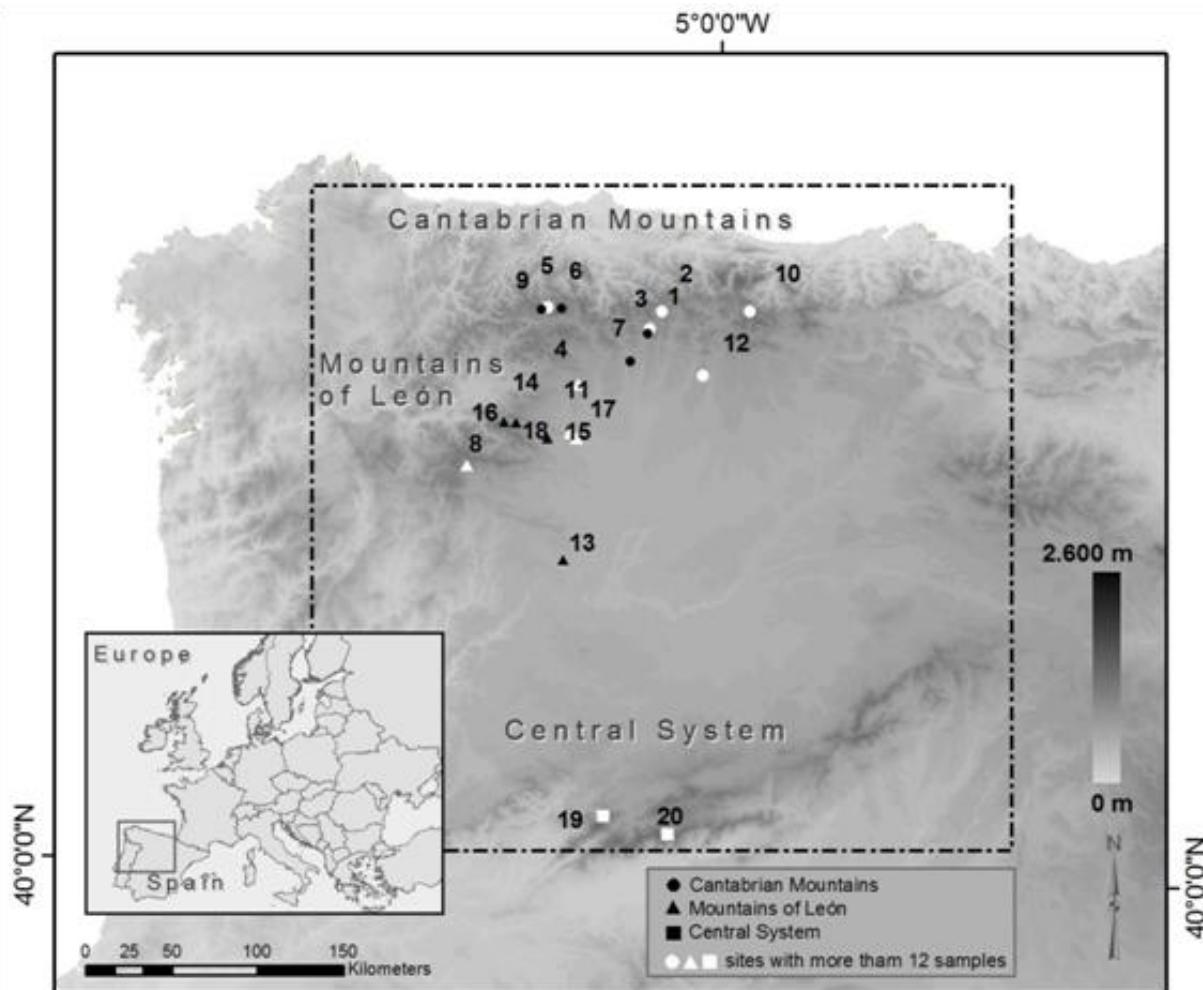
Figure 3.1. Limit of the study area, fixed considering the current breeding range of the subspecies in the Iberian Peninsula. The figure shows the localities sampled for genetic analyses. See Table 3.1 for correspondence between site numbers and names. Symbols represent different mountain regions within the breeding range of *Luscinia svecica azuricollis* (circles: Cantabrian Mountains, triangles: Mountains of León, squares: Central System). Symbols in white indicate sites with more than 12 samples. The dashed rectangle delimits the area where the habitat suitability model was fitted.
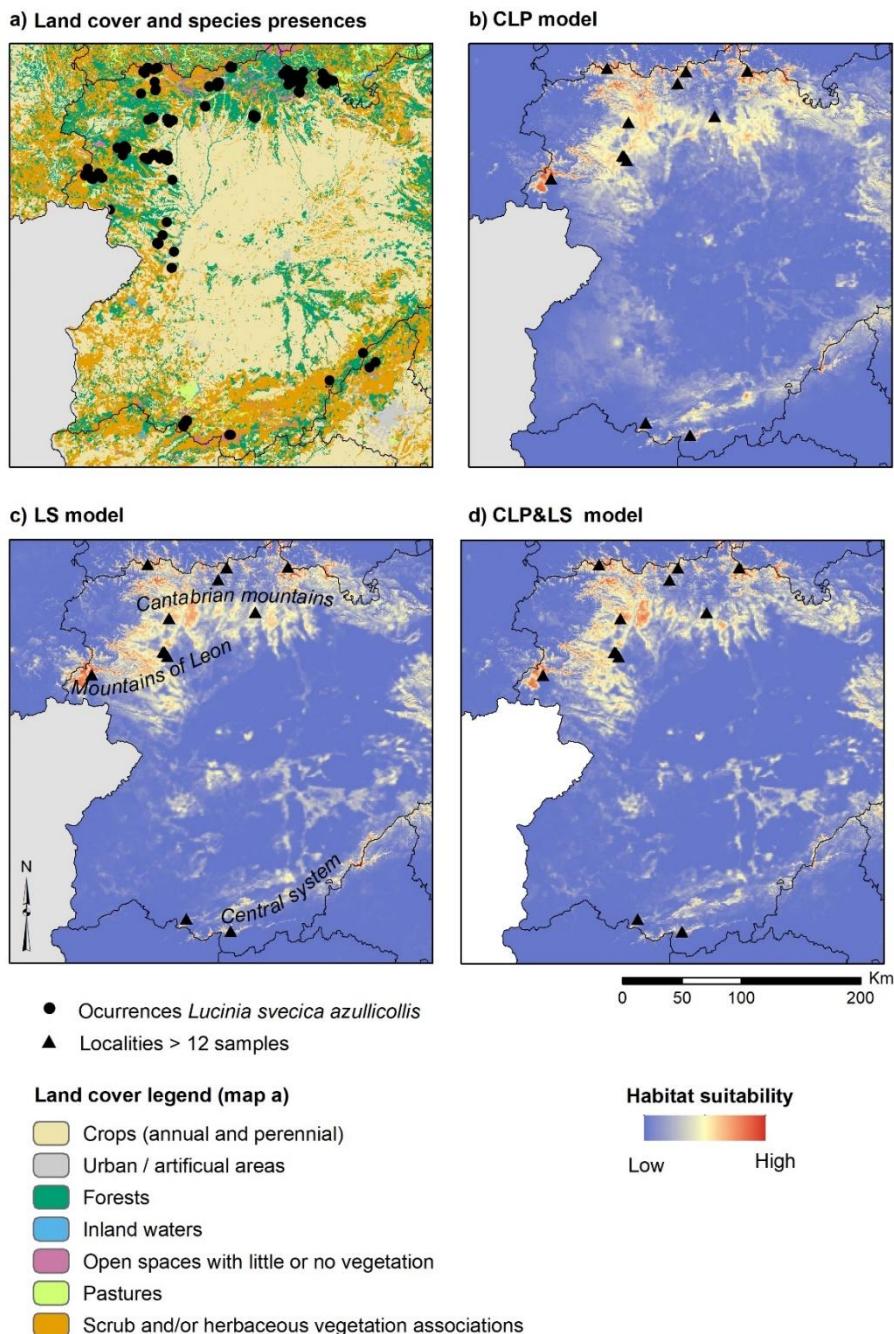
Figure 3.2. a) Occurrence data (black points) of *Luscinia svecica azuricollis* used to fit the MaxEnt model in the Iberian Peninsula. The map also shows the main land cover types present in the study area (source: CORINE land cover 2006 - CLC2006: https://land.copernicus.eu/pan-european/corine-land-cover/clc-2006). Note the large extension of agricultural crops in between the mountain ranges where the species breeds. b) Habitat suitability map based on current landscape patterns (CLP model output. c) Habitat suitability map based on recent past landscape stability (LS model output). d) Habitat suitability map considering the additive effect of current landscape patterns and landscape stability (CLP&LS model output). Maps b), c) and d) show the localities where more than 12 samples were collected (used for both genetic and connectivity analyses).

Table 3.1. List of predictors accounting for climate, topography and vegetation that have been considered for modelling the habitat suitability of Iberian bluethroats.

Variable	Description	Resolution (m)
Climate		
*TEMP$_{SPRING}$	Average mean temperature in spring (March, April and May)	200
TEMP$_{SUMMER}$	Average mean temperature in summer (June, July and August)	200
TMIN$_{SPRING}$	Averaged minimum temperatures of spring (March, April and May)	200
TMIN$_{SUMMER}$	Averaged minimum temperatures of summer (June, July and August)	200
*BIO4	Seasonality: Annual temperature variation based on the standard deviation of monthly temperature averages	200
*RAI	Total annual rainfall	200
Topography		
*SLO	Slope expressed in degrees from a digital elevation model (Spanish Geographic Institute)	200
*SOUTH	Southness. Measure of the aspect normalized to a -1 to 1 scale. Calculated as: Cos (((aspect)180º) DIV degrees)	200
*RAD	Total annual solar radiation	200
Land cover		
*NDVI$_{2010}$	Annual Mean NDVI values for 2010 derived from a series of monthly Maximum Value Composites of Normalised Difference Vegetation Index images (NDVI-MVC)	1000‡
*NDVI$_{CV}$	Coefficient of variation of NDVI values of an annual series of Maximum Value Composites of Normalised Difference Vegetation Index (NDVI-MVC) for the period 1987 -2009	1000‡

Low correlated variables ($r_{Pearson}$ < 0.7) that were retained for modelling analyses are preceded by the symbol *.
‡ NDVI values were assumed constant within all 200 m cells contained in each km

Landscape resistance surfaces

We converted the habitat suitability maps achieved from CLP, LS and CLP&LS models into resistance surfaces using different transformation functions to test different hypotheses about the potential resistance of landscape attributes to species dispersal (Figure 2S3 from Appendix S3). At one end, we assumed that the larger amount and connectedness of suitable habitat between two localities, the smaller the genetic distance (i.e. a linear relationship between habitat suitability h and landscape resistance R, where $R = 1 – h$). At the other end, we assumed that landscape resistance is minimum at mid - high values of habitat suitability, but very high when habitat suitability is low (i.e. a negative exponential function $R = h^{-1}$). Between these two extremes, following Trainor *et al.* (2013), we used Equation 1 to estimate resistance surfaces that asymptotically approached both the linear and negative exponential extremes depending on the value of a single rescaling parameter c.

Eq. 1 $R = 100 - 99 \frac{1-\exp(-c \, x \, h)}{1-\exp(-c)}$

Considering different values of c, from 0.25 to 16, we generated seven additional resistance layers for each habitat suitability model. Values in all resistance layers ranged from 1 to 100, representing minimum and maximum resistance values, respectively.

We also created a resistance layer assuming homogeneous resistance to movement across the territory (R =1) to test for the effect of geographic distance on bluethroat genetic differentiation (IBD hypothesis).

Connectivity modelling analysis

We used the eight-neighbour cell-connection scheme settings in CIRCUITSCAPE v.4.0 (McRae 2006; McRae & Shah 2011) to evaluate pairwise distances between localities with more than 12 samples based on: (i) IBD; (ii) isolation by landscape resistance derived from current landscape patterns (IBCR), isolation by landscape resistance derived from landscape stability (IBLSR) and isolation by landscape resistance derived from both current landscape and stability (IBC&LSR). The IBCR, IBLSR and IBC&LSR hypotheses were evaluated using the multiple resistance layers derived from CLP, LS and CLP&LS models, respectively. CIRCUITSCAPE is a tool that incorporates the principles of the electric circuit theory to model gene flow and differentiation in plant and animal populations (McRae *et al.* 2008). It estimates a unique value of pairwise resistance between two locations by integrating dispersal through grid cells with different resistances to species movement over all potential dispersal paths. It has been successfully used to model genetic connectivity between populations in heterogeneous landscapes (Phillipsen & Lytle 2012, Amos *et al.* 2014).

## Effect of geographic and landscape resistance on genetic differentiation

To assess the relationship between genetic distance (FST values) and landscape resistance to species dispersal (IBD, IBCR, IBLSR and IBC&LSR), we fitted Maximum-Likelihood Population Effects models (MLPE) accounting for the non-independence of pairwise distance data (Clarke *et al.* 2002; Van Strien *et al.* 2012). These models perform well in landscape genetics model selection approaches when compared to other methods able to accommodate pairwise distances, such as Mantel tests (Shirk *et al.* 2017). Models were fitted using the ResistanceGA R Package (Peterman, 2018), setting REML=FALSE in the mlpe_rga () function. This results in an unbiased Akaike Information Criterion (AIC; Burnham & Anderson, 2002) score fit on maximum likehood estimates. Candidate models were compared using the AICRMLE score and two R2 estimates (marginal R2 GLMM(m) and conditional R2 GLMM(c)), which measure the variance explained by the fixed effects parts of the MLPE model only (i.e. the pairwise IBD, IBCR, IBLSR or IBC&LSR resistance distance matrices) or by both the fixed and random effects, respectively (Nakagawa *et al.* 2013, 2017, Johnson 2014). R2 estimates were calculated using the r.squaredGLMM function in the R.package MuMin (Barton 2019). We compared models based on a single predictor, either IBD or the multiple variations of IBCR, IBLSR and IBC&LSR resistance matrices (Figure 3.3).

Results
Genetic patterns and processes

The GENELAND Bayesian clustering analysis evidenced an optimal genetic structure of four clusters (Figure 3.4; Figure Chap.3.1S2 from Appendix Chap.3.S2). Genetic differentiation was significant among localities after Bonferroni adjustement (global F_{ST} = 0.022, p=0.0002) in 41 out 66 pairwise comparisons (62%, significant F_{ST}=0.002-0.091; Table Chap.3.1S2 from Appendix Chap.3.S2). Genetic diversity parameters, however, did not differ significantly among genetic clusters or localities (ANOVA, p>0.05) (Table 3.2 and Table Chap.3.2S2 from Appendix Chap.3.S2). The estimates of the inbreeding coefficient (F_{IS}) differed among clusters (p=0.0013, 800 randomizations).
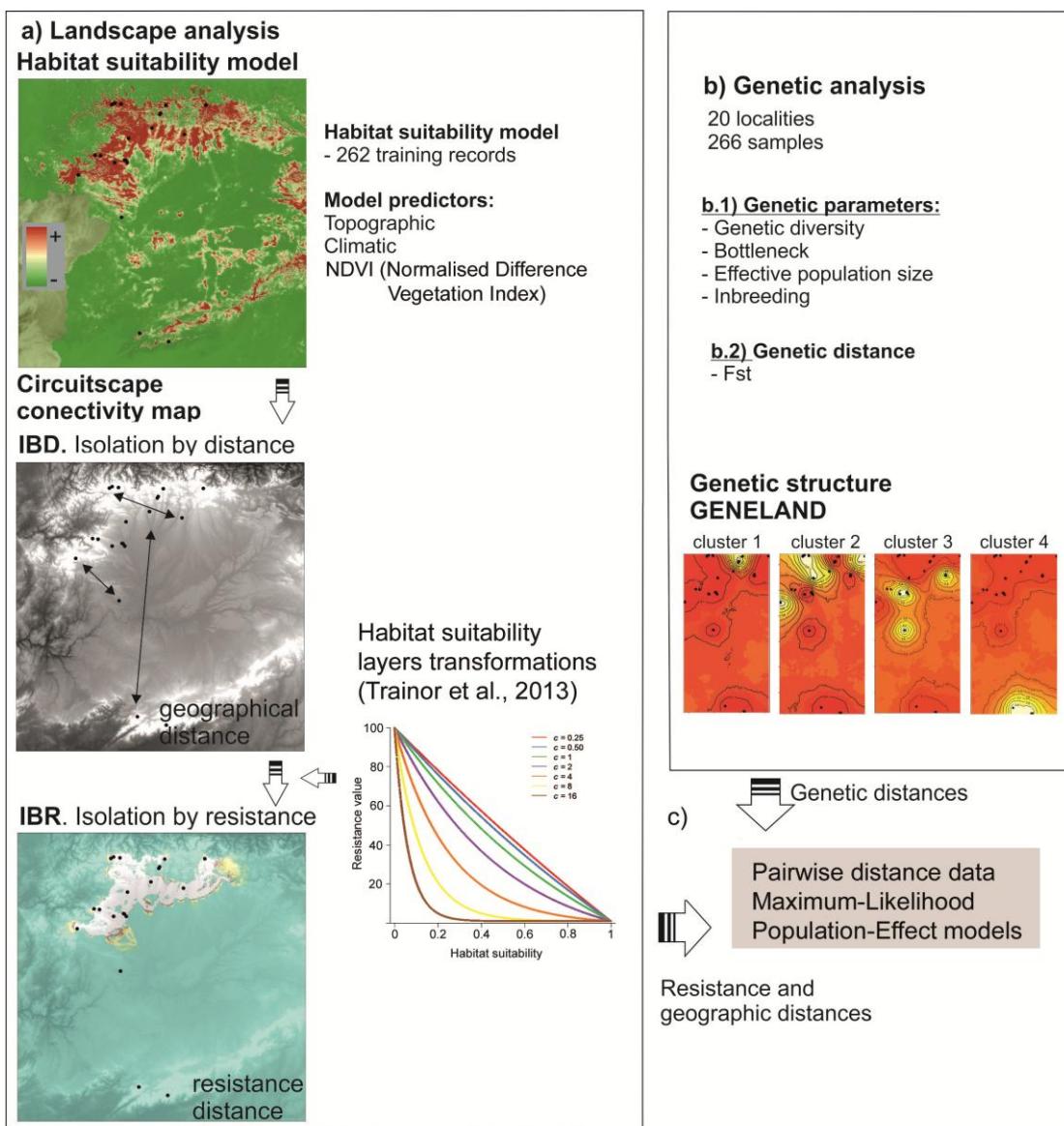
Figure 3.3. Flow chart showing the methodological steps carried out in the study: a) Landscape permeability analysis included: habitat suitability modelling, generation of resistance surfaces, Circuitscape connectivity assesment based on Isolation-By-Distance and Isolation-by-Resistance (IBD and IBR, respectively) and pairwise distances calculation. b) Genetic analysis encompassed genetic parameters estimation and quantification of genetic distances among populations based on F_{ST} values. c) Correlations of pairwise landscape resistance and geographic distance with genetic distance were evaluated with Maximum-likelihood population-effect models.

The local inbreeding coefficient (F_{IS} value over all loci) was positive and significantly different from zero in clusters K1 (F_{IS}= 0.071, p=0.0300) and K4 (F_{IS}= 0.072, p=0.0075), as well as in a locality sited in the Central System (F_{IS}= 0.101, p=0.0098). F_{IS} values were significantly positive for three loci in K1, one locus in K2, three loci in K3 and two loci in K4 (Table 3.2; Tables Chap.3.2S2 and Chap.3.3S2 from Appendix Chap.3.S2). The mean effective size (Ne) of the breeding population of Iberian bluethroats was 522 individuals (Jackknife 95% CI = 304-1371). No significant excess of heterozygosity was detected in any of the genetic clusters, indicating no evidence of recent bottlenecks. However, a significant heterozygosity excess was found in three localities. (p<0.05, Table 3.2).
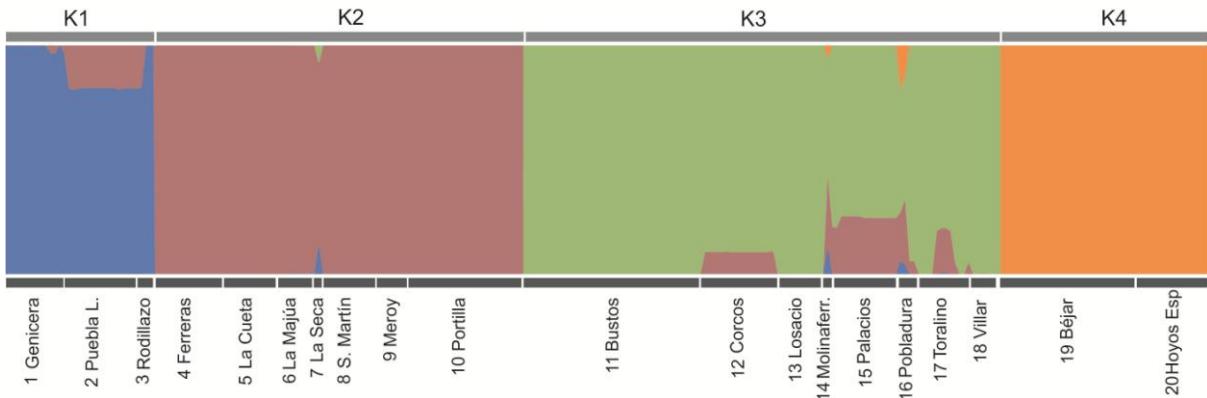
Figure 3.4. Individual bayesian assignment of 20 sampling localities and 266 individuals to each GENELAND cluster.

Habitat suitability models

The predictive performance of CLP (AUC mean ± SD = 0.948 ± 0.015), LS (AUC mean ± SD = 0.951 ± 0.009) and CLP&LS (AUC 0.957±0.011) habitat suitability models was high, indicating good model discrimination. Spring temperature, annual rainfall and vegetation (NDVI) explained more than 85% of the variance in all models (Table 3.3). Despite the temperature being the most relevant variable in all cases, vegetation played a major the role in defining breeding habitat suitability, explaining 30% of the variance in CLP model. Topographic predictors (slope, radiation, southness) and seasonality were relatively unimportant in the models (contribution <6%). The highest values of habitat suitability for the focal species were found at the Northern and Eastern part of the study area (Figure 3.2b, c and d). These correspond to areas with low/medium NDVI coefficient of variation (LS and CLP&LS models: areas where vegetation has remained mostly stable during two decades prior to bird surveys), low annual precipitation and low spring average mean temperatures (see Figure 3S3, 4S3 and 5S3 in Appendix S3 for the response curves of the predictors). The AIC values for the CLP, LS and CLP&LS models were 7037, 7011 and

6951, respectively. These values suggest that CLP&LS model, considering the additive effect of current attributes and temporal stability of landscape), was the best at explaining bluethroat occurrence.

Effect of geographic and landscape resistance on genetic differentiation

Genetic distance pattern was better supported by landscape resistance than by Euclidean distance (ΔAIC_{Euc} >5; Table 3.4). IBCR, IBLSR and IBC&LSR models explained between 62% and 76 % of the variation in genetic distances, with small differences among models, depending on the transformation function considered. The best performing transformation functions were those assuming a linear or quasi-linear relationship (c= 0.25 - 2; Figure 2S3 from Appendix S3) between habitat suitability and landscape resistance. The negative exponential and the transformation functions approximating the negative exponential (c =8, 16) performed only slightly better than the Euclidean function. This suggests that subtle differences in intermediate or high-quality habitat strongly influence species dispersal.

Discussion

This study showed evidences of a significant genetic structure in Iberian breeding bluethroats that correlated better landscape resistance than to geographic distance. Regardless geographic distance, functional connectivity (and hence, gene flow) between breeding areas was higher when the intervenient landscape among populations consisted of temporally-stable patches of highly suitable breeding habitat, being the relationship between gene flow and presence of intervenient habitat linear or quasi-linear.

Table 3.2. Genetic diversity in the 12 localities with 12 or more individuals genotyped and BOTTLENECK results testing the null hypothesis of mutation-drift equilibrium under the two-phase model, using the Wilcoxon test (TPM, 95% single step mutations and 5% multiple step mutations). Na: Average number of alleles per locus; H_{obs} and H_{exp}: average observed and expected heterozygosity; F_{IS}: inbreeding coefficient. Figures in bold indicate F_{IS} values significantly different from zero and rejection of null hypothesis of mutation drift equilibrium (p<0.05).

	Genetic diversity				Bottleneck		
Locality	Na	H_{obs}	H_{exp}	F_{IS}	One tail, heterozygosity deficiency	One tail, heterozygosity excess	Two tail, both outcomes
01Genicera	4.5	0.594	0.613	0.073	0.903	0.116	0.232
02Puebla de Lillo	4.9	0.623	0.642	0.060	0.947	0.065	0.131
04Ferreras de Cepeda	4.9	0.624	0.626	0.034	0.984	**0.042**	0.084
05La Cueta	4.6	0.607	0.612	0.048	0.997	**0.005**	**0.010**
08San Martín de Castañeda	4.9	0.716	0.625	-0.103	0.722	0.313	0.625
10Portilla de la Reina	5.2	0.626	0.638	0.039	0.958	0.053	0.106
11Bustos	5.9	0.644	0.648	0.020	0.947	0.065	0.131
12Corcos	5.2	0.601	0.600	0.031	0.615	0.423	0.846
15Palacios de la Valduerna	5.2	0.626	0.634	0.049	0.813	0.216	0.432
17Toralino	4.9	0.669	0.638	-0.012	0.991	**0.012**	**0.024**
19Béjar	5.8	0.596	0.619	0.055	0.688	0.348	0.695
20Hoyos del Espino	4.8	0.507	0.542	**0.101**	0.278	0.754	0.557

These results suggest that despite the high vagility of bluethroats, structural habitat connectedness between landscape patches showing low temporal variability in vegetation condition play an important role at facilitating Iberian bluethroat breeding dispersal. However, it should be highlighted that our models explained only a part of the genetic variation (< 72 % in all cases). A reason could be that dispersal is not only related to landscape resistance or geographic distance, but is also associated to other factors not considered in this study, including behavioural traits (mating systems and philopatry), demographic parameters (effective population size and variations in breeding success between populations), physiological tolerance or dispersal ability. All these factors

can interact and shape genetic structure at different spatio-temporal scales (Anderson *et al.* 2010, Athrey *et al.* 2012). For instance, strong site fidelity could explain the small rates of gene flow of populations found at low- mid latitudes (FitzSimmons *et al.* 1997, Van Bekkum *et al.* 2006) in comparison with the northernmost grounds, where breeding site fidelity is much lower (Both & Visser 2001, Martin & McKay 2004). The interaction between breeding site fidelity, habitat fragmentation and small population size might result in an increase of genetic drift (Rousset 1997). The fact that the model considering the potential additive effect of current landscape patterns and recent past landscape stability did not explain more genetic

Table 3.3. Permutation importance of each environmental variable in Maxent models. The values of each environmental variable are randomly permuted, in turn, on training presence and background data.

Variable	CLP model	LS model	CLP&LS model
TEMP$_{SPRING}$	**47.0**	**55.2**	**43.5**
BIO4	6.0	5.7	6
RAI	**21.6**	**17.6**	**16.2**
SLO	2.4	3.5	2.7
SOUTH	0.6	0.6	0.5
RAD	0.1	0	0.2
NDVI$_{2010}$	**22.4**	-	**11.6**
NDVI$_{CV}$	-	**17.4**	**19.2**

The model is reevaluated on the permuted data and the resulting drop in training AUC is shown in the table, normalized to percentages. Variables are: average temperature in spring (TEMPSPRING), annual seasonality (BIO4), total annual rainfall (RAI), slope in degrees (SLO), southness (SOUTH), total annual radiation (RAD), annual mean NDVI for 2010 (NDVI2010) and coefficient of variation of NDVI for the period 1987-2009 (NVDICV). CLP model: model accounting current landscape attributes; LS model: model accounting for recent past landscape stability; CLP&LS model: model accounting for both current landscape and recent past landscape stability. In bold variables contributing more than 10%.

variation than models based on each of these terms separately (current *vs.* recent past landscape stability) also supports this idea. Another explanation may relate to the fact that we used NDVI values as subrogates of vegetation composition and vigour, may show limitations to capture completely what makes a particular site especially suitable for the species in terms of habitat conditions. Likely, NDVI resolution (1 km) did not allow to detect subtle differences in the within-pixel grassland availability, where bluethroats require less foraging effort (the species forages for food on the ground). Moreover, climatic predictors explained most of the variance in all habitat suitability models (CLP, LS and CLP&LS; 60%; Appendix S3), which might partly explain why all models explain similar amounts of genetic variation. The maintenance of suitable breeding habitat for bluethroats depends on both human activities and natural processes, such as

secondary succession after disturbance (Calvo *et al.* 2002). The structure of these habitats is inherently variable and, consequently, their population carrying capacity changes constantly. In fact, many landscape patches holding suitable habitat may disappear because of cutting and burning or intensive cattle farming (Morán-Ordóñez *et al.* 2013). Following disturbance, patches may rapidly recover due to auto-succession processes and, then, be re-occupied by bluethroats in an estimated period of five to ten years (authors, unpublished data). At the other end, current patches of suitable habitat may become permanently unsuitable for the focal species in the short mid-term if shrublands evolve into forest habitats, as a result of secondary succession, a prevailing process in the mountain systems under study (Morán-Ordóñez *et al.* 2013, Álvarez-Martínez *et al.* 2014). This problem is especially relevant for the bluethroat populations breeding at the foothills of the Cantabrian Mountains and Mountains of León, where the abandonment of livestock farming is more pronounced and the populations of wild ungulates are less numerous. Nevertheless, this situation can be rapidly reversed by disturbance processes like fire that, in the study area, is a management tool widely used by local farmers to facilitate the regeneration of pastures supporting extensive grazing (Viedma *et al.* 2006).

Iberian breeding populations are isolated from each other (especially those in the Central System) and also from other European populations (note that the closest populations are over 500 km away, in France, and correspond to the subspecies *L. s. namnetum;* Johnsen *et al.* 2006). Peripheral populations often present low levels of genetic diversity and high inbreeding in comparison with populations located at the core of the species distribution range (Hoffman & Blows 1994, Hampe & Petit 2005). This is the case, for example, of *L. s. namnetun* and *L. s. azuricollis*, whose breeding areas are the most geographically isolated, showing the lowest allelic richness and heterozygosity and the highest inbreeding coefficients within the subspecies complex (Johnsen *et al.* 2006). We found significant values of inbreeding for Iberian

populations, being consistent with isolation and small population size. In these situations, mating is non-random and occurs among close relatives, resulting in an increase in the rate of homozygotic descendants in a few generations. This fact may have severe implications for subspecies persistence, as it would reduce its capacity to respond against future environmental changes (Keller & Waller 2002, Hoffman & Sgrò 2011). Indeed, the estimated effective population size was very small for the subspecies as a whole, as well as for the genetic clusters. In some cases, it was lower than 500 individuals, the minimum value considered necessary to guarantee long-term genetic integrity (Franklin & Frankham 1998). The bottleneck episodes detected in three of the evaluated localities, together with the reduced effective population size in some of the genetic clusters, suggest recent declines in the Iberian bluethroat breeding population size, at least locally. The explicit consideration of bottleneck episodes is of great relevance because they may lead to a high expression of recessive alleles, reducing individual fitness, genetic diversity and population viability, which make populations less capable to adapt against environmental changes (Bouzat 2010, Velando *et al.* 2015).

Implications for conservation

Since the second half of the last century, the Iberian mountains where bluethroats inhabit are being affected by rural abandonment that leads to vegetation encroachment, landscape homogenisation and afforestation (Herrando *et al.* 2014, Lasanta *et al.* 2016). These changes are reducing the availability of semi-natural breeding habitats in mountain areas of the Iberian Peninsula, which, according to the results of our study, may have severe consequences on bluethroat genetic structure and connectivity.

However, within the subspecies range, there are still many unoccupied suitable areas, which suggests that habitat suitability is not enough to understand the genetic composition and structure of this subspecies and that, managing bluethroat populations still needs of improved knowledge on the behavioral traits of this subspecies. However, with the current

knowledge, habitat management is the main action that can be practically implemented to conserve bluethroat populations across the study area.

Current habitat management policies related to shrublands and heathlands, as those related to livestock farming, wild herbivorous pressure, wildfire prevention or land abandonment, can have a significant impact on the Iberian bluethroat population dynamics in the mid- and long-term. Our results suggest that promoting the spatial connectedness and temporal stability of suitable habitat (shrub/heath – pasture mosaics) in heterogeneous human-shaped landscapes would improve gene flow and, consequently, species persistence.

These management interventions should be prioritized in the areas with the highest risk of habitat loss (due to either land abandonment and natural succession or to habitat removal for intensification) and higher potential impact for the subspecies. In particular, our results suggests especial conservation efforts should be made to preserve the Central System bluethroat population, which is located at the southernmost limit and highest altitude of the subspecies range, and shows the highest genetic and phenotypic divergence and isolation, as well as the smallest population size. On the other hand, in this area there is a high risk of habitat loss associated to tourist pressure (skiing and mountain tourism) and wildfires. As it is not possible to implement measures to improve functional connectivity between this and the populations in the north of the Peninsula, due to distance (150 km) and the lack of suitable intervenient habitat (Figure 3.2), management should be focused on promoting habitat connectivity between localities.

Management actions that could prove beneficial to increase suitable habitat between localities include for example, the maintenance of extensive livestock farming because livestock maintain open spaces where prey diversity is highest (García-Tejero *et al.* 2013) and limit forest expansion and landscape homogeneization (Lasanta *et al.* 2016, Guadilla-Sáez *et al.* 2019).

Table 3.4. Results of the maximum-likelihood population effects models (MLPE) for the relationship between genetic distances (F_{ST}) and each of the species dispersal hypotheses tested: "Current" for isolation by current landscape resistance (IBCR), "Landscape stability" for isolation by landscape resistance derived from landscape stability (IBLSR), "CurrentLS" for isolation by landscape resistance derived from both current landscape and stability (IBC&LSR) and "Euclidean distance" for isolation by distance (IBD). The field "resistance" indicates the rescaling approach used to convert habitat suitability values into resistance distances (linear, negative exponential – Exp^{-1} – or values of the C rescaling factor of 0.25, 0.5, 1, 2, 4, 8, 16 in Eq. 1: Figure 2S3 in Appendix S3). AIC is the Akaike's Information Criterion generated for each MLPE model. ΔAIC_{null} is the difference between the AIC value generated for each MLPE model and the AIC of a 'null' model with the same structure of random effects and no explanatory fixed predictors. ΔAIC_{Euc} is the difference of AIC between landscape resistance and Euclidean distance models. $R^2_{GLMM(m)}$ and $R^2_{GLMM(c)}$ are the marginal and conditional R^2 values of the fitted MLPE model, respectively.

Landscape	Resistance	AIC	ΔAIC_{null}	ΔAIC_{Euc}	$R^2 GLMM(m)$	$R^2 GLMM(c)$
Current	2	127.72	-61.12	-16.69	0.66	0.76
Landscape stability	2	127.90	-60.95	-16.51	0.66	0.73
Landscape stability	1	128.05	-60.80	-16.36	0.65	0.73
Current	1	128.18	-60.66	-16.23	0.66	0.75
Landscape stability	0.5	128.47	-60.38	-15.94	0.65	0.72
Landscape stability	Linear	128.72	-60.12	-15.69	0.65	0.72
Current	0.5	128.76	-60.08	-15.65	0.65	0.74
Landscape stability	0.25	128.77	-60.08	-15.64	0.65	0.72
Current	4	129.08	-59.76	-15.33	0.66	0.76
Current	0.25	129.14	-59.71	-15.27	0.65	0.74
Current	Linear	129.18	-59.66	-15.23	0.65	0.74
Landscape stability	4	129.54	-59.31	-14.87	0.65	0.73
CurrentLS	2	131.86	-56.99	-12.55	0.64	0.72
CurrentLS	1	132.21	-56.63	-12.20	0.63	0.72
CurrentLS	0.5	132.65	-56.19	-11.76	0.63	0.71
CurrentLS	4	132.70	-56.15	-11.71	0.64	0.73
CurrentLS	0.25	132.94	-55.91	-11.47	0.63	0.71
CurrentLS	Linear	132.98	-55.87	-11.43	0.62	0.71
Current	8	134.08	-54.77	-10.34	0.64	0.74
Landscape stability	8	134.32	-54.53	-10.09	0.63	0.73
CurrentLS	8	135.91	-52.94	-8.50	0.63	0.72
CurrentLS	Exp-1	136.64	-52.21	-7.78	0.62	0.72
Current	16	137.00	-51.85	-7.41	0.62	0.72
Landscape stability	Exp-1	137.32	-51.53	-7.10	0.62	0.72
Landscape stability	16	137.57	-51.27	-6.84	0.62	0.72
Current	Exp-1	137.83	-51.01	-6.58	0.62	0.72
CurrentLS	16	137.98	- 50.87	- 6.43	0.62	0.72
Euclidean		144.41	-44.44	0.00	0.54	0.61

Also, the maintenance of other frequent low-intensity disturbances in the landscape are also a key to maintain bluethroat habitat: experimental treatments in mountain areas in the north of the Iberian Peninsula showed shrubland communities recover their original state nine years after burning and clearing, with trees starting to encroach shrublands 15 years after the disturbances (Calvo *et al.* 2002). Thus, in areas with presence of bluethroat populations where extensive farming has been abandoned, conservation interventions such as frequent shrub-clearing with machinery or controlled-burning promoting the maintenance of shrubland-pasture mosaics (Morán-Ordóñez *et al.* 2013) should be adequately planned and implemented if the species is to be preserved. Investments in the promotion of traditional management (e.g. through subsidies to extensive livestock practices) to improve the conservation status of bluethroats could be more cost-effective if, besides improving the condition of the subspecies habitat, the maintenance of semi-natural mosaics can also contribute to achieve other environmental goals such as reduce fire risk, mitigate soil erosion, increase pasture quality or improve wildlife habitat (Lasanta *et al.* 2016). For example, in the study area, management for promoting semi-natural shrub/heath- pasture mosaics will also benefit other species of EU Community Conservation Interest (Birds Directive 2009/147/EC; Anon, 2009), such as the grey partridge *Perdix perdix* subsp. *hispanensis* or the broom hare *Lepus castroviejoi*. Along with the bluethroat, these are the flagship species of the species community inhabiting these semi-open shrub/heath-pasture mosaics in northernmost Iberian mountain system.

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SUPPORTING INFORMATION (Chapter 3)

APPENDIX Chap.3.S1

Table Chap.3.1S1. Climatic features in the three Iberian mountain ranges where *Luscinia svecica azuricollis* breeds.

Mountain range	Climate	Mean annual rainfall	Mean annual temperature
Cantabrian Mountains	Oceanic	1200 mm	8.7 ºC
Mountains of León	Mediterranean	500 mm	10.7 ºC
Central System	Mediterranean	1150 mm	9.0 ºC

Table Chap.3. 2S1. Sampling localities: name, mountain range, number of samples (n), habitat type and mean altitude (m a.s.l.).

Locality name	Mountain range	n	Habitat	Altitude (m a.s.l.)
1-Genicera	Cantabrian Mountains	14	Brooms	1790
2-Puebla de Lillo	Cantabrian Mountains	17	Brooms	1727
3-Rodillazo	Cantabrian Mountains	2	Brooms	1639
4-Ferreras de Cepeda	Mountains of León	17	Heathlands	976
5-La Cueta	Cantabrian Mountains	13	Brooms	1557
6-La Majúa	Cantabrian Mountains	6	Brooms	1832
7-La Seca	Cantabrian Mountains	1	Heathlands	1129
8-San Martín de Castañeda	Mountains of León	12	Brooms	1711
9-Meroy	Cantabrian Mountains	7	Brooms	1591
10-Portilla de la Reina	Cantabrian Mountains	26	Brooms	1830
11-Bustos	Mountains of León	39	Holm oak	845
12-Corcos	Cantabrian Mountains	16	Heathlands	1024
13-Losacio	Mountains of León	11	Holm oak	815
14-Molinaferrera	Mountains of León	1	Heathlands	1239
15-Palacios de la Valduerna	Mountains of León	15	Holm oak	810
16-Pobladura de la Sierra	Mountains of León	2	Brooms	1706
17-Toralino	Mountains of León	14	Holm oak	838
18-Villar de Golfer	Mountains of León	7	Heathlands	980
19-Béjar	Central System	31	Brooms	1970
20-Hoyos del Espino	Central System	15	Brooms	2026

APPENDIX Chap.3.S2. Genetic patterns and processes

We investigated the genetic structure of the breeding population of *Luscinia svecica azuricollis* using the spatially explicit hierarchical Bayesian clustering program GENELAND v. 4.0.4 (Guillot *et al.* 2005). First, we ran 10 independent Markov Chain Monte Carlo (MCMC) simulations using the correlated allele frequency and spatial models for 5×10^5 iterations (thinning every 500th iteration) allowing the number of clusters (K) to vary between 1 and 10. We selected the model with the highest negative log-likelihood which, in all cases, corresponded to K = 4. Second, we ran 20 MCMC simulations using the spatial, correlated allele frequency model with K fixed to the value identified in the first step. Each run consisted of 5×10^5 iterations (thinning every 500th iteration) and a 10% burn-in period. MCMC convergence was checked by computing average posterior probabilities from the output of each independent run. Finally, we used the run with the highest average posterior probability to estimate the geographic limits of K and assigned individuals to populations based on their probability of membership for each pixel of the spatial domain (150 × 300 pixels). The analysis was performed on the sample of 266 individuals (Figure 3.1a).

Cluster K1 included individuals from the central area of the Cantabrian Mountains. Cluster K2 grouped the remaining Cantabrian individuals, together with those of the Mountains of León. Cluster K3 included birds from the foothills of both the Mountains of León and Cantabrian Mountains. Individuals from the Central System formed the cluster K4. All individuals, except four, were assigned to the corresponding genetic cluster with a probability value higher than 75% and none of them were assigned to more than one genetic cluster. Localities belonging to cluster K4 presented the highest values of genetic differentiation when compared to those included in clusters K1, K2 and K3.

Figure Chap.3.1S2. Spatial output from GENELAND. Colour gradient represents high (yellow) to low (red) posterior probabilities of belonging to each inferred genetic cluster. K1: Central Cantabrian Mountains; K2: Cantabrian Mountains, except the central area, and Mountains of León; K3: Foothills of the Cantabrian Mountains and Mountains of León, K4: Central System. Black dots correspond to sampling localities.
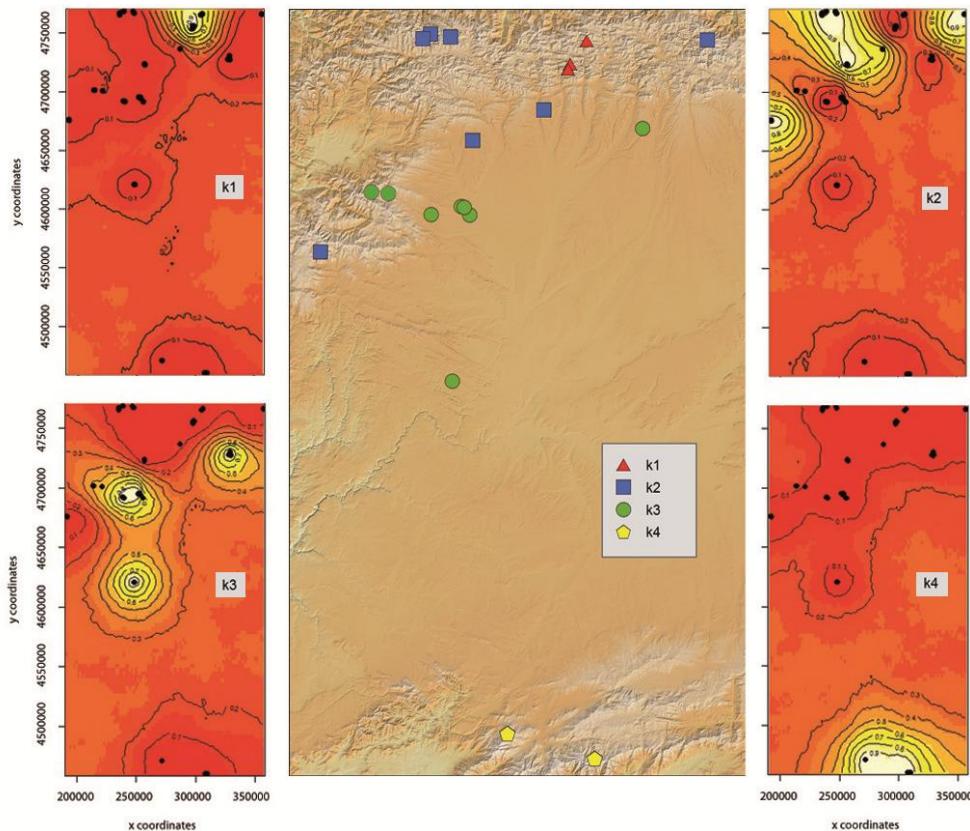
Table Chap.3.1S2. Pairwise F_{ST} values between localities

	K1. Genicera	K1.Puebla de Lillo	K2. Ferreras	K2. La Cueta	K2. San Martín C.	K2. Portilla R.	K3. Bustos	K3. Corcos	K3. Palacios V.	K3. Toralino	K4. Béjar	K4. Hoyos del Esp.
K1. Genicera		0.008	**0.026**	**0.014**	**0.036**	0.012	**0.039**	**0.032**	0.015	**0.013**	**0.024**	**0.064**
K1. Puebla de Lillo	0.1503		**0.016**	0.007	0.013	**0.012**	**0.025**	**0.043**	0.015	-0.003	**0.051**	**0.079**
K2. Ferreras	0.0032	0.0113		0.001	-0.001	**0.008**	**0.025**	**0.024**	0.009	**0.010**	**0.053**	**0.091**
K2. La Cueta	0.0295	0.1808	0.5350		-0.004	-0.003	**0.020**	**0.094**	0.001	0.010	**0.003**	**0.073**
K2. San Martín C.	0.0013	0.0621	0.2768	0.6361		0.001	**0.013**	0.013	-0.004	0.000	**0.051**	**0.076**
K2. Portilla R.	0.0816	0.0092	0.0168	0.5008	0.1711		**0.015**	**0.016**	-0.001	**0.004**	**0.034**	**0.054**
K3. Bustos	0.0003	0.0003	0.0003	0.0095	0.0405	0.0003		**0.002**	0.003	0.001	**0.033**	**0.041**
K3. Corcos	0.0032	0.0003	0.0021	0.0203	0.0584	0.0011	0.0226		0.002	0.015	**0.038**	**0.058**
K3. Palacios V.	0.0895	0.1074	0.1126	0.5282	0.6082	0.6229	0.3097	0.3147		-0.008	**0.015**	**0.033**
K3. Toralino	0.0029	0.3045	0.0116	0.1884	0.8916	0.0211	0.5405	0.0497	0.8647		**0.022**	**0.035**
K4. Béjar	0.0003	0.0003	0.0003	0.0003	0.0003	0.0003	0.0003	0.0003	0.0005	0.0003		0.008
K4. Hoyos del Esp.	0.0005	0.0003	0.0003	0.0003	0.0003	0.0003	0.0003	0.0003	0.0008	0.0003	0.1976	

Pairwise F_{ST} values (above diagonal) and their respective p-values (below diagonal) between localities. In bold, significant values after sequential Bonferroni correction.

Table Chap.3. 2S2. Inbreeding coefficients.

| Locus	K1 F_{IS} (p-value)	K2 F_{IS} (p-value)	K3 F_{IS} (p-value)	K4 F_{IS} (p-value)
PmaC25	0.228 (0.1038)	0.001 (0.5600)	0.109 (0.1088)	0.091 (0.2888)
Ppi2	**0.257** (0.0125)	0.062 (0.1825)	**0.098** (0.0450)	-0.177 (0.9950)
Ptc2	0.011(0.6388)	-0.089 (0.8188)	-0.046(0.7300)	0.139 (0.1900)
Ase19	0.155 (0.1563)	0.024 (0.4338)	**0.127** (0.0100)	0.150 (0.1263)
Cuu4	-0.120 (0.9475)	0.084 (0.1000)	0.003 (0.5350)	0.089 (0.1338)
PATMP2	-0.114 (0.8888)	0.065 (0.2575)	-0.033 (0.7588)	-0.039 (0.7513)
Cuu10	**0.552** (0.0013)	**0.242** (0.0288)	**0.265** (0.0038)	**0.652** (0.0013)
Hru7	0.006 (0.5500)	-0.016 (0.6788)	-0.081 (0.9850)	0.001 (0.6038)
Mcy4	-0.010 (0.6113)	-0.080 (0.9350)	-0.031 (0.7663)	0.114 (0.1800)
Phtr2	-0.018 (0.6838)	-0.035 (0.8525)	-0.010 (0.6650)	-0.025 (0.7025)
All locus	**0.071** (0.0300)	0.013 (0.2688)	0.025 (0.0625)	**0.072** (0.0075)

Inbreeding coefficient (F_{IS}) per locus in each cluster and p-value based on 800 randomizations estimated by Fstat. Bold values denoted significant F_{IS} (p<0.05).

APPENDIX Chap.3.S3. Habitat suitability model

Figure Chap.3.1S3. Bivariate Pearson´s correlations between candidate environmental predictors of Maxent models.
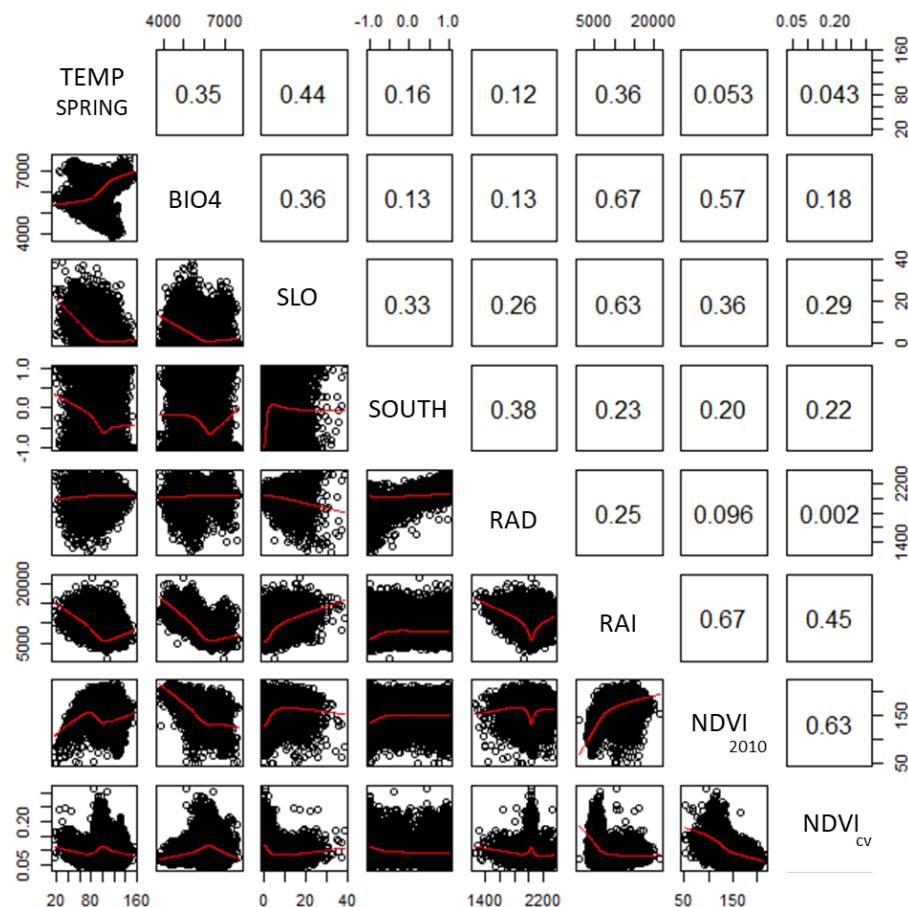

Figure 2S3. Curves used to transform Maxent model habitat suitability values into friction values.
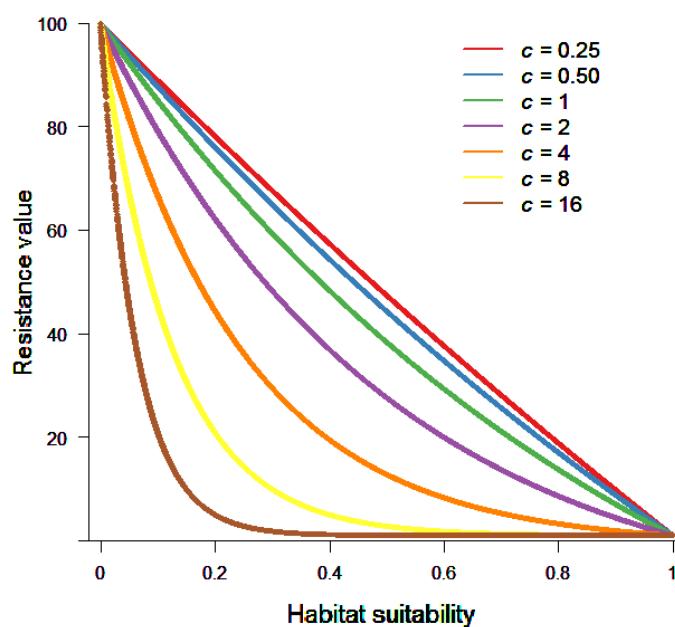
Figure Chap.3.3S3. Bluethroat response curves identified by Maxent for the CLP model. The curves show the effect of each predictor on the logistic response when keeping all other environmental variables at their average sample value (Phillips *et al*. 2006). Each curve represents the average response (red line) and the standard deviation (blue area) corresponding to the cross-validated model. Variables are: average temperature in spring (March, April and May; TMEAN$_{SPRING}$), annual seasonality (BIO4), total annual rainfall (RAI), slope in degrees (SLO), southness (SOUTH), total annual radiation (RAD) and annual mean NDVI for 2010 (NDVI$_{2010}$).

TMEAN$_{SPRING}$

BIO4

RAI

SLO

SOUTH

RAD

NDVI$_{2010}$
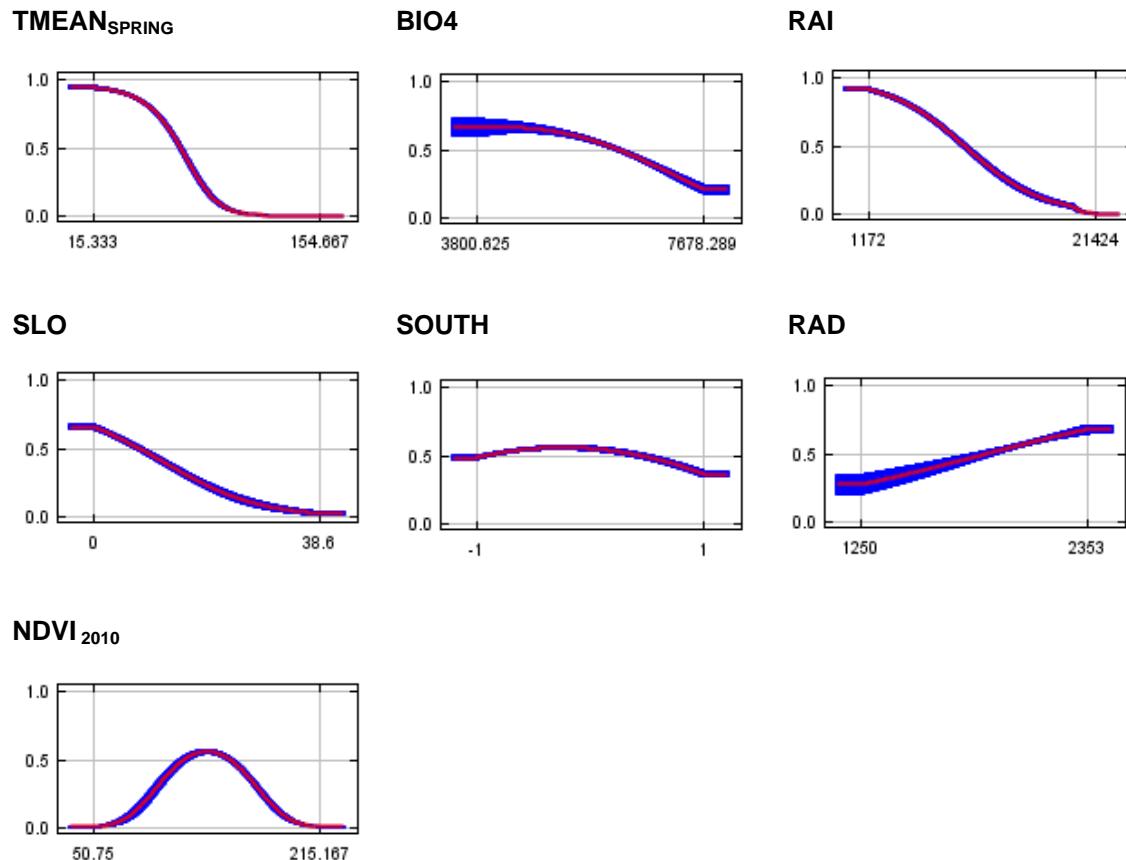
Figure Chap.3.4S3. Bluethroat response curves identified by Maxent for the LS model. The curves show the effect of each predictor on the logistic response when keeping all other environmental variables at their average sample value (Phillips *et al*. 2006). Each curve represents the average response (red line) and the standard deviation (blue area), corresponding to the cross-validated model. Variables are: average temperature in spring (March, April and May; $TMEAN_{SPRING}$), annual seasonality (BIO4), total annual rainfall (RAI), slope in degrees (SLO), southness (SOUTH), total annual radiation (RAD) and coefficient of variation of NDVI for the period 1987-2009 ($NVDI_{CV}$).

TMEAN_{SPRING}

BIO4

RAI

SLO

SOUTH

RAD

NDVI_{CV}
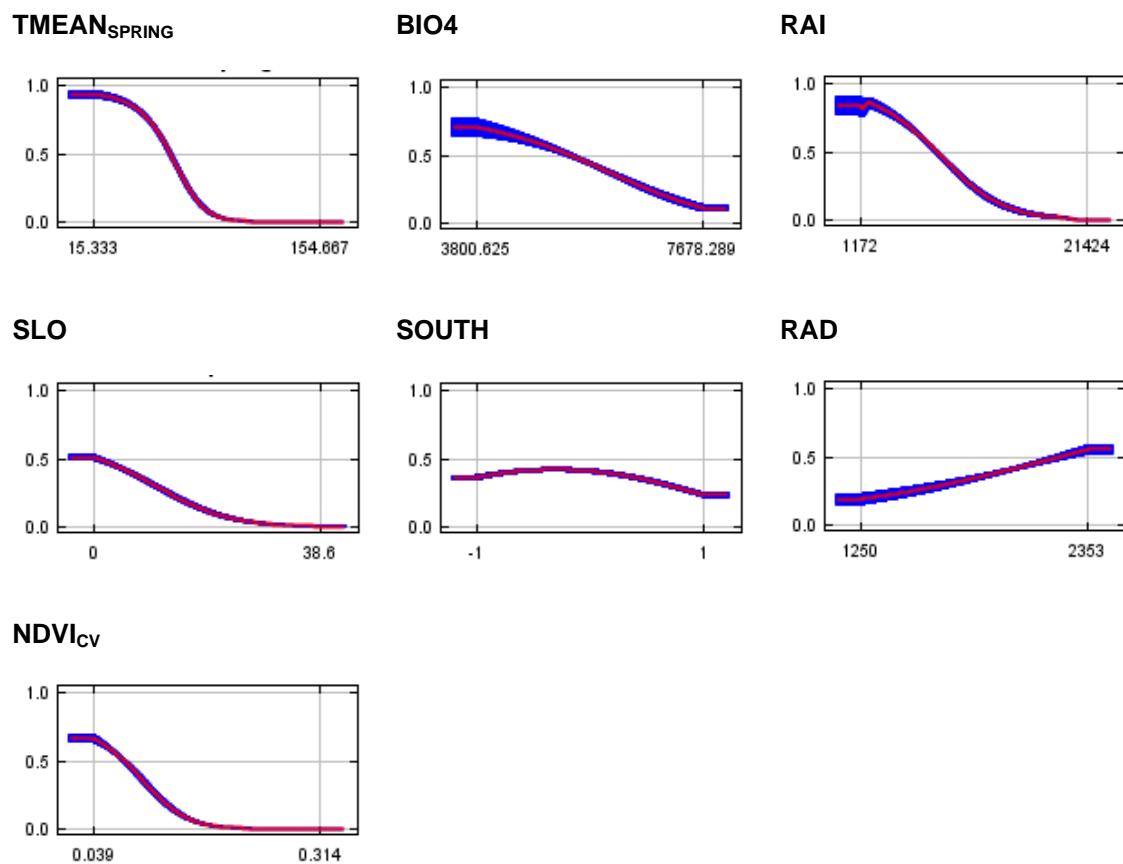
Figure Chap.3.5S3. Bluethroat response curves identified by Maxent for the additive model (CLP&LS) The curves show the effect of each predictor on the logistic response when keeping all other environmental variables at their average sample value (Phillips *et al.* 2006). Each curve represents the average response (red line) and the standard deviation (blue area), corresponding to the cross-validated model. Variables are: average temperature in spring (March, April and May; $TMEAN_{SPRING}$), annual seasonality (BIO4), total annual rainfall (RAI), slope in degrees (SLO), southness (SOUTH), total annual radiation (RAD), the coefficient of variation of NDVI for the period 1987-2009 ($NVDI_{CV}$) and the annual mean NDVI for 2010 ($NDVI_{2010}$).
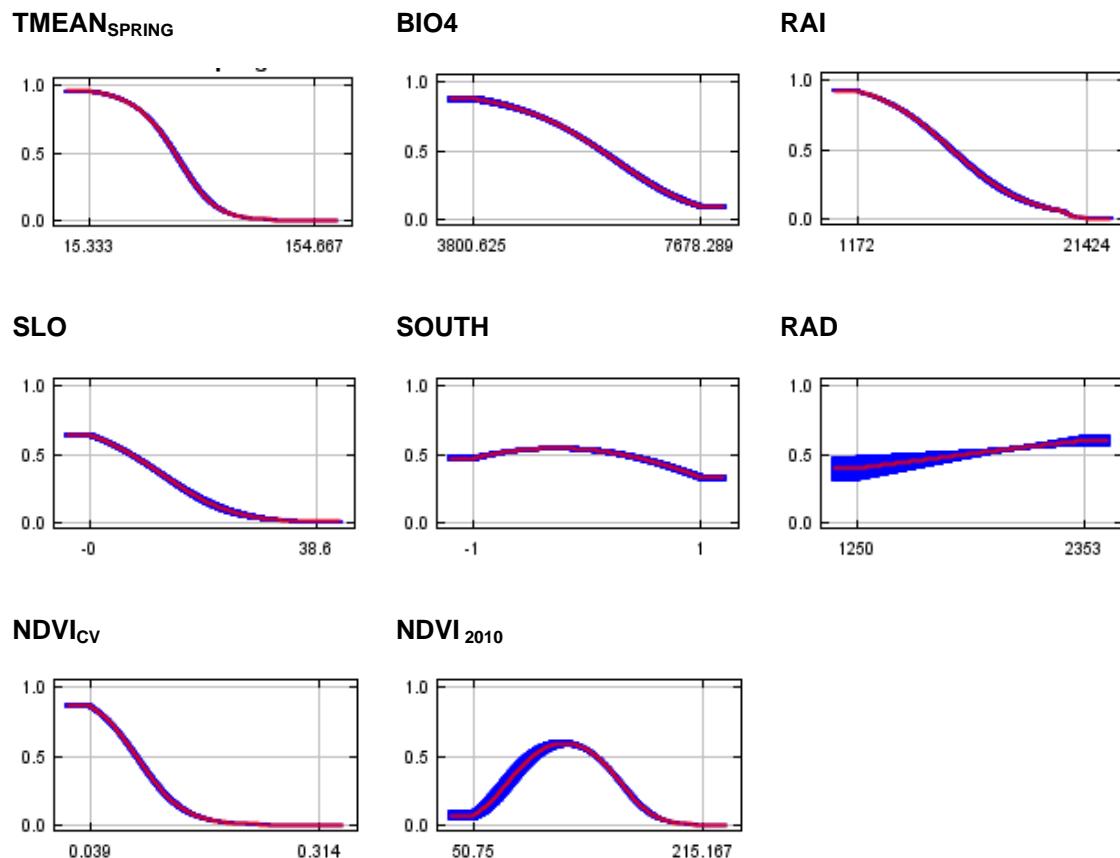
CAPÍTULO 4

Morphological differentiation patterns of Iberian breeding bluethroat *Luscinia svecica azuricollis* across geographic and environmental gradients in temperate mountains of southern Europe.

Abstract

In mountain ranges, sharp spatial variations in habitat heterogeneity and climate across geographic gradients provide an excellent scenario to assess how gene flow drives genetic and phenotypic differentiation in bird populations. We analyze morphological differentiation patterns, in correlation with genetic differentiation and geographic isolation, across geographic and environmental gradients for the case of the Iberian bluethroat *Luscina svecica azuricollis*, a migratory bird breeding in NW Iberian mountains. We collected a sample of 625 bluethroats across its whole distribution area to measure wing length, tarsus length, body weight and wing pointedness. Morphological differentiation across geographic (latitude and altitude) and environmental (climate and vegetation) gradients was assessed using GLMMs. Additionally, the role of genetic distance and geographic isolation as drivers of morphological differentiation was evaluated with Mantel tests. Bird morphology varied significantly with latitude and altitude, but not with climate or vegetation. For the case of latitude, morphological differences were contrary to what was expected according to Bergmann's rule. Wing lenght and body weight were both correlated to geographical distance, while only the last varied with genetic differentiation. All biometric variables diverged significantly among genetic clusters, wing pointedness varying also between localities. The greatest genetic and phenotypic differentiation was detected in the southernmost mountain range that holds the most geographically isolated genetic group. We conclude that Iberian breeding bluethroats show latitudinal phenotypic differentiation, as well as strong morphological d that correlates with geographic isolation and genetic drift. Some deviances from this pattern occur for biometric variables with greater selective pressure, which points out to local adaptations related to migratory strategies.

Key words: Bergmann's rule, body size, geographic isolation, phenotypic plasticity.

Introduction

The assessment of different factors underlying spatial patterns of morphological differentiation is useful to test theoretical and applied questions in ecology and conservation (Morgan 2004), including adaptation to migration (Mönkkönen, 1995), microhabitat food selection, new habitat colonization or survival rate (Forstmeier & Keßler 2001, Hall *et al.* 2004). Morphological differentiation is a complex phenomenon in which diverse factors such as geographical isolation, random stochastic phenomena, including genetic drift (Wright 1931), and selective forces (Hughes 2000, Millien *et al.*. 2006) can participate simultaneously (Coyne & Orr 2004, Orsini *et al.* 2013). Therefore, the joint analysis of morphological and genetic differentiation is relevant to decipher whether differentiation occurs solely due to geographical or functional isolation or, instead, other selective forces are at work (Clegg *et al.* 2002). Additionally, both morphological and genetic differentiation may be determined, to a large extent, by dispersion capacity, philopatry and habitat fragmentation (Slatkin 1985). In specific, different habitat characteristics, as physiography and vegetation, might imply different selection patterns across individuals. A strong habitat selection might be associated to a high differentiation level, but a low adaptation capability to different environments. Phenotypic plasticity could be, therefore, a suitable strategy in changing environments given that selective pressures often vary (Millien *et al.* 2006; Siepielski *et al.* 2009).

Mountain ranges of temperate regions are an ideal scenario for exploring diversification processes. They frequently give rise to highly variable climatic conditions, operating at macro and micro-scale level, which hence promote the existence of a high richness of habitats. On the other hand, temperate mountains were a refuge during the last glaciation, and today many species remain isolated in heterogeneous habitats with island-like conditions (Hewitt 1996). Consequently, these mountain bird populations may show morphological adaptations that vary soundly across geographic gradients even at relatively small geographic scales. For instance, there are two morphological traits than can vary substantially at intra-specific level in mountain birds and, therefore, constitute a good tool to investigate diversification processes: body size and wing pointedness.

Differences in body size across geographic gradients were first codified in Bergmann's rule, which postulates that body size increases with latitude (Bergmann 1847, Mayr 1956; Lindsey 1966; Huston & Wolverton 2011). Since the underlying mechanism of this rule is thermoregulation (i.e., the increase in body size is a response to a decrease in temperature; Rodríguez *et al.* 2006), it has been also associated with altitude (Teplitsky & Millien 2014). Despite the rule was originally defined inter-specifically (Blackburn *et al.* 1999), it has been commonly applied afterwards to populations within a single species. In fact, the mechanisms behind the relationship size–latitude are primarily limited to intra-specific scales (Berke *et al.* 2013). Bergmann's rule has held true for many taxa throughout the world (Hamilton 1961, Blackburn & Gaston 1996; Gaston & Blackburn 1996, Ashton 2002), but it is best supported in endotherms, particularly in birds, where a trend of increasing body size with latitude was found in more than 70% of species (Meiri & Dayan 2003, Millien *et al.* 2006). However, deviations occur, probably due to phylogenetic history, species range size, reproductive factors or competition, as well as altitudinal variability in climate, soils and habitat (Watt *et al.* 2010, Huston & Wolverton 2011).

Wing pointedness can be directly linked to migratory behaviour, which is a major driver of reproductive isolation and speciation (Winker 2010). This morphological trait may be used as a proxy of energy-efficient flight and migratory movement magnitude. This relationship was systemized as the Seebohm's rule (Seebohm 1901, Mönkkönen 1995, Lockwood *et al.* 1998, Voelker 2001, Forschler & Bairlein 2011), which establishes that migrants have relatively longer and more pointed wings than resident birds. Wing shape has been also related to breeding ground altitude (Bears *et al.* 2008), habitat use and foraging behaviour (Marchetti *et al.* 1995;

Forstmeier & Keβler 2001), density of obstacles (vegetation) (Alatalo *et al.* 1984), sexual selection (Hedenström & Møller 1992) and predation risk (Swaddle & Lockwood 1998).

In this study, we aim to assess morphological differentiation patterns, in correlation with genetic differentiation and geographic isolation, across geographic and environmental gradients in the breeding grounds of a long distance migrant passerine, the bluethroat *Luscinia svecica*. This is a small (14-20 cm) polytypic passerine breeding from Iberia to Alaska (Cramp 1988, Meijer & Stastny 1997). The westernmost European populations overwinter in southwestern Europe, northerwestern Africa and western Sahelian belt; Arizaga *et al.* 2015). The phenotypic (Arizaga *et al.* 2006, Hogner *et al.* 2013, García *et al.* 2017) and genotypic (Johnsen *et al.* 2006, Johnsen *et al.* 2007) variation of this species constitutes a complex mosaic associated to differences in geographical morphs and life history, where Iberian breeding bluethroats *L. s. azuricollis* are considered among the most ancestral forms (Johnsen *et al.* 2006). Iberian bluethroats are distributed across strong geographic and environmental gradients in NW Iberian mountains, where they show a moderate degree of genetic differentiation (Alda *et al.* 2013, García *et al.* 2020). The subspecies breeds along an altitudinal gradient ranging from 750 to 2400 m a.s.l., under considerably different climatic conditions (i.e. mean annual temperature spans from 0ºC to 12 ºC and mean annual rainfall from 400 to 1800 mm). Main habitats consist of shrublands, from degraded holm oak shrublands (*Quercus rotundifolia* and *Cistus* spp.) in the lowest areas to heathlands (*Erica* spp. and *Calluna vulgaris*) and brooms (*Cytisus* spp. and *Genista* spp.) in the uppermost lands (García *et al.* 2000, Arizaga *et al.* 2011, Alda *et al.* 2013). Across this gradient, the prey assemblage that constitute the diet of the species also differ substantially (García-Tejero *et al.* 2013). According to Bergmann's rule, we would expect birds living at the highest and northernmost areas having larger body size than those living in the lowest and southernmost areas. Additionally, we envisage a certain degree of differentiation in wing pointedness among

populations, given the selective forces of variables related to migratory strategy and phenology (Delmore *et al.* 2015) and the fact that Iberian breeding populations spend the winter in different areas of the Iberian Peninsula and tropical Africa (Arizaga *et al.* 2015). Finally, as bluethroat populations are genetically well differentiated and occupy highly fragmented and geographically isolated habitats, we hypothesize a good correlation between genetic and morphological differentiation patterns that could respond to local adaptation and genetic drift.

Methods

Sampling area and data collection

A total of 625 Iberian bluethroat breeding males were sampled for morphological evaluation between 1998 and 2012. They were captured using mist nets and spring traps throughout 23 localities covering the whole altitudinal and environmental range where the subspecies is known to breed in the Cantabrian Mountains, Mountains of León (with foothills) and Central System (Figure 4.1; Table S1 in the Supplementary Material). Since sampling (March-September) partially coincided with the migration period of European populations included in other subspecies, we applied a habitat use criterion to ensure that all captured individuals corresponded to the local subspecies. Therefore, we avoided sampling in habitats exploited by bluethroat migrants, i.e. wet areas with dominant halophytic communities, that are not used by local birds (Bermejo & De la Puente 2004, Arizaga *et al.* 2006, Arizaga *et al.* 2010; Arizaga & Tamayo 2013). Measurements were taken by a team of three skilled ringers that used the same protocols. All data were standardized to a single observer. Recaptures were excluded to avoid pseudo-replication (only data from the first capture event of each bird were used).

For each bird, we measured a set of biometric variables: wing length (maximum chord) ($\pm$ 0.5 mm), primary feathers length (from the base to the tip of the feather, $\pm$ 0.5 mm; P2 to P9, numbered descendently), tarsus length ($\pm$ 0.1 mm, measured with a digital caliper) and body

weight (± 0.1 g, measured with a digital balance). Regarding P2 to P9, the measurement of the total length of the feather provides more statistical consistency in comparison with the distance between the feather tips, as it allows for a lower measurement error (distances are much shorter than primary lengths) and a higher repeatability (Lessels & Boag 1987; Swaddle & Witter 1994). Lengths of P2 to P9 were used to calculate an index of wing pointedness (Lockwood *et al.* 1998). In specific, this morphometric variable was estimated on the basis of the measurement of the primary feathers

(P2 to P9 -in descending order-) through a size-constrained component analysis (SCCA).

Additionally, we determined bird sex and age, classifying individuals as either first-/second-year bird (FY/SY, EURING code 3 and 5) or adult (AD, EURING code 4 and 6). FY birds undergo a partial moult after fledging, before the autumn migration, whilst AD do a complete moult. The existence of a moult limit, which is normally found in great coverts, reveals that a bird is a FY (or SY, if captured during its second year of life, before its first complete moult after breeding for the first time); otherwise, the bird is classified as AD (no moult limit).
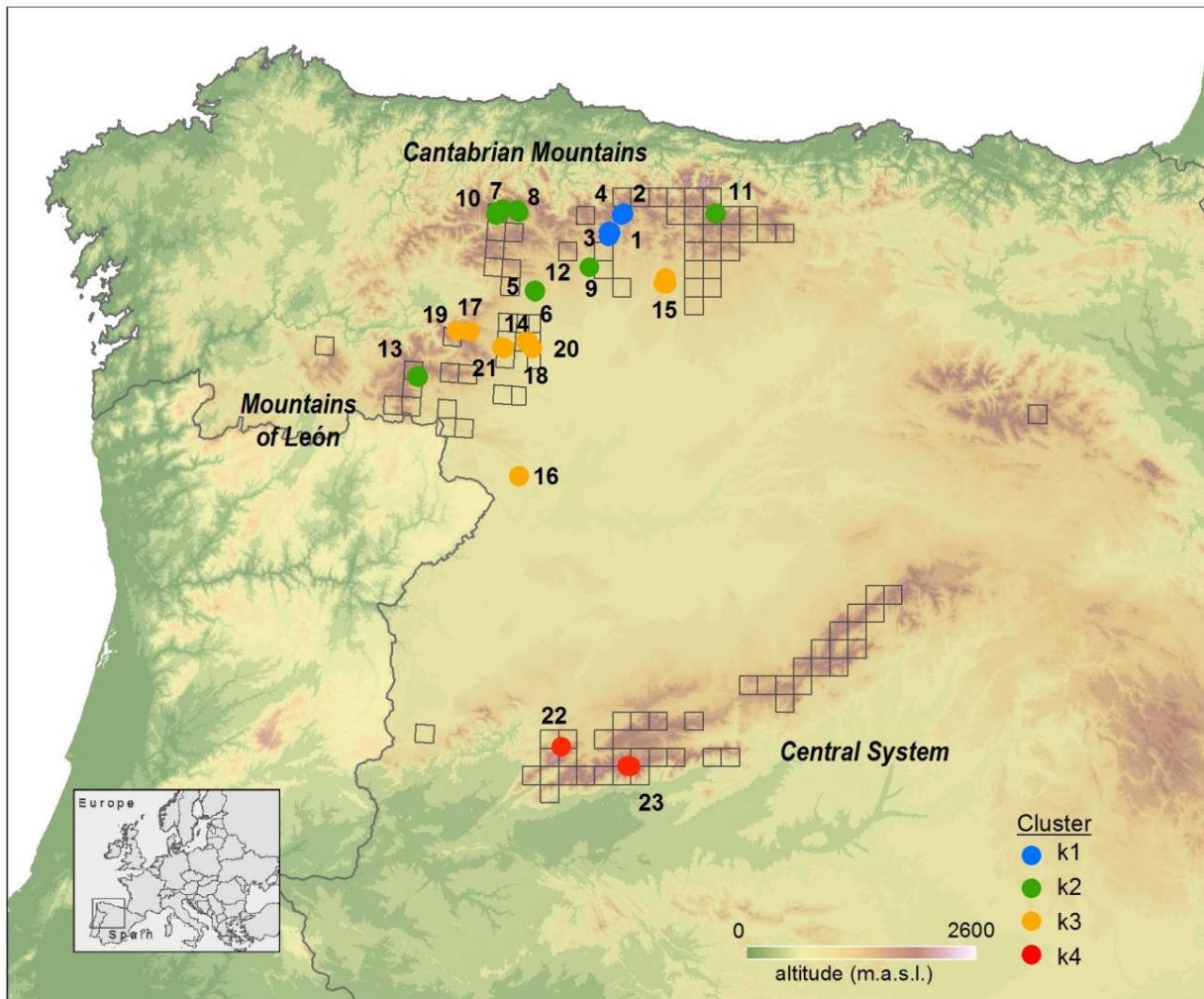
Figure 4.1. Study area. Black grid represents the breeding range of bluethroat in the Iberian Peninsula, according to Martí & Del Moral (2003). Dots correspond to capture localities (n=23) where morphological measurements and genetic data were collected. Color indicates the genetic cluster identified for each locality: K1-Central Cantabrian Mountains. K2- Cantabrian Mountains (except central area) and Mountains of León. K3-Foothills of Cantabrian Mountains and Montes of León, K4-Central System. See Table S1 in the Appendix for more information about the sampling localities.

To account for geographic and environmental variation, we recorded, for each sampling locality: (i) latitude and altitude; (ii) dominant vegetation type, i.e. brooms (*Cytisus* spp. and *Genista* spp), heathlands (*Erica* spp. and *Calluna vulgaris*) or holm oak shrublands (*Quercus rotundifolia* and *Cistus* spp.); and, (iii) averaged mean temperature and rainfall of spring (March, April and May; corresponding to species arrival to breeding sites, mating and nesting) and summer (June to August; corresponding to fledging and post breeding months prior to migration). Climatic data were retrieved from Ninyerola *et al.* (2005) that provides data for the period 1950–1999 at 200m resolution. In order to summarize the main characteristics of each locality, climatic variables were entered into a Principal Component Analysis (PCA), where the first principal component ($PC1_{cli}$) accounted for 74.75% of the variance.

Genetic divergence: DNA extraction, microsatellite genotyping, genetic structure and differentiation

A subsample of 266 bluethroats, from the whole set of individuals captured for morphological assessment, was used for genetic analysis. Genomic DNA was extracted from blood samples, being all samples genotyped for 12 microsatellite loci (Alda *et al.* 2013). In a previous study, García *et al.* (2020) used the same data sample to investigate genetic distance (F_{ST} values) and structure of this breeding population by means of R- GENELAND v. 4.0.4 (Guillot *et al.* 2005), a package that applies a Bayesian clustering method to infer a number of clusters (K) of individuals. The four main clusters identified in that study were considered for further analyses (Figure 4.1).

Morphological differentiation across geographic and environmental gradients

To evaluate morphological differentiation across geographic and environmental gradients and Bergmann's rule, we calculated an index of body size as the first Principal Component ($PC1_{morph}$) of a PCA based on the biometric variables wing length, tarsus length and body weight (Freeman & Jackson 1990, Schauble 2004, Milá *et al.*

2009). $PC1_{morph}$ showed high and positive factor loading for all the input variables and accounted for 54.3% of the variance. On the other hand, we evaluated wing pointeness across geographic and environmental gradients. In both cases, we built a matrix Euclidean distance among pairs of localities (scores of $PC1_{morph}$ and wing pointedness values). Thereafter, we built General Linear Mixed Models (GLMM) using, as object variable, the index of body size ($PC1_{morph}$) in each locality and the wing pointedness values, and, as explanatory variables, the geographic (latitude and altitude) and environmental (climate and vegetation) variables. Locality was considered as a random effect and the age of the birds (juveniles *vs*. adults) was included as a fixed effect because it may likely affect morphometric traits (Arizaga *et al.* 2006). GLMM is an appropriate method for modelling clustered and autocorrelated data (Pinheiro & Bates, 2000) and, therefore, for dealing with spatial pseudorreplication. Prior the analysis, we evaluated the Pearson's pairwise correlations between all predictors in order to minimize multi-collinearity problems. When two or more explanatory variables were strongly correlated (r> 0.7) we kept the variable with a more direct interpretation from the point of view of the ecology of the species. The most parsimonious models were selected by means of Akaike's Information Criterion (Burnham & Anderson 2002) using the dredge function of MuMIn R library. All statistical tests were implemented in R, version 3.6.3 (R Core Team 2020).

Geographical isolation and genetic distance as drivers of morphological differentiation

First, we tested differences on biometric (wing length, tarsus length and body weight) and morphometric (wing pointedness) traits among genetic clusters and localities using ANOVA and post-hoc Tukey test. Then, we evaluated whether the differentiation of these biometric and morphometric traits was driven by geographic isolation and /or by genetic differentiation by means of Mantel tests (Mantel, 1967). Morphological and genetic differentiation, as well as geographic isolation, were calculated as Euclidean distances between all pairs of sampling localities.

RESULTS

The most parsimonious model obtained evaluating body size differentiation across geographic and environmental gradients included latitude, altitude and age. Trend in body size were in the opposite direction to that expected according to Bergmann's rule. Furthermore, wing pointedness varied in relation to latitude and age in the best model (Table 4.1). Bluethroats located at the southernmost breeding areas were larger and had the more rounded wings than northern birds.All biometric variables varied significantly among genetic clusters, with Tukey-HSD post hoc analysis showing significant differences among the Central System (genetic cluster K4) and the rest of breeding nuclei (genetic cluster K1, K2 and K3) (Table S2 and Table 4.2). Wing length ($F_{22,629}=8.24$, p<0.001), tarsus length ($F_{22,595}=2.21$, p<0.01) and body weight ($F_{22, 579}=5.57$, p<0.001) also diverged among sampling localities. Regarding wing pointedness, significant differences were observed between genetic clusters ($F_{3, 349}=9.11$, p<0.001) and localities ($F_{20, 426}=6.01$, p<0.001). Bluethroats in the northern mountain clusters (K1 and K2) had more pointed wings than those in lower altitudes (K3) and further south, in the Central System) (Table S2, Figure S1 in the Supplementary Material). The differentiation of wing length and body weight was significantly correlated to geographical distance (Figure 4.2), whilst only body weight was driven by genetic distance (Figure 4.3).

Table 4.1. Best models tested by Mixed Generalized Linear Models including geographic and environmental drivers of biometric (body size) and morphological (wing pointedness) differentiation. Significant factors (P < 0.05) are shown in boldface type in each model.

| Model/explanatory variable		Estimate	SE	Significance	ΔAIC
BODY SIZE					
Latitude + Altitude + Age					0.00
	Intercept	0.4689	0.0923	p<0.001	
	Latitude	**-0.7784**	**0.0701**	**p<0.001**	
	Altitude	**0.1415**	**0.0715**	**p=0.0487**	
	Age (young)	-0.2015	0.1138	p=0.0775	
Latitude + Altitude					1.09
Latitude					1.72
Latitude + Age					1.87
Age					15.09
Altitude + **Age**					15.96
WING SHAPE					
Latitude + Age					0.00
	Intercept	1.1237	0.0112	**p<0.001**	
	Latitude	**-0.0388**	**0.0064**	**p<0.001**	
	Age (young)	**0.0521**	**0.0137**	**p<0.001**	
Latitude + Altitude + Age					1.91
Latitude + Vegetation + **Age**					2.46
Latitude + Vegetation +Altitude + **Age**					2.51
Age					4.79
Altitude + **Age**					6.84

DISCUSSION

The analysis of bluethroat morphological differentiation along geographical and environmental gradients showed clear differences for latitude and, at a lesser extent, for altitude. Birds nesting further south were larger than those nesting in northern latitudes, which is the contrary to what Bergmann's rule states. Although this rule is applicable to a considerable number of species (Hamilton 1961, Blackburn & Gaston 1996), there are many other species or populations in which it is not (see, for example, Salewski *et al.* 2010). Morphological differences not explained by latitude are usually attributable to local variations in productivity that depend on soil fertility, water availability and temperature (Strickland & Demarais 2008, Huston & Wolverton 2011). In our case, the fact that Bergmann's rule is not fulfilled, showing indeed the opposite trend, may be related to effect of altitude, as birds nesting in the southernmost mountain range, the Central System, are found at the highest altitude. This mountain system also has the highest Mediterranean influence among the target mountain ranges, thus birds need to search for resources at higher altitude, where climatic conditions and available resources are more favorable. The results suggest that stochastic factors, such as genetic drift and geographical isolation, together with deterministic forces, such as ecological selection mechanisms, have contributed to morphological divergence at different spatio-temporal scales.

These differences between bluethroat populations have already been highlighted and linked to migration patterns and overwintering areas (Arizaga *et al.* 2015). Nevertheless, some divergences occur in this general pattern when biometric variables are analysed separately, with no correlation between morphological divergence and genetic differentiation for wing and tarsus length. This lack of correlation between genetic divergence and morphological differentiation could be associated to different factors. First, the isolation process is recent and the time passed might be was not enough for reproductive isolation to be reflected in neutral molecular markers (such as the microsatellites used) (Nice & Shapiro 1999). This delay in reflection in molecular markers is usually more obvious when values for morphologic divergence are compared with genetic divergence calculated via mitochondrial DNA given that the mutation rate is lower than for microsatellites (Ortego *et al.* 2012). Second, if strong selective forces intervene, they could act more or less intensively over adaptation processes to specific local conditions, for instance through differences in diet and habitat selection (Fiedler 2005, Förschler & Bairlein 2011). Third, perhaps the different factors and selective forces are acting antagonistically, thus masking differentiation. In any case, wing and tarsus length are subjected to strong selective pressures and represent a trade-off between other conflicting selective pressures.

Table 4.2. P-values of Tukey-HSD post hoc analysis of wing length, tarsus length, body mass and wing pointedness. K1-Central Cantabrian Mountains. K2- Cantabrian Mountains (except central area) and Mountains of León. K3-Foothills of Cantabrian Mountains and Montes of León, K4-Central System.

|	K1-K2	K1-K3	K1-K4	K2-K3	K2-K4	K3-K4
Wing length	0.841	0.703	*	*	***	***
Tarsus length	0.600	0.694	0.750	0.963	**	***
Body mass	0.338	0.896	***	0.307	***	***
Wing pointedness	0.979	*	***	*	***	0.210

(*$p<0.05$, **$p<0.01$, ***$p<0.001$)
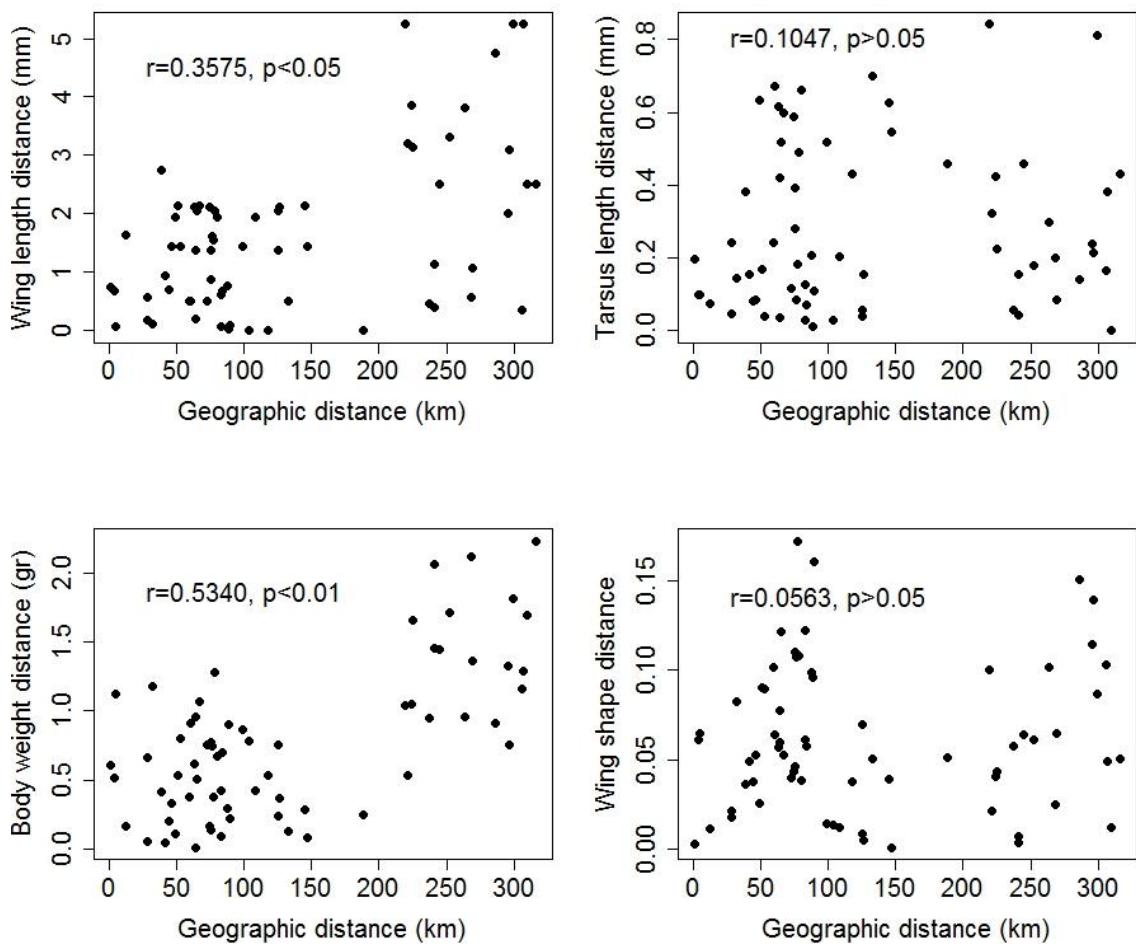
Figure 4.2. Mantel correlations between morphological trait differentiation and geographic distance among localities.

For instance, wing length has been linked, in the case of bluethroat, to different migration distances (Winkler & Leisler 1992, Arizaga *et al.* 2006), habitat use and foraging behaviour (Marchetti *et al.* 1995, Forstmeier & Keβler 2001) or sexual selection (Hedenström & Møller 1992). Indeed, as bluethroat forages on the ground, tarsus length, as other morphological characters, could change rapidly when is subjected to natural selection (Salewski *et al.* 2014). The pattern described for bluethroats is consistent with that of one species that inhabits changing environments affected by perturbations and is able to colonize new areas (Yeh & Price 2004) or remain and adapt to those changes via phenotypic plasticity or small microevolutionary changes (González *et al.* 2013).

The analyses of biometric and morphometric variables amongst genetic clusters also strengthened the idea that morphological differentiation is not only due to genetic drift. Individuals from the Central System (K4) are noticeably the largest, which is reflected in all biometric variables considered. Nevertheless, for the case of wing pointedness, this trend does not occur, with the most pointed wings for individuals from the Northern Mountains (K1 and K2). Differences in wing morphology might be related to differences in migration strategy, but no directly, as this variable vary according to other factors, such as the age of the individuals that may not be associated to overwintering areas, but to selective pressures as flight tactic against predation (Mönkkönen 1995), amongst other factors.
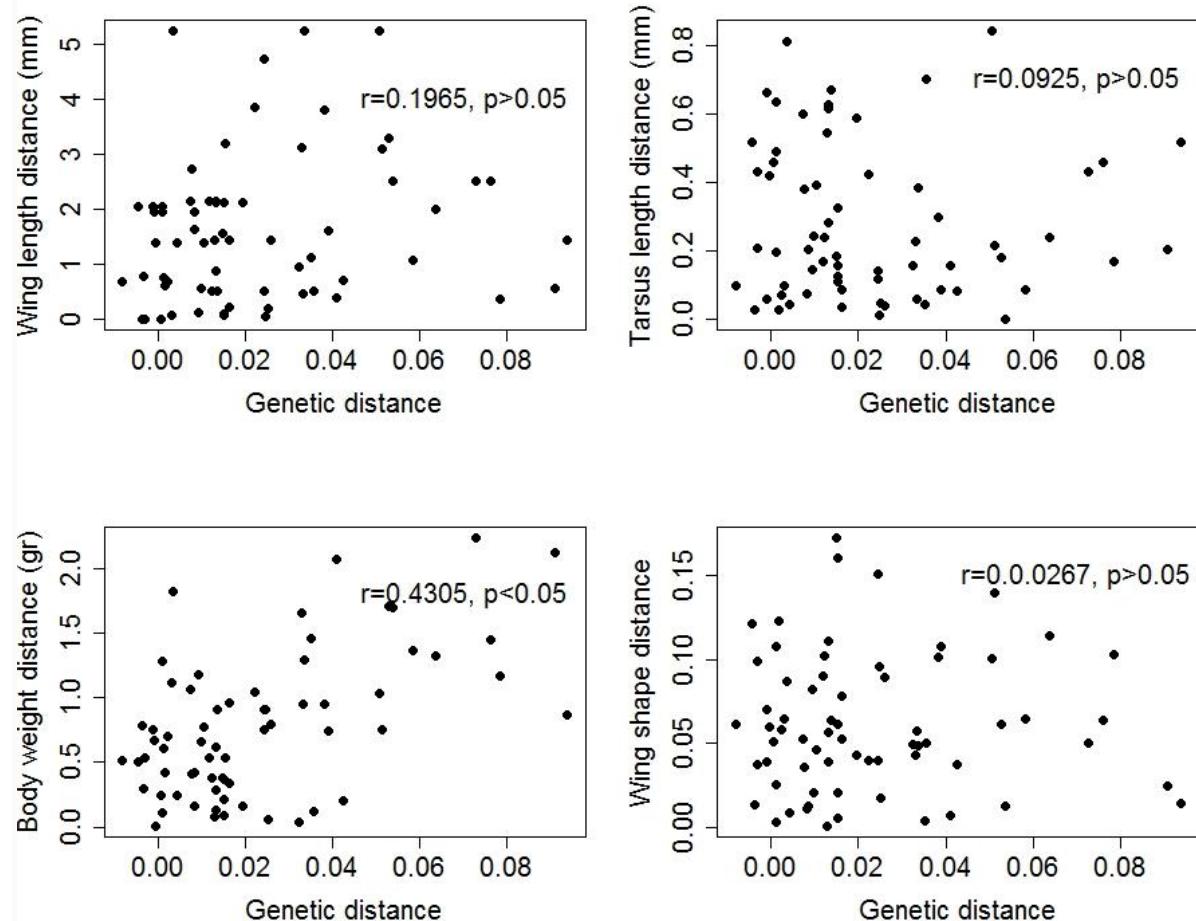
Figure 4.3. Mantel correlations between morphological trait and genetic differentiation among localities.

Furthermore, the comparative analyses amongst clusters and the assessment of the effect of geographic factors on bird size indicate that there is no evolutionary confluence. On the one hand, the largest birds were found at the highest altitude and lowest latitude and, the other hand, birds occupying territories at high altitude and exhibiting similar phenology with regard to occupation of territories in spring had not similar wing morphology. In this sense, migration phenology has been highlighted as a notable selective force (Delmore *et al.* 2015) and a divergent migratory behavior could involve genetic and phenotypic divergence (Irwin, 2009, Winkler & Leisler 2005). This may be a basic question in genetic differentiation, as it could act as a barrier to gene flow. For the case of different populations of Iberian bluethroats, they

are supposed to do not share overwintering areas and arrival at breeding areas and reproductive phenology do not coincide in time (Casagrande *et al.* 2006). In this sense, differences in the date of arrival from winter quarters to Iberian breeding areas are remarkable, with birds reaching the foothills of the Northern Mountains towards the end of February or beginning of March and birds arriving to areas at higher altitude (over 2000 m a.s.l.) by May.

Within the subspecific complex of bluethroat, significant differences have been found in wing morphology amongst populations of southern (*L. s. namnetum, L. s. azuricollis*) and central Europe (*L. s. cyanecula*) that overwinter in the Iberian Peninsula and Africa and the subspecies *L. s. svecica*, which migrates

from Scandinavia to Indo-European overwintering areas (Lislevand *et al.* 2015). Also, it has been considered that Iberian populations could follow different migration patterns than the other subspecies (Arizaga *et al.* 2006). Differences in wing shape have been observed in species with both migratory and sedentary populations (Mulvihill & Chandler 1990, Pérez-Tris & Tellería 2001), being migratory distance also related to different subspecies (Förschler & Bairlein 2011). In general, in species with migrating and resident populations, the migrating populations tend to have more pointed wings (Mulvihill & Chandler 1990, Senar *et al.* 1994, Fiedler, 2005). However, Fudickar & Partecke (2012) did not find any difference in wing morphology in a population of European blackbird, which also has migratory and sedentary populations, indicating that differentiation may be restricted by other selective forces, such as habitat. Förschler & Bairlein (2011) found that the larger subspecies of northern wheatear, which flies long distances crossing large stretches of open sea, presented the most marked morphological adaptations, while the smaller subspecies, distributed in

breeding areas in the northernmost latitudes, in Siberia, showed similar adaptations to long distance migration. In our study, the northernmost populations, in the Cantabrian Mountains, had smaller but more pointed wings than the others. Unfortunately, there is no accurate information on the overwintering grounds of Iberian populations and even less on the existence of differentiated areas for each population.

In conclusion, the study of morphological differentiation in populations that inhabit fragmented habitats across geographical and environmental gradients can be very useful to understand the early stages of speciation processes. Iberian bluethroat breeding populations show a strong morphological differentiation that correlates with geographic isolation and genetic drift. Some divergences from this general pattern occur in biometric variables with greater selective pressure, which points out to local adaptations related to differentiate migratory strategies.

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SUPPORTING INFORMATION (Chapter 4)

Table Chap.4.S1.1. Mean ±SE of wing length, tarsus length, body mass and wing pointedness of bluethroat males in each genetic cluster. Sample size is shown within brackets. (*p<0.05, **p<0.01, ***p<0.001, n.s.= non-significant). K1-Central Cantabrian Mountains. K2- Cantabrian Mountains (except central area) and Mountains of León. K3-Foothills of northern mountains, K4-Central System.

|	K1	K2	K3	K4	ANOVA
Wing length (mm)	74.16±1.70 (46)	75.24±1.69 (33)	75.35±1.82 (316)	78.67±1.39 (203)	***
Tarsus length (mm)	26.91±0.61 (48)	26.86±0.74 (38)	26.84±0.79 (325)	27.16±0.73 (206)	***
Body mass (gr.)	17.68±0.87 (47)	17.40±0.96 (36)	17.73±1.11 (313)	18.81±1.12 (205)	***
Wing pointedness	1.10±0.12 (28)	1.12±0.10 (63)	1.18±0.14 (316)	1.20±0.11 (203)	

Toma de medida del tarso en un ejemplar de pechiazul.

Table Chap.4.S1.2. Sampling localities: mean altitude (m.a.s.l.), genetic cluster and number of samples (n=625).

Sampling locality	Altitude (m a.s.l.)	Genetic cluster	Number of samples (morphological data)
1-Genicera	1790	K1	12
2-Puebla de Lillo	1727	K1	14
3-Rodillazo	1639	K1	2
4-Villaverde de la Cuerna	1762	K1	6
5-Brañuelas	1057	K2	3
6-Ferreras de Cepeda	976	K2	12
7-La Cueta	1557	K2	6
8-La Majúa	1832	K2	5
9-La Seca	1129	K2	1
10-Meroy	1591	K2	6
11-Portilla de la Reina	1830	K2	20
12-Requejo-Corús	1060	K2	5
13-San Martín de Castañeda	1711	K2	5
14-Bustos	845	K3	51
15-Corcos	1024	K3	13
16-Losacio	815	K3	8
17-Molinaferrera	1239	K3	1
18-Palacios de la Valduerna	810	K3	235
19-Pobladura de la Sierra	1706	K3	2
20-Toralino	838	K3	9
21-Villar de Golfer	980	K3	8
22-Béjar	1970	K4	202
23-Hoyos del Espino	2026	K4	5

Figure Chap.4.S1.1. Differences in primary feather length among males corresponding to different genetic clusters: K1-Central Cantabrian Mountains, K2-Cantabrian Mountains and Mountains of León, K3-Foothills of the Cantabrian Mountains and Mountains of León and K4-Central System.
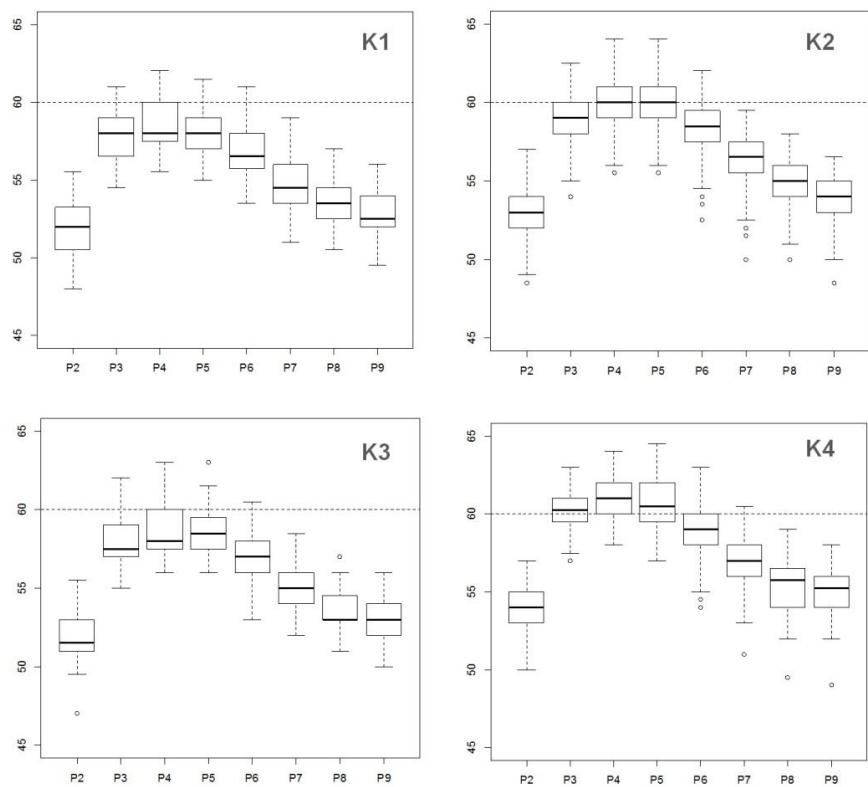
Forma de ala en un ejemplar de pechiazul. Montes de León. Se muestra la numeración de las primarias.

CAPÍTULO 5

Environmental fit prevails over chance effects in explaining song differentiation among populations of an upland passerine.

Abstract

Understanding the influence of environmental, social and intrinsic factors in sexual signaling variation is crucial to evaluate the adaptive potential of animal communication and identify early indicators of habitat deterioration in the wild. In this context, integrative approaches targeting all these influences are required for investigating the diversity of signals that are especially complex, such as bird song. Here, we assess whether individual indicators of song performance reflect population quality indicators and contrast deterministic (adaptations to environment and social context, allometric constraints) *vs.* stochastic (differentiation by geographic isolation) explanations for song spatial differentiation. We studied the song of Bluethroat *Luscinia svecica azuricollis* across its patchy upland distribution in Spain. To account for song structural variation, we measured a pool of spectral and temporal variables, being repertoire differentiation used as an indicator of song complexity. We fitted song data to parameters related to genetic structure and morphological differentiation of populations, habitat type and quality, as well as social context (species density and song bird community composition) by means of Generalized Linear Models. Then, using Mantel tests, we analyzed the influence of drift (differentiation by isolation) *vs.* environment (differentiation by environmental fit) in determining acoustic spatial variation. Our results highlighted a significant association of song spectral structure with vegetation type, body size and genetic structure and a significant connection between individual song complexity and habitat suitability. Mantel tests did not reveal a general relationship between song differentiation and genetic or geographic distances, but highlighted a significant relationship between song and environmental differentiation. Our study demonstrates that deterministic variation, in the form of adjustment to local habitat, prevails in shaping variation of song structure across the patchy distribution of the montane-dwelling study species. Part of variation among populations is also explained by body size and is associated with the genetic identity of populations. Moreover, song complexity emerges as a good population-level indicator of habitat quality for the species. These results suggest adaptive variation of song structure and highlight an emerging pattern from individual to population level that may be useful in conservation planning.

Introduction

Understanding the processes beyond the extraordinary diverse design of animal signals is a crucial challenge in both ecological and evolutionary research. Signals mediate many fitness-related animal behaviors implicated in intra and interspecific interactions and play an essential role in the differentiation of lineages (Wilkins *et al*. 2013). Birdsong is an emblematic example in this context. Serving in territorial defense, it acquires ecological relevance in space partitioning and species coexistence (Tobias *et al*. 2010, Tobias *et al*. 2014). Additionally, being implicated in mate attraction and intraspecific recognition (Collins 2004), it may fuel speciation processes by restricting gene flow (West-Eberhard 1983, Wilkins *et al*. 2013, Slabbekoorn & Smith, 2002, Aplin 2019). One of the most prominent features of birdsong is its high diversity, not only among species, but also within species. The song is acquired through social learning in songbirds (Seddon *et al.* 2013, Danchin *et al*. 2004, Osiejuk *et al*. 2019) and subjected to both random drift and local adaptation processes (Seddon 2005, Sorjonen 1986, Collins 2004, Ritchie 2007, Grant & Grant 2010). It is a target of sexual selection and may undergo correlated evolution with other phenotypic traits, such as body size (Turcoková *et al*. 2010). Therefore, integrative perspectives accounting both for extrinsic (environmental) and intrinsic (genetic or allometric) factors are required to disentangle the forces that shape it and contribute to its diversity (Gil & Gahr 2002, Derryberry *et al*. 2018).

In passerine birds, song complexity (repertoire size) depends not only on genetic factors, but also on the social or ontogenetic context (Nordby *et al.* 1999). For instance, high density of conspecifics (Laiolo *et al*. 2008), high species richness (Kelley *et al*. 2008, Grant & Grant 2010) or the abundance of resources during development (MacDonald *et al*. 2006) promote the diversity of individual repertoires. Some studies have identified bird song complexity to be an honest signal of individual quality, with individual variation in song traits advertising about male condition (Rowe & Houle 1996,

Buchanan & Catchpole 1997). Song frequency, often negatively correlated with bird body size (Podos 2001, Price 2008), is another example, although this feature also depends on habitat structure. Low frequency sounds in fact transmit better across dense vegetation than those at high frequency (Richard & Wiley, 1980; Bradbury & Vehrencamp 1998, Tobias *et al*. 2010).

The main objective of this study was to test alternative hypotheses on the role of allometry with body size, environmental fit (to vegetation type), habitat quality, social factors (density and composition of the song bird community) and chance (differentiation by geographic distance) in determining song spatial variation and song structure differentiation. The latter was evaluated in terms of complexity and structure, using as case study the Iberian breeding populations of *Luscinia svecic*a *azuricollis*, a specialist of montane shrublands. Iberian bluethroats are distributed across a wide geographical and environmental gradient where they present a moderate genetic differentiation (Alda *et al*. 2013). Thus, it represents an ideal model to assess whether song variation display patterns compatible with drift (variation through geographic distance) or adjust to environmental variation (environmental fit) and, at the same time, to test whether song covaries with intrinsic factors (genetic or biometric variation). If drift prevails, we would expect to find increasing song differences with geographic distance, as well as spatial differences in song structure varying in accordance to neutral genetic structure (Illera *et al*. 2014). We also predict a negative correlation between song frequency and body size, if allometry constrains song output (Price 2008), and an influence of vegetation type on spectral and temporal traits if song structure reflects a local adaptation to its habitat (Buchanan & Catchpole 1997, Grava *et al*. 2012). Given that Bluethroats imitate not only their congeners, but also other species, we expect that the homo- or hetero-specific social context will influence song output (Laiolo *et al*. 2011), thus song complexity to be related to conspecifics' abundance and bird species richness. Finally, the role of territory habitat quality on song complexity was assessed

through spatially explicit modelling approaches integrating different habitat components. Understanding the role of chance and of extrinsic and intrinsic factors on song spatial variation is crucial to evaluate the consequences of population isolation and to identify a reliable behavioral indicator of habitat deterioration or social environment in the wild.

Materials and methods

Study model

The bluethroat is a small migrant passerine (length 14-20 cm) that forms a complex of subspecies that has diverged recently (Hogner *et al.* 2003). Within the complex, the Iberian breeding population *Luscinia svecica azuricollis* represents the most ancestral subspecies that split from the rest 350,000 years ago in the Pleistocene (Hogner *et al.* 2003), showing a high genetic differentiation from the other subspecies (Alda *et al.* 2013). This subspecies breeds in mountain landscapes, where livestock and forestry activities allow the long-term maintenance of successional habitats (Morán-Ordóñez *et al.* 2013). It has a patchy distribution, occurring in three mountain ranges of northwestern Iberian Peninsula (Cantabrian Mountains, Mountains of León and Central System; Figure 5.1A) across an altitudinal range of 800 - 2400 m a.s.l. Sharp environmental differences exist among localities. They present a complex orography and are separated by a matrix of unsuitable habitats. The species inhabits shrublands dominated by *Quercus rotundifolia* and *Cistus* spp. at low-altitude, as well as heathlands (*Erica* spp. and *Calluna vulgaris*) and brooms (*Cytisus* spp. and *Genista* spp.) at high altitude (García *et al.* 2017). These landscapes are threatened by land use change (i.e., intensification and abandonment) that may modify significantly landscape connectedness and, thus, Bluethroat dispersal and genetic differentiation (Alda *et al.* 2013).

Bluethroat males usually sing in prominent areas (i.e., bush top), but also from inside the vegetation and, sometimes, in flight. Territorial song consists of phrases of 10 to 20 seconds, with loud sweet short whistles, grating buzzing sounds, dry trills, liquid trills and jingling notes (Figure 2) (Sorjonen 1986, Naguib & Kolb 1992, Turcoková *et al.* 2010). Bluethroats mimic sounds of different bird species, but also of amphibians and insects (Cramp 1988). In the Iberian range, it has been proved that, at least, they imitate to Red-legged Partridge *Alectoris rufa*, Common Quail *Coturnix coturnix* and Rufous Nightingale *Luscinia megarhyncos* (Corley & Bernis 1954, Corley 1959).

Song recording, spectrographic measurements and syllable classification

Songs were recorded in 15 localities distributed across the Iberian subspecies range (Figure 5.1A) three hours after the sunrise and three hours before the sunset, from March to June of 2011 and 2012. We used a Zoom H4n recorder and a Røde NTG3 microphone (frequency response 40-20kHz) to record the song of 138 male individuals from a distance of 5-40 m, for at least 2 minutes. This was the minimum time lag in which we could record an individual before it changed position or hid in the vegetation after noting our presence. Since many recorded birds were not marked, we could not assure individual identity for longer periods.

We used Avisoft SASLabPro software (Raimund Specht, Berlin, Germany) for sound analyses (sampling frequency 22,050 Hz, FFT length 512, time resolution 17.9 ms, frequency resolution 43Hz and Bartlett Window Function). We quantified acoustic parameters from waveforms (temporal parameters), spectra (frequency parameters) and sonograms (syllable identification). We measured a pool of variables that we consider could represent the complexity and structure of song strophes and syllables (Table 5.1). For strophes, we measured strophe duration, the number of original (different) syllables within a strophe, the overall number of syllable (irrespective if they were repeated or original), and, on average spectra, the lowest, maximum and dominant frequencies of the whole strophe, being the latter the harmonic that carries the greatest energy.
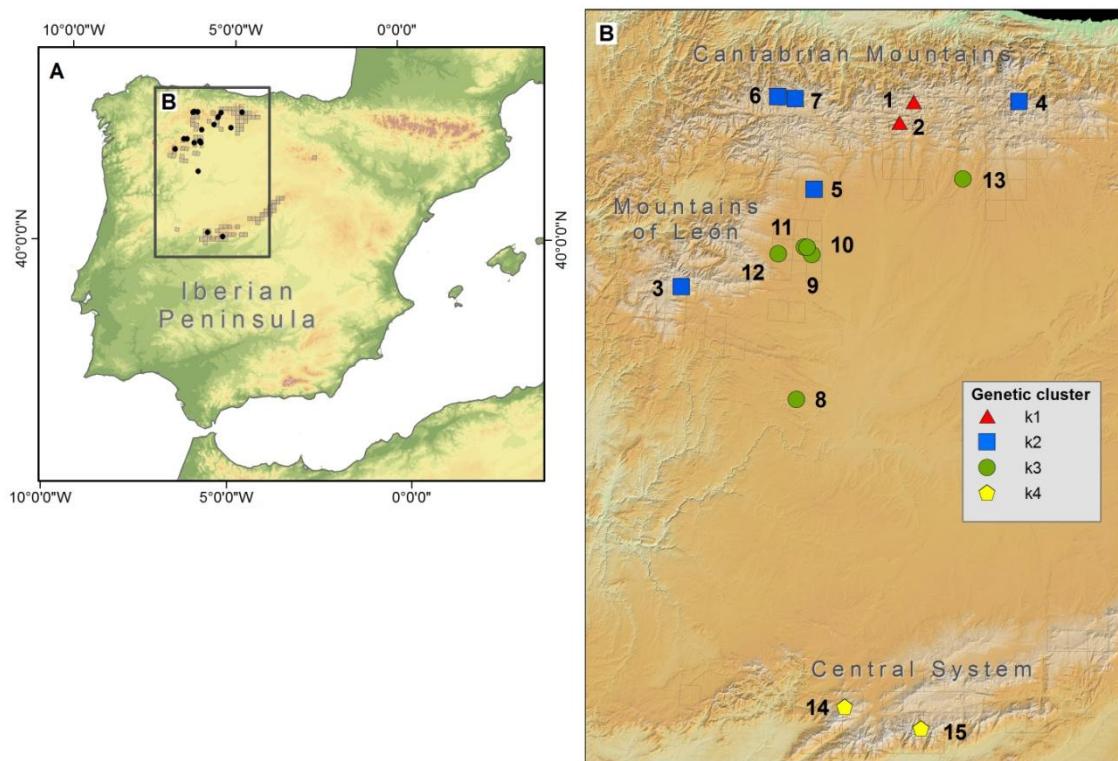
Locality	Mountain range	Number of song samples
1- Puebla Lillo	Cantabrian Mountains	7
2- Genicera	Cantabrian Mountains	9
3- San Martín de Castañeda	Mountains of León	3
4- Portilla de la Reina	Cantabrian Mountains	7
5- Ferreras de Cepeda	Mountains of León	8
6- La Cueta	Cantabrian Mountains	3
7- La Majúa	Cantabrian Mountains	9
8- Losacio	Mountains of León	3
9- Palacios de la Valduerna	Mountains of León	7
10- Toralino	Mountains of León	12
11- Bustos	Mountains of León	17
12- Villar del Golfer	Mountains of León	13
13- Corcos	Cantabrian Mountains	14
14- La Covatilla	Central System	13
15- Gredos	Central System	13

Figure 5.1. Study area. (A) Bluethroat breeding distribution in the Iberian peninsula: Black dots represent the song sampling localities and grey squares the breeding distribution (10 x 10 km grid) according to Martín & Del Moral (2003). (B) Song sampling localities: Symbols represent different genetic clusters (k1-k4) according to García *et al.* (2020).

Table 5.1. Song traits measured for this study on the song of Iberian Bluethroat breeding populations. Hz:Hertz, sec: seconds.

Type	Variable code	Description
Raw variables Spectral (Hz)	Fmin	Lowest frequency of the song phrase
	Fmax	Maximum frequency of the song phrase
	Fdom	Dominant frequency of the song phrase
	FminFirst	Lowest frequency of the first syllable
	FmaxFirst	Maximum frequency of the first syllable
	FdomFirst	Dominant frequency of the first syllable
	FminSec	Lowest frequency of the second syllable
	FmaxSec	Maximum frequency of the second syllable
	FdomSec	Dominant frequency of the second syllable
	FminSecLast	Lowest frequency of the second last syllable
	FmaxSecLast	Maximum frequency of the second last syllable
	FdomSecLast	Dominant frequency of the second last syllable
	FminLast	Lowest frequency of the last syllable
	FmaxLast	Maximum frequency of the last syllable
	FdomLast	Dominant frequency of the last syllable
Raw variables Temporal (sec)	DurPhr	Duration of the song phrase
	DurFirst	Duration of the first syllable
	DurSec	Duration of the second syllable
	DurSecLast	Duration of the second last syllable
	DurLast	Duration of the last syllable
Raw variables Repertoire	OrSyl	Number of original syllables per phrase
	TotSyl	Total number of syllables per phrase
Derived variables	Song frequency range	Difference between the maximum and the Minimum of frequencies of the strophe
	Syllable frequency range	Difference between the maximum and the minimum frecquencies of the first, second, second last and last syllables
	Syllable average duration	Average duration of the first, second, second last and last syllables
	Syllable rate	Relation between the number of different syllables and the duration of the strophe
	Average syllable dominant frequency	Average of dominant frequency of the first, second, second last and last syllables
	Average syllable minimum frequency	Average of minimum frequency of the first, second, second last and last syllables
	Average syllable maximum frequency	Average of maximum frequency of the first, second, second last and last syllables

At the syllable level, we selected the first, second, second last and last syllables, as representative of a strophe, and we measured their lowest, maximum and dominant frequencies, and their duration. Syllables were classified by visual inspection of the spectrograms (Botero *et al.* 2008). A syllable was considered as a continuous stroke of elements separated by less than 25 ms (Briefer *et al.* 2008) and a stroke was included in the same syllable when it had similar characteristics of timing, frequency and shape (Palmero *et al.* 2012). The two measures of repertoire size we selected (the number of different syllables and the overall number of syllable per strophe) were

chosen to allow objective comparisons among individuals. The classification of syllables was restricted to the strophe and we did not attempt to derive a measure of overall complexity of the Bluethroat repertoire, within and across individuals, to avoid subjectivity in classification. From the above variables, we calculated a number of derived variables that embraced comprehensively the song variation of the species in order to reduce the redundancy of raw variables in further analyses. We, therefore, measured: song frequency range (expressing the difference between the maximum and the minimum of frequencies of the strophe), average syllable frequency range (as above, but calculated averaging the frequency range of the four syllable measured), syllable average duration (calculated averaging the duration of the four syllables), average syllable dominant frequency (calculated averaging the dominant frequency of the four syllables) and syllable rate (overall number of syllable divided by song duration).

Genetic structure and genetic differentiation

To account for Bluethroat genetic differentiation indices, we used information on genetic distances among localities and genetic clusters, as published by García *et al.* (2020). These authors performed DNA extraction and microsatellite genotyping from the samples of 266 individuals captured in the same localities where song was recorded.

Bird morphology

Bluethroat body size was estimated by means of three variables: wing length-maximum chord (± 0.5 mm), tarsus length (± 0.1 mm) and body weight (± 0.1 g), all measured on a sample of 353 adult males that were captured between 2010 and 2011 in the same localities where song was recorded. We performed a Principal Component Analysis entering measurements of individuals from all localities. The value of the first principal component was taken as an index of overall body size, as recommended for small passerines (Freeman & Jackson 1990, Milá *et al.* 2009). Then, an average value of this PC per location was calculated.

Vegetation type and habitat quality

In each sampling point where birds were recorded, we noted the type of dominant vegetation. We considered three categorical vegetation types according to their structure (stem height and total vegetation cover) and species composition: brooms (*Cytisus* spp. and *Genista* spp), heathlands (*Erica* spp. and *Calluna vulgaris*) and holm oak shrublands (*Quercus rotundifolia* and *Cistus* spp.).

Habitat quality was assessed by means of a species distribution modelling (SDM) approach based on the species-environment relationship. We applied MaxEnt 3.3.3k (Phillips *et al* 2006; Phillips & Dudík, 2008) to a sample of 262 Bluethroat occurrence locations and a set of predictors including climate, topography and land cover at 200 m of spatial resolution that are likely to influence the suitability of breeding sites for Bluethroats. The output of the model is a habitat suitability index that can be interpreted as a habitat quality index ranging from 0 (null habitat quality) to 1 (high habitat quality) (García *et al.* 2007). Model predictive performance was assessed by means of the area under the receiver-operator characteristic curve (AUC), adapted for use with presence-background samples, using a ten-fold cross-validation. See García *et al.* (2020) for more technical details.

Composition of bird community

We conducted field surveys during the breeding season (from 1st May to 15th June) of 2010, 2011 and 2012, three hours after the sunrise and under good weather conditions (without rainfall or strong wind), to evaluate the composition of the bird community in each locality. The sampling method was a point transect sampling method (Bibby *et al.* 2000, Buckland *et al.* 2001) that allowed the estimation of: (i) Bluethroat abundance (number of individuals per sampling point); (ii) presence of other bird species (number of species per locality). After preliminary surveys and analyses on species' effective detection radius (Buckland *et al.* 2001), a minimum distance of 250 m between sampling points was fixed in order to avoid double counting of the same individuals (Buckland 2006).

Table 5.2. List of models including extrinsic and intrinsic driver of song differentiation as tested by Generalized Linear Models. Significant factors (P < 0.05) are shown in boldface type in each model. *Best models (ΔAIC≤3) in each set.

Response variable	Model	ΔAIC
OrSyl	**Hab_Qual**	0.0*
OrSyl	**Hab_Qual** + Bird_Rich	1.8*
OrSyl	**Hab_Qual** + Bluetr_Ab	2.0*
OrSyl	**Hab_Qual** + Bluetr_Ab + Bird_Rich	3.8
OrSyl	**Hab_Qual** + Cluster	4.2
DurPhr	**Hab_Qual**	0.0*
DurPh	**Hab_Qual** + Bird_Rich	1.9*
DurPh	**Hab_Qual** + Bluetr_Ab	2.1*
DurPh	**Hab_Qual** + Cluster	3.3
DurPh	Bird_Rich	4.0
Fdom	**Veg_Type** + **Body_Size** + Hab_Qual + **Cluster**	0.0*
Fdom	**Veg_Type** + **Body_Size** + **Cluster**	1.7*
Fdom	**Veg_Type** + **Body_Size**	5.1
Fdom	**Veg_Type** + **Cluster**	5.4
Fdom	**Body_Size** + Hab_Qual + **Cluster**	5.5
Song frequency range	**Veg_Type** + **Body_Size**	0.0*
Song frequency range	**Veg_Type** + **Body_Size** + Hab_Qual	1.9*
Song frequency range	Veg_Type + **Body_Size** + Cluster	5.6
Song frequency range	**Veg_Type** + **Body_Size** + Hab_Qual + Cluster	6.9
Song frequency range	**Body_Size** + **Cluster**	8.0
Syllable frequency range	**Veg_Type** + **Body_Size**	0.0*
Syllable frequency range	**Veg_Type** + Body_Size + Hab_Qual	0.9*
Syllable frequency range	**Veg_Type** + **Body_Size** + Cluster	4.4
Syllable frequency range	**Body_Size** + Hab_Qual + **Cluster**	4.8
Syllable frequency range	Veg_Type + **Body_Size** + Hab_Qual + Cluster	5.1
Syllable average duration	*Null model*	0.0*
Syllable average duration	**Cluster**	0.2
Syllable average duration	**Cluster** + Hab_Qual	2.0*
Syllable average duration	Veg_Type	2.1*
Syllable average duration	Hab_Qual	2.1*
Syllable rate	*Null model*	0.0*
Syllable rate	Bird_Rich	1.1*
Syllable rate	**Cluster**	1.2*
Syllable rate	Hab_Qual	1.3*
Syllable rate	Bluetr_Ab	2.1*
Average syllable dominant frequency	**Body_Size** + **Cluster** + **Hab_Qual**	0.0*
Average syllable dominant frequency	**Body_Size** + **Veg_Type**	2.3*
Average syllable dominant frequency	**Body_Size** + Veg_Type + Cluster + **Hab_Qual**	2.7*
Average syllable dominant frequency	**Body_Size** + **Veg_Type** + Hab_Qual	2.9*
Average syllable dominant frequency	**Body_Size** + **Cluster**	5.4

OrSyl:Number of original syllables per phrase; DurPhr: Duration of the song phrase; Fdom: Dominant frequency of the song phrase: Bird_Rich: Number of bird species in each locality; Bluetr_Ab: Bluethroats per sampling point in each locality; Body_Size: Scores of principal component analysis including the morphometric traits measured in males; Cluster: estimated using GENELAND; Hab_Qual: Output values of MAXENT habitat suitability model; Veg_Type: Brooms, Heathlands or Holm oak shrublands.
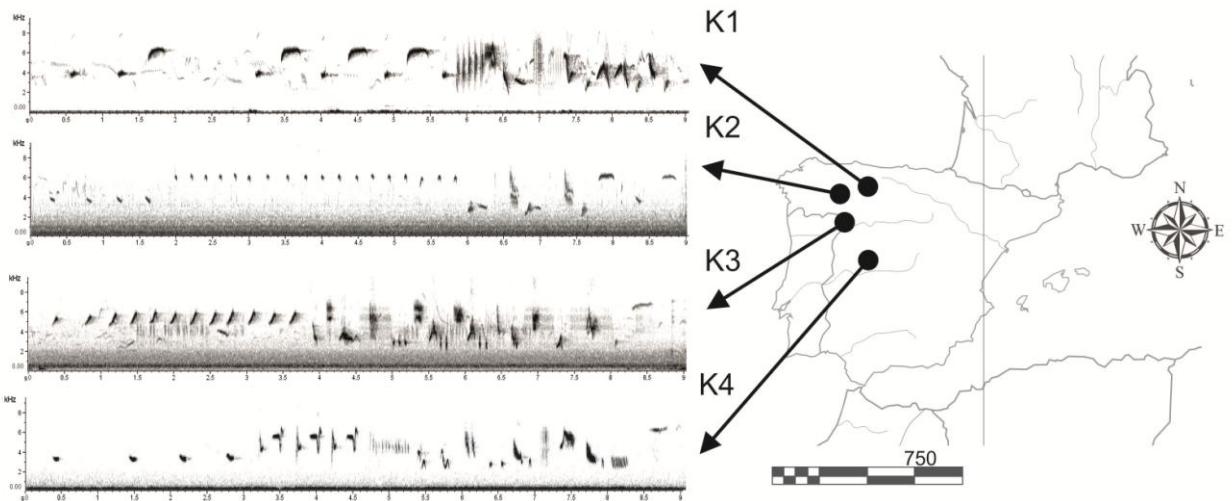
Figure 5.2. Song spectrogram examples of a Bluethroat in each mountain range. 1 y 2: Cantabrian Mountains; 3: Foothills of the Mountains of León, 4: Central System.

In each sampling point, we recorded all visual or acoustic contacts during five minutes, after waiting a minute for birds to resume normal behaviour (Bibby *et al.* 2000). The number of sampling points was 312 and each point was surveyed once.

Statistical analyses

Since the full set of 29 acoustic variables (Table 5.1) displayed different degree of bivariate correlation among them (Figure Chap.5.1S1 from the Appendix Chap.5), we used an expert criterion to select the pool of non-correlated variables, among raw and derived, that best summarize song variation. Therefore, in order to analyze the drivers of variation, we considered the number of original syllables per phrase as a proxy of song complexity, which we expect could correlate with habitat quality (Laiolo *et al.* 2008, Briefer *et al.* 2010, Grava 2012, Grava *et al.* 2012, Van Oort *et al.* 2006) or bird community diversity (Laiolo *et al.* 2011). We also selected the dominant frequency of song phrase and syllables as indicators of body size (Podos 1997, 2001) or vegetation structure (Richard & Wiley 1980), the latter also potentially influencing other target variables as song frequency range, syllable frequency range, phase and syllable durations and syllable rate. Finally, we chosen duration parameters that, together with

frequency parameters, can be population specific (Grant & Grant 2010, Illera *et al.* 2014). All further analyses were performed at the locality level, thus we averaged the values of variables collected at individual level (acoustic variables and body size) and vegetation type for each locality. Other variables were solely estimated in locality (genetic cluster, abundance, species richness, habitat suitability).We assessed the influence of genetic, morphologic, environmental and social factors on these selected song variables by means of Generalized Linear Models (GLMs). As predictors we considered male body size, habitat quality index, vegetation type, Bluethroat abundance and bird species richness, as well as genetic cluster (k1 to k4; García *et al.* 2020). Models were selected following both the criteria of Akaike's Information and model parsimony (Burnham & Anderson 2002) and also using the dredge function of MuMIn R library (R Core Team 2017).

We then analyzed whether song differentiation was associated with geographical, genetic distances (F_{ST}) and environmental dissimilarity between localities using Mantel tests. Song dissimilarity among localities was calculated as the Euclidean distance between song structure variables (including all raw variables and the derived ones described in Table 5.1; Figure Chap.5.1S2 from the Appendix). Environmental

dissimilarity was quantified considering habitat quality (continuous variable) and type (categorical variable) as the Gower distance (Gower 1971), that was estimated with the daisy function of the R package cluster (Maechler *et al.* 2019). Additionally, we used a Mantel correlogram (Legendre & Fortin, 2010) to examine if the relationship between song divergence and geographical distance was spatially structured. All statistical tests were performed using R 3.4.3 (R Core Team 2017).

Results

GLM analyses revealed that the mean number of different syllables per phrase and the duration of the song phrase were strongly and positively associated with the index of habitat quality, being this variable consistently included as a significant factor in all best models (Table 5.2). Populations made of larger individuals sang song phrases with lower average dominant frequency. Both song and syllable frequency range were driven by vegetation type, body size and genetic cluster. On average, males from holm oak shrublands sang with a higher dominant frequency than those from heathlands and brooms. Finally, average syllable dominant frequency was significantly associated with body size, cluster, habitat quality and vegetation type. No variable affected syllable average duration and syllable rate and no social feature entered in the best models of any acoustic variable (Table 5.3).

We found differences between clusters and localities in almost all frequency variables, but not in duration and complexity (number of syllables) song parameters (Table Chap.5.1S1 from the Appendix Chap.5). However, Mantel tests considering dissimilarity in all song variables did not reveal a significant relationship with genetic distance (F_{ST}) (r = 0.153, p = 0.119). There was no pattern of song differentiation by geographic distance across the entire distribution of the subspecies (r = 0.081, p = 0.236), but we identified a significant pattern at short distance categories (up to a distance of 70 km) (Figure Chap.5.1S1). Finally, song and environmental dissimilarity (r = 0.223, p = 0.045) were

significantly linked. This relationship persisted when controlling for the effect of geographic distance with partial Mantel test (r = 0.227, p = 0.036) (Figure 3).

Discussion

The action of deterministic and stochastic forces in driving character evolution becomes especially overt in populations with upland distributions, where adaptations to local conditions may be promoted by the reduction of gene flow among isolated populations, but, at the same time, drift effects are fueled by founder effects and small population sizes (Pato *et al.* 2019). Iberian breeding Bluethroat populations apparently display environmental fit in their song traits, supporting hypotheses of acoustic adaptation to the local habitat, in spite allometric constraints also control the direction of variation. The hypothesis of variation by isolation (and drift) can be partially dismissed: geographic distance had a limited effect and this effect disappeared when environmental dissimilarity was entered in the models as a predictor of song differentiation. Social biotic factors, such as conspecific density or local species richness, also played no role in the differentiation of the species repertoire at large scale in this study. This result has also been reported by Turcoková *et al.* (2010) in the same species and it seems rather that habitat quality is the predictor that best explains song complexity, revealing an emerging connection between individual repertoire and habitat suitability at the locality level.

The results of this study, emphasizing influences of body size, genetic cluster, vegetation type and habitat quality on song structure, support the idea that variability in song traits is due to a combination of forces (Gil & Gahr 2002). In particular, we found that variables related to song complexity were mainly influenced by habitat quality, so that Bluethroats with a more complex song occupy the best quality habitats. High habitat quality may assure the proper conditions for bird neural and cognitive development and determine that, on average, individuals produce songs with greater complexity.

Table 5.3. Generalized Linear Models selected to explain Iberian Bluethroat song traits in relation to a set of explanatory variables including habitat type, habitat quality, body size and genetic cluster. Significant factors (P < 0.05) are shown in boldface type.

Song trait (response variable)	Explanatory variable	Estimate	SE	Significance
OrySyl (Number of original syllables per phrase)	Intercept	9.435	0.266	
	Habitat Quality	**0.774**	**0.267**	**<0.01**
DurPhr (Duration of the song phrase)	Intercept	5.646	0.166	
	Habitat Quality	**0.343**	**0.167**	**<0.05**
Fdom (Dominant frequency of the song phrase)	Intercept	4253.31	243.81	
	Veg_Type. Holm oak	**412.53**	**133.99**	**<0.05**
	Veg_Type. Broom	124.07	208.15	0.55
	Body size	**-1.106**	**0.05**	**<0.001**
	Habitat Quality	-112.27	57.99	0.055
	Cluster k2	-181.78	207.96	0.386
	Cluster k3	-283.00	263.93	0.285
	Cluster k4	**-677.96**	**185.20**	**<0.001**
Song frequency range	Intercept	5008.78	82.36	
	Veg_Type. Holm oak	**215.83**	**120.25**	0.075
	Veg_Type. Broom	**-436.52**	**110.52**	**<0.001**
	Body size	**-96.14**	**30.07**	**<0.005**
Syllable frequency range	Intercept	2000.17	86.83	
	Veg_Type. Holm oak	126.99	126.77	0.312
	Veg_Type. Broom	**-701.81**	**116.52**	**<0.001**
	Body size	**113.97**	**31.70**	**<0.001**
Syllable average duration	Null model (intercept only)	0.135	0.003	
Syllable rate	Null model (intercept only)	1.762	0.047	
Average syllable dominant frequency	Intercept	4021.84	104.51	
	Body size	**-76.00**	**23.38**	**<0.005**
	Habitat Quality	**-115.80**	**42.33**	**<0.05**
	Cluster k2	233.58	127.57	0.069
	Cluster k3	**298.35**	**117.17**	**<0.05**
	Cluster k4	-255.60	140.92	0.071
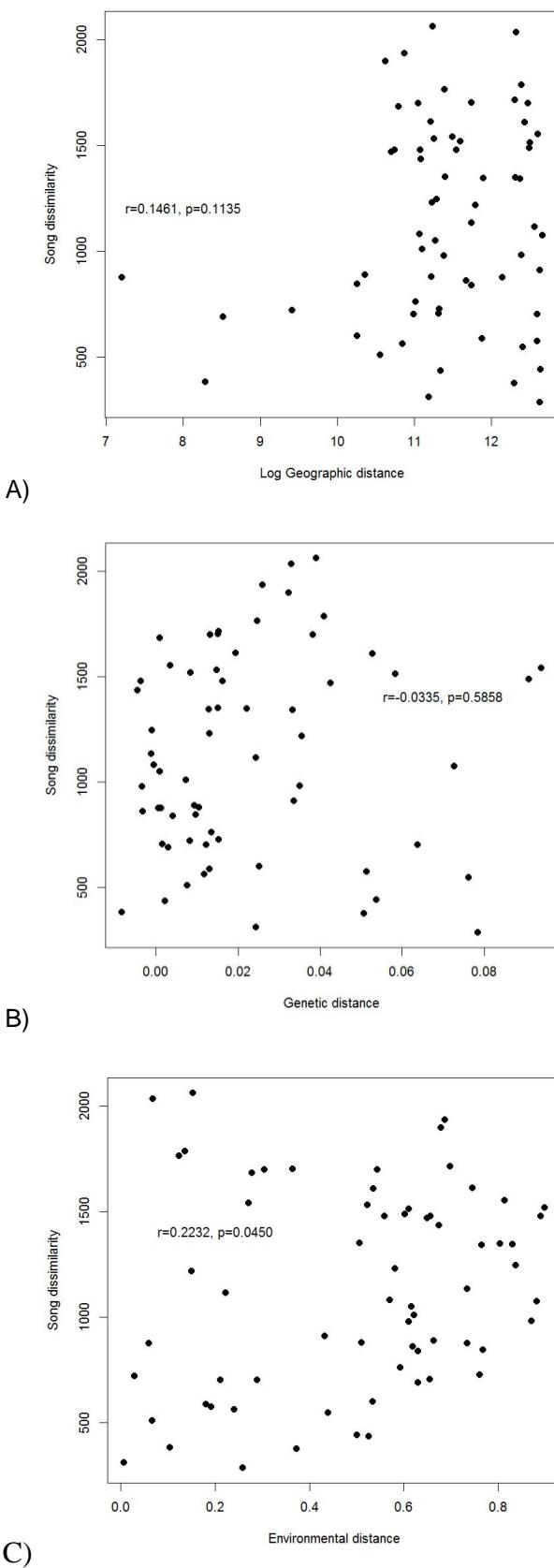
Figure 3. Mantel test results and plots of: A) song dissimilarity and geographic distance; B) song dissimilarity and genetic distance; C) song dissimilarity and environmental distance.

This interpretation, which should however be confirmed by experimental evidence, is in line with that obtained for other species with patchy distribution (MacDonald *et al.* 2006; Pijanowski *et al.* 2011). Bluethroats show high site fidelity (Alda *et al.* 2013). Therefore, males probably sing close to the site they were born and partially learn their song there, thus contributing to the maintenance of the song pool of the population across generations (see also Laiolo *et al.* 2008).

Dominant frequencies and frequency ranges were strongly correlated with body size, but spectral variables also varied among genetic clusters (Table Chap.5.1S2). This result can be partly explained by the covariation of genetic and biometric differentiation in the Bluethroat (Turcoková *et al.* 2010), as also observed in other bird species (Ryan & Brenowitz 1985, Martin *et al.* 2011, Illera *et al.* 2014). Allometric relationships represent, therefore, a mechanistic explanation useful for interpreting the genetics - acoustics association found in this study. Body size - song frequency relationships have been reported in a large number of studies among and within species (Ryan & Brenowitz 1985, Podos 2001, Price 2008), although exceptions also exist (Patel *et al.* 2010, Illera *et al.* 2014).

The observed cline of song and morphometric variation did not impede song to match vegetation structure variables. Vegetation type and habitat are considered to be main factors shaping the evolution of birdsong (Boncoraglio & Saino 2007, Goretskaia *et al.* 2018, Phillips *et al.* 2020). In particular, dense vegetation has been shown to promote the evolution of songs with lower frequencies, narrower frequency ranges and longer intervals between sound elements (Morton 1975). In our study system, bluethroats inhabiting vegetation patches with higher vegetation cover and structural complexity, as holm oak shrubs, emitted higher frequencies than those living in patches of brooms and heaths. The latter two form very dense shrublands, where not only leaves, but also an intricate structure of branches likely damper song transmission and, thus, favour individuals with lower frequency songs. The environment has an overring role in song differentiation across the entire range of Iberian Bluethroats also when considering all acoustic variables. Results of Mantel tests supports a pattern of differentiation by adaptation, promoted by environmental variation, over a pattern of differentiation by distance engendered by drift (Pato *et al.* 2019). Song differentiation increased with geographic distance, but only between localities up to a distance of 70 km. This threshold distance may reflect both patch size and the range of movements of the species. Individuals may share their repertoire with neighbours and, then, progressively differentiate from father individuals up to a certain threshold distance (Laiolo 2008). Assortative mating, i.e. females preferring to breed with males singing local songs (Podos 2010), may contribute to the maintenance of the similarity among local songs, together with phylopatry (Alda *et al.* 2013). Philopatric behaviour has been identified as decisive in the appearance of dialects in other bird species (Andrew 1962, Baker & Cunningham 1985), being the white-crowned sparrows *Zonotrichia leucophrys* a good example of it (MacDougall-Shackleton & MacDougall-Shackleton 2001).

In conclusion, this study demonstrates the effects of intrinsic and extrinsic drivers in shaping variation of song structure, even in species with patchy distribution in which differentiation due to isolation and chance effects should prevail. Patterns of variation are consistent with the acoustic adaptation hypothesis and with basic allometric rules between song frequencies and body size. Moreover, the relationship of song structure and complexity with habitat corroborates the functional importance of song for shedding light on the ecological and evolutionary processes beyond individual adaptation (Gasc *et al.* 2013, Pijanowski *et al.* 2011). All in all, the study of individual behavioural traits across large distributions connects the individual and the population level and may provide useful information for evolutionary research as well as conservation planning.

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Macho de pechiazul en un jaral de la comarca de Aliste (Zamora).

SUPPORTING INFORMATION (Chapter 5)

Appendix Chap.5.

Table Chap.5.1S1. Values (mean ± SD) of song variables measured and ANOVA tests results among genetic clusters of Iberian Bluethroats (García *et al.* 2020) and sample localities. Significant results highlighted in bold.

Variable	k1 (n=16)	k2 (n=30)	k3 (n=66)	k4 (n=26)	ANOVA Cluster	ANOVA Locality
Fmin	2205.37 ± 191.64	1863.20 ± 386.47	1742.06 ± 264.85	2098.30 ± 332.75	**<0.001**	**<0.001**
Fmax	6663.75 ± 453.10	6772.40 ± 503.50	6879.66 ± 462.43	6634.00 ± 671.59	0.153	0.237
Fdom	4296.00 ± 674.23	4059.00 ± 579.03	4236.60 ± 570.65	3768.84 ± 423.17	**<0.01**	**<0.001**
FminFirst	3480.75 ± 174.78	3698.68 ± 767.65	3921.42 ± 929.55	3297.30 ± 722.15	**<0.01**	**<0.05**
FmaxFirst	4150.62 ± 258.64	5071.20 ± 948.00	5482.51 ± 683.43	4269.07 ± 846.52	**<0.001**	**<0.001**
FdomFirst	3782.50 ± 234.50	4418.00 ± 740.05	4769.75 ± 764.66	3814.38 ± 775.26	**<0.001**	**<0.001**
FminSec	3530.25 ± 286.62	3471.28 ± 714.01	3709.24 ± 1007.61	3236.38 ± 716.84	0.106	0.050
FmaxSec	4294.12 ± 342.14	4829.13 ± 779.97	5395.84 ± 745.99	4368.53 ± 769.00	**<0.001**	**<0.001**
FdomSec	3898.50 ± 358.96	4244.66 ± 591.29	4626.54 ± 789.32	3819.46 ± 732.32	**<0.001**	**<0.001**
FminSecLast	3196.25 ± 499.18	2983.26 ± 744.54	2643.06 ± 587.29	2965.92 ± 501.49	**<0.01**	**<0.01**
FmaxSecLast	5001.62 ± 716.52	5012.86 ± 726.92	5383.33 ± 825.89	4652.38 ± 523.30	**<0.001**	**<0.01**
FdomSecLast	4044.50 ± 591.30	3948.13 ± 668.06	3987.24 ± 585.38	3809.61 ± 521.51	0.550	0.260
FminLast	3251.37 ± 562.61	3080.86 ± 665.31	2867.12 ± 642.16	3015.46 ± 472.97	0.103	**<0.05**
FmaxLast	4788.75 ± 621.13	5050.40 ± 769.99	5188.90 ± 709.85	4715.61 ± 499.07	**<0.05**	0.07
FdomLast	4045.25 ± 535.99	4129.26 ± 653.37	3983.15 ± 630.00	3923.15 ± 475.91	0.590	0.779
DurPhr	5.03 ± 1.82	5.90 ± 2.29	5.79 ± 2.11	5.36 ± 1.09	0.398	0.502
DurFirst	0.08 ± 0.02	0.11 ± 0.06	0.11 ± 0.06	0.11 ± 0.05	0.216	0.399
DurSec	0.09 ± 0.02	0.13 ± 0.08	0.12 ± 0.09	0.11 ± 0.05	0.382	0.267
DurSecLast	0.11 ± 0.03	0.12 ± 0.04	0.14 ± 0.06	0.15 ± 0.05	0.090	0.055
DurLast	0.14 ± 0.05	0.15 ± 0.08	0.15 ± 0.06	0.18 ± 0.08	0.246	0.456
OrSyl	9.07 ± 2.35	9.56 ± 3.58	9.70 ± 3.18	8.63 ± 3.24	0.518	0.193
TotSyl	16.90 ± 4.98	19.70 ± 7.06	19.40 ± 7.06	17.70 ± 4.47	0.357	0.339

Figure Chap.5.1S1. Bivariate Pearson´s correlations between the song variables considered in this study.
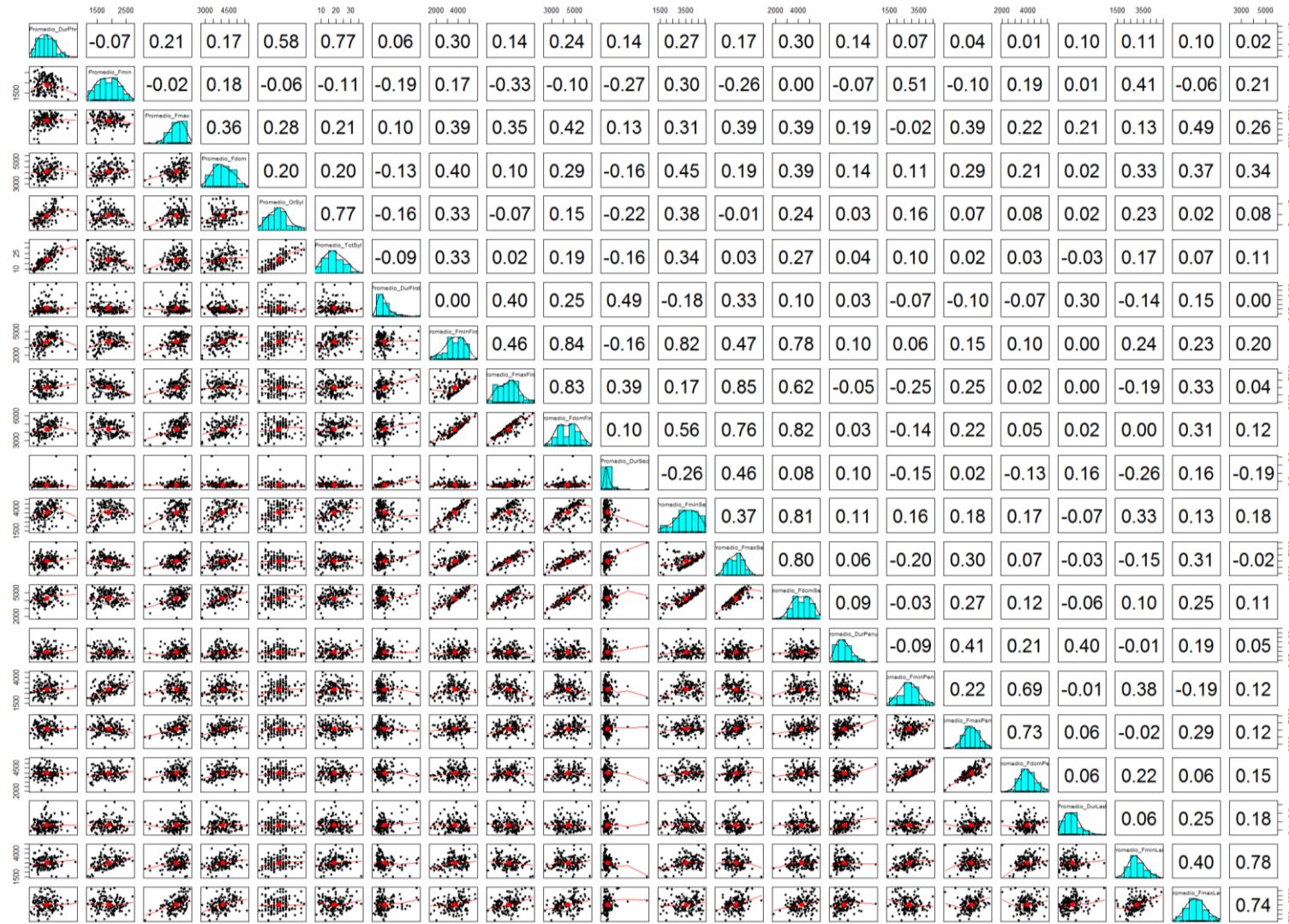
Figure Chap.5.2S1. Mantel correlogram showing spatial correlation in Iberian Bluethroat song structure. A solid square indicates that correlations between song differentiation and geographic distance were significant at α=0.05 with a Bonferroni correction and a square above the red line represents positive spatial correlation. The coefficient of correlation is represented in the Y-axis, and geographic distance (in m) in the X-Axis.
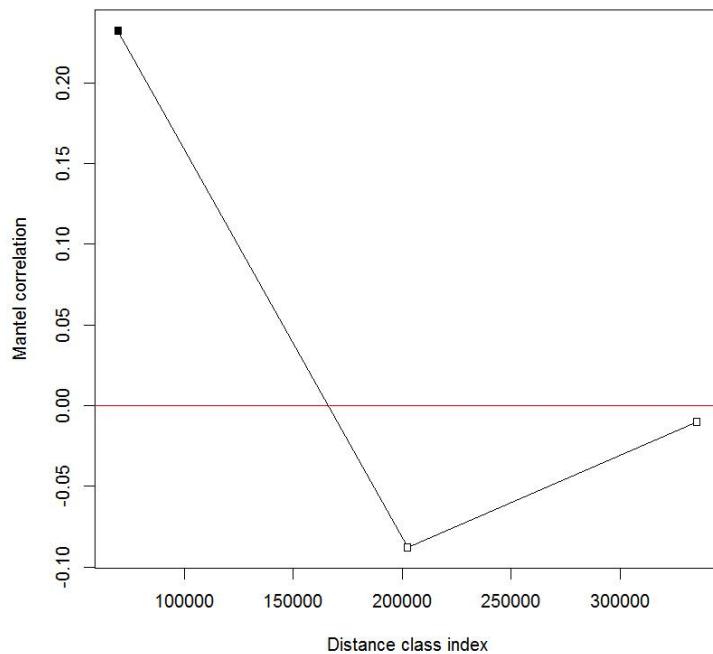
Equipo de grabación de canto. Sanabria. Zamora

5 Discusión general

5 DISCUSIÓN GENERAL

En esta tesis se ha demostrado la influencia de la heterogeneidad del paisaje sobre los procesos de diferenciación genética y fenotípica ocurridos en una especie de ave paseriforme migratoria, cuestión que resulta básica para entender fenómenos claves en Ecología como la especiación y la diversidad biológica. En el capítulo 1 se evidenció la singularidad de las poblaciones ibéricas de *Luscinia svecica azuricollis* en el contexto del complejo subespecífico del pechiazul, así como la importancia de conservar estas poblaciones para garantizar niveles de diversidad genética suficientes que aseguren la persistencia de la especie en un contexto de cambio global. En los capítulos 2 y 3 se demostró que la estructura genética de los pechiazules ibéricos, evaluada mediante el uso de marcadores genéticos neutrales (microsatélites), presenta una mayor correlación con la resistencia del paisaje, evaluada mediante técnicas de modelado espacialmente explícito basadas en series temporales de datos de Teledetección, que con la distancia geográfica. A pesar de la capacidad de movimiento de la especie, la conexión funcional existente entre los parches de hábitat favorable desempeña un importante papel en la dispersión reproductora (capítulo 3). Finalmente, en los capítulos 4 y 5 se puso de manifiesto la diferenciación morfológica de los machos nidificantes de *Luscinia svecica azuricollis* a lo largo de gradientes ambientales y geográficos, en función del aislamiento geográfico y la diferenciación génica, identificándose un efecto importante de la latitud, aunque en sentido contrario a lo que afirma la Regla de Bergmann (capítulo 4). Por último, los análisis de estructura del canto indicaron que la diversificación de este rasgo cultural está modelada por un conjunto de fuerzas determinísticas y estocásticas, a lo que se suman adaptaciones locales, favorecidas a su vez por procesos de deriva génica (capítulo 5).

5.1 Relevancia de las poblaciones ibéricas de pechiazul

La determinación de la posición taxonómica de las poblaciones de las especies debe reflejar la divergencia evolutiva de las mismas y los procesos de adaptación a las diferentes condiciones ambientales. Las poblaciones nidificantes ibéricas de pechiazul han estado tradicionalmente clasificadas erróneamente en la subespecie *cyanecula*, que se distribuye por Europa Central. Sin embargo, actualmente está ampliamente reconocido que deben ser asignadas a la subespecie *azuricollis* (Hogner *et al*. 2013, Clements *et al*. 2016). Los resultados obtenidos en esta tesis (capítulo 1) reflejan la singularidad de las poblaciones ibéricas respecto de las subespecies como *cyanecula*, *namnetum* y *magna* con marcadas diferencias biométricas y de plumaje. El análisis de las características biométricas en las diferentes subespecies europeas permitió encontrar diferencias significativas en variables biométricas como el ala, el tarso y el pico. Se estima que las diferencias en el ala pueden estar relacionadas con estrategias migratorias diversas entre las distintas subespecies, como ya se ha demostrado anteriormente analizando la morfología del ala (Arizaga *et al*. 2006). En cuanto al plumaje, las características más distintivas de los machos de la subespecie ibérica son la ausencia de mancha blanca en el pecho en un porcentaje más elevado que en el resto de subespecies, a excepción de *magna*, y la ausencia completa de banda de color blanco en el pecho. En este sentido, debe tenerse en cuenta que la ornamentación del plumaje del pecho está fuertemente relacionada con la diferenciación genética de las subespecies de pechiazul (Johnsen *et al*. 2006) y que éste es un factor que puede limitar el cruzamiento entre subespecies. Este patrón de clasificación filogenética basado en el plumaje y la genética es concordante con el obtenido para otras características estudiadas en las poblaciones

europeas, tales como la morfología espermática, que es un factor determinante en los procesos de especiación a través de la selección sexual postcópula (Hogner *et al.*, 2013).

5.2 Efectos del paisaje en la diferenciación genética entre las poblaciones ibéricas de pechiazul

El análisis de la diferenciación genética basado en marcadores neutrales en varias localidades donde nidifica el pechiazul en los macizos montañosos del NW de la península Ibérica permitió establecer la existencia de un grado de diferenciación genética moderado y claras evidencias de limitaciones en el flujo génico, además de signos de erosión genética. Se identificaron cuatro clústeres genéticos, tres en el norte de España (Montes de León, cordillera Cantábrica oriental y occidental y cordillera Cantábrica central) (capítulo 2) y uno más en el sistema Central (capítulo 3). La divergencia genética entre ellos fue muy similar, lo que indica la existencia de factores que restringen el flujo génico a escala local (Clark *et al.* 2008). Los valores de diferenciación genética entre los clústeres del norte de España fueron superiores a los encontrados entre subespecies de Escandinavia reconocidas como *Luscinia svecica svecica* y *Luscinia svecica gaetkei* (Hellgren *et al.* 2008). Este grado de diferenciación genética, junto con los bajos valores de diversidad genética obtenidos, más bajos en relación con el resto de poblaciones euroasiáticas (Johnsen *et al.* 2006, Hellgren *et al.* 2008), podría relacionarse con los efectos de una deriva génica por aislamiento genético y geográfico (Frankham *et al.* 2002). Además, la diferenciación genética a escala local en la península Ibérica puede relacionarse con una acusada fidelidad a las áreas de cría, un fenómeno que reduce el flujo génico y que ha sido considerado para explicar el grado de diferenciación de especies migradoras y con una capacidad de desplazamiento muy alta, como es

el caso del pechiazul (FitzSimmons *et al.* 1997; Van Bekkum *et al.* 2006).

El análisis espacial de la conectividad funcional entre localidades, evaluada como la resistencia que ofrece el paisaje al intercambio genético, puso de manifiesto la importancia de aplicar técnicas de modelado espacialmente explícito basadas en datos de observación remota (imágenes de satélite) en Genética de Paisaje. Especialmente relevante fue la utilización de series temporales del índice de vegetación de diferencia normalizada (NDVI), producto espectral que ha permitido reflejar la naturaleza y estabilidad de los hábitats considerados. Se encontró que la estructura genética actual de las poblaciones nidificantes Ibéricas de pechiazul está condicionada por los atributos actuales del paisaje, así como por su estabilidad, por lo que se estima un efecto de la permeabilidad del paisaje en la estructura genética actual de las poblaciones de pechiazul estudiadas (capítulo 3). Los resultados obtenidos también muestran un efecto del aislamiento geográfico en la diferenciación genética, al contrario de lo que se ocurre cuando se analizan únicamente las poblaciones norteñas, cordillera Cantábrica y Montes de León, como se obtuvo en el capítulo 2. Por otra parte, se observó que la conectividad funcional entre las distintas localidades y, por tanto, el flujo génico, es más elevado cuanto mayor es la conectividad estructural del hábitat idóneo, lo que apoya la idea de que las características del paisaje influyen en la dispersión y el flujo génico entre poblaciones (Bruggeman *et al.* 2010, Coulon *et al.* 2010, Milá *et al.* 2010).

En el Capítulo 3 se encontró que la mayor idoneidad de hábitat para el pechiazul corresponde a zonas ocupadas por matorral que no ha estado sometido a niveles elevados de perturbación, se ha mantenido más estable a lo largo del tiempo y ha sufrido cambios limitados de estructura de hábitat. El mantenimiento del hábitat de nidificación del pechiazul depende tanto de las actividades humanas, como de los

procesos naturales de sucesión secundaria. La estructura de estos hábitats de matorral es inherentemente variable y, por tanto, su capacidad de carga cambia de manera constante. De esta forma, no solo las perturbaciones extremas de origen antrópico, como los incendios o los desbroces, pueden destruir el hábitat del pechiazul, sino también una evolución por sucesión secundaria de los hábitats de matorral hacia hábitats forestales también puede suponer una pérdida de hábitat para la especie.

Además del aislamiento geográfico y paisajístico (Moore *et al.* 2005, Porlier *et al.* 2009), otros muchos factores pueden subyacer tras los patrones de estructura genética observados, tales como diferencias en rasgos biológicos relacionados con el tamaño de población, el patrón migratorio, el área de invernada, el sistema de emparejamiento y la filopatría hacia las áreas de nidificación. Estos factores pueden interactuar a diferentes escalas temporales y espaciales. Otras circunstancias, como el hecho de situarse en el extremo meridional de su distribución, podrían explicar el alto grado de endogamia y la baja diversidad genética encontrados (Hoffman & Blows 1994, Hampe & Petit 2005). En general, las poblaciones ibéricas de pechiazul mantienen un pequeño tamaño de población efectiva y unos valores de diversidad genética reducida, lo que puede comprometer su futuro al perder capacidad de adaptación frente a los cambios presentes y futuros (Reed & Frankham 2003, Bouzat 2010).

5.3. Diferenciación fenotípica en relación con las características genéticas y el paisaje

Diferenciación morfológica

En el capítulo 4 se encontraron diferencias morfométricas entre los individuos correspondientes a las diferentes localidades y grupos genéticos, así como una correlación entre algunas de las variables morfológicas (ala y peso) con la diferenciación genética existente o con el aislamiento geográfico (capítulo 4). La mayor diferenciación, tanto genética como fenotípica, se detectó en las localidades del macizo montañoso del sistema Central, que alberga el grupo genético más meridional y aislado geográficamente. Los resultados obtenidos sobre diferenciación morfológica son concordantes, en general, con procesos de deriva génica fruto del aislamiento geográfico, aunque la diferencia detectada en las variables sobre las que existe a priori mayor presión selectiva, longitud de tarso y ala, apunta a la existencia de adaptaciones locales. En otras especies, este tipo de adaptaciones se ha relacionado con el hábitat, la dieta y los patrones migratorios (Fiedler 2005, Förschler & Bairlein 2011). En el caso del pechiazul, las diferencias observadas en el índice de apuntamiento del ala podrían relacionarse con diferentes estrategias de migración en los grupos genéticos establecidos. En este sentido, Arizaga *et al.* (2006) encontraron diferencias similares en la morfología alar entre las poblaciones del sur y centro de Europa, que fueron corroboradas posteriormente mediante análisis conjuntos de isótopos y morfología (Arizaga *et al.* 2015). En definitiva, los resultados de los análisis de las variables biométricas y de apuntamiento del ala fortalecen la idea de que la diferenciación morfológica no es únicamente fruto de la deriva génica, sino que se debe al efecto de fuerzas selectivas, incluso antagonistas. Así, los ejemplares del sistema Central tienen un tamaño claramente superior que se refleja en todas las variables biométricas consideradas. Sin embargo, no ocurre lo mismo con el índice de apuntamiento del ala, detectándose una clara diferenciación respecto a las poblaciones de las montañas norteñas, pero no respecto de las poblaciones de las áreas de piedemonte de los macizos montañosos del norte. No obstante, las diferencias en la morfología alar pueden no estar relacionadas únicamente con la estrategia migratoria, dado que ésta varía en función de

otros factores, como la edad de los ejemplares, pudiendo haber otras presiones selectivas, como el hábitat o la forma de vuelo frente a la depredación (Mönkkönen 1995).

Por otra parte, respecto a la diferenciación morfológica a lo largo de gradientes geográficos y ambientales, únicamente se detectaron diferencias en función de la latitud, pero en sentido contrario a lo esperado de acuerdo con la regla de Bergmann. Aunque la regla de Bergman se ha comprobado en muchos grupos de especies (Hamilton 1961, Blackburn & Gaston 1996), también existen multitud de ejemplos de especies o poblaciones en las que la no se cumple (Salewski *et al.* 2010). El hecho de que la regla de Bergman no se cumpla en el caso de esta especie en la península Ibérica, puede deberse a que las aves que nidifican a mayor altitud se sitúan en el macizo más meridional y con más influencia mediterránea, lo que empujaría a las aves a buscar sus condiciones climáticas más favorables a mayor altitud. Por otra parte, los resultados obtenidos parecen indicar que no existe una confluencia evolutiva en el tamaño de las aves. Las aves más grandes se localizan en latitudes inferiores y a mayor altitud, no detectándose que las que nidifican a mayor altitud tengan una morfología alar similar.

Diferenciación en el canto

Se encontraron notables diferencias en las características del canto a lo largo del área de distribución del pechiazul en la península Ibérica, tanto entre distintas localidades como entre grupos genéticos (capítulo 5). Este patrón espacial se asoció con variaciones en la calidad del hábitat, la diferenciación genética y el aislamiento geográfico, así como con las condiciones particulares de cada localidad, como el tipo de vegetación, y el tamaño corporal de las aves. Los resultados apoyan la idea de que una multitud de fuerzas determinísticas y estocásticas están involucradas en la estructura y complejidad del canto de las aves y lo modulan

con diferente dirección e intensidad. El aislamiento y las adaptaciones locales promueven la reducción del flujo génico y esto condiciona la diferenciación fenotípica favorecida por efectos de la deriva génica, como el reducido tamaño de población o el efecto fundador. Al contrario de lo esperado, la riqueza de otras especies de canto en cada localidad y la abundancia de congéneres no supusieron una mayor riqueza del repertorio, a pesar de que el pechiazul imita las señales acústicas de otras especies (Cramp 1988). Por su parte, la fragmentación de los hábitats dificulta la transmisión del canto entre poblaciones distantes o no conectadas. Los resultados muestran que es más probable que los individuos de un mismo parche de hábitat compartan su repertorio y que este repertorio sea progresivamente más diferente en individuos más alejados o aislados, como se ha demostrado en otras especies que utilizan hábitats fragmentados (Laiolo 2008). La diferenciación del canto puede acelerarse mediante el apareamiento asortivo entre individuos con una estructura de canto común, lo que provoca un refuerzo de las diferencias de canto entre poblaciones y una reducción del flujo genético entre los diferentes grupos dialectales. Además, el fuerte comportamiento filopátrico de los pechiazules ibéricos, que ha contribuido a su diferenciación genética (Alda *et al.* 2013), puede contribuir también a la diferenciación del canto. En este sentido, la filopatría ha sido identificada como factor determinante en la aparición de dialectos en otras especies de aves (Andrew 1962, Baker & Cunnigham 1984). De acuerdo con los resultados obtenidos, la complejidad del canto está influenciada por la calidad del hábitat, de modo que los pechiazules con un canto más complejo ocuparían los mejores hábitats. Esta relación entre la complejidad del canto y la calidad del hábitat es concordante con la teoría de que el canto es una señal honesta de la calidad de los territorios (Catchpole 1986; Gil & Gahr, 2002).

Entre las variables de canto consideradas en este estudio, se han detectado diferencias en los valores de las frecuencias máxima, mínima y dominante de las frases analizadas y de las primeras sílabas, mientras que apenas existen diferencias en las frecuencias de las últimas sílabas de cada estrofa ni en las variables de las notas y frases, ni en la complejidad. Este mayor grado de diferenciación de las primeras sílabas puede producirse porque estas notas se perciben en primer lugar y pueden permitir la identificación individual entre territorios adyacentes, como se ha observado en otras especies (Nottebohm 1975), Palmero *et al.* 2012). Por otro lado, la estructura más común del canto del pechiazul se inicia con la repetición continua de una o dos notas (Cramp 1988, Naguib & Kolb 1992). Por otra parte, se detectó que las diferencias de frecuencia entre clusters y localidades están fuertemente correlacionadas con el tamaño de las aves, tal y como se ha observado en otras especies de aves (Ryan & Brenowitz, 1985; Martin *et al.* 2011) y también en otras subespecies europeas de pechiazul (Turcoková *et al.* 2010). Estas diferencias de frecuencias entre los diferentes grupos genéticos pueden aumentar el aislamiento reproductivo de las poblaciones y su diferenciación genética (Grant & Grant, 1996). En cuanto a la estructura del hábitat, la frecuencia del canto fue superior en localidades donde la vegetación presenta una estructura vertical más compleja (encinares degradados). La estructura del hábitat se considera uno de los factores que determinan la evolución del canto (Boncoraglio & Saino 2007) y la transmisión a larga distancia depende, en gran medida, de las circunstancias ambientales (Hansen 1979). El hecho de que exista un patrón general de degradación sonora en función del hábitat llevó a la hipótesis de adaptación acústica (Morton 1975) que propone que, en áreas de vegetación densa, las aves emiten en frecuencias más bajas, rangos de frecuencia más estrechos e intervalos más largos entre los elementos sonoros. Los resultados apoyan la idea de que el efecto de factores intrínsecos y

extrínsecos afecta a la variación de la estructura del canto, incluso en especies como el pechiazul con distribución irregular, donde podría pensarse que la diferenciación se debiera al aislamiento y a los efectos de azar. Además, la relación de la estructura y la complejidad del canto con el hábitat corroboran la importancia funcional del canto para arrojar luz sobre los procesos ecológicos y evolutivos que van más allá de la adaptación individual.

5.4 Implicaciones para la conservación

El diseño de políticas acordes con la conservación de la diversidad genética de las especies debe considerar la posición taxonómica de las poblaciones (Alström *et al.* 2007) y, por tanto, debe tener en cuenta las características tanto genéticas como fenotípicas. En este sentido, resulta útil la identificación de "unidades significativamente evolutivas" (ESUs; Ryder 1986, Moritz 1994, Crandall *et al.* 2000, Luck *et al.* 2003) para determinar las zonas que mantengan una mayor diferenciación, dando prioridad en las políticas de conservación a espacios que incluyan gradientes ambientales, de forma que aseguren la viabilidad de las poblaciones y de su potencial adaptativo (Meffe & Carrol 1997, Moritz 2002, Holderegger *et al.* 2006). Dado que las poblaciones ibéricas habitan en el límite latitudinal y altitudinal de la especie, en los macizos del noroeste peninsular, se considera que, en un escenario de cambio climático (Thomassen *et al.* 2010), es muy probable que se produzca una reducción en el hábitat disponible, dado que en muchas áreas no hay posibilidad de desplazamiento de las actuales poblaciones a nuevos emplazamientos a mayor altitud (Walther *et al.* 2002, Förschler *et al.* 2011). Esto pone de relieve que, en el caso de las montañas ibéricas, se debe favorecer la conexión funcional entre poblaciones, de forma que se permita el intercambio genético, la conservación de la diversidad genética y, por tanto, la preservación del potencial evolutivo y el mantenimiento de la resiliencia de las especies ante los cambios ambientales futuros. Para ello,

deben desarrollarse programas de manejo de los hábitats de matorral que tengan en consideración su conectividad a gran escala. Y por otra parte, deben llevarse a cabo estudios específicos sobre las comunidades faunísticas y florísticas que permitan evaluar los efectos de las políticas de conservación así como el efecto de las diferentes actividades económicas desarrolladas en los principales núcleos de distribución de la especie.

Se estima que la consideración explícita de las diferencias genéticas y fenotípicas existentes a nivel de subespecie es clave para el diseño de estrategias de conservación realistas a diferentes escalas geográficas. De acuerdo con esto, las poblaciones ibéricas deben considerarse como prioritarias para preservar la diversidad genética en el contexto europeo.

A pesar del importante valor ecológico de las poblaciones de *azuricollis*, existen serios problemas de conservación, tales como los incendios forestales, el uso intensivo del territorio por actividades agroganaderas y forestales y la falta de aplicación de criterios conservacionistas en la gestión del territorios, entre otros. Así pues, la sostenibilidad de sus poblaciones depende de políticas de conservación que tengan en cuenta la importancia de considerar cuestiones como la prevención de incendios forestales y el manejo del matorral a través del pastoreo como principal actividad agroganadera tradicional. En general, las actividades agroganaderas deben suponer perturbaciones de escasa intensidad que eviten cambios drásticos en la composición actual de los hábitats (Bunce *et al.* 2004). Además, dado que el pechiazul se distribuye actualmente en espacios de la Red Natura 2000, su conservación implicaría la preservación de multitud de hábitats prioritarios y de especies amenazadas en zonas de montaña, incluidos algunos endemismos ibéricos.

6 Conclusiones

6 CONCLUSIONES

1. Las poblaciones ibéricas de pechiazul *Luscinia svecica azuricollis* deben considerarse como prioritarias en el diseño de políticas de conservación encaminadas a la preservación de la diversidad genética y la resiliencia de la especie frente a cambios ambientales a largo plazo en el contexto euroasiático.

2. El análisis de la estructura genética de las poblaciones ibéricas de pechiazul, basado en marcadores neutrales (microsatélites), evidenció la existencia de cuatro clústeres genéticos que mostraron un grado de diferenciación genética moderada. Este grado de diferenciación fue superior al identificado entre subespecies europeas.

3. Se encontraron evidencias de limitaciones en el flujo génico entre clústeres, un tamaño efectivo de población reducido y signos de erosión genética, lo que se relaciona con procesos de deriva génica por aislamiento reproductor y con una acusada fidelidad a las áreas de cría.

4. La calidad del hábitat de nidificación del pechiazul ibérico se asocia fundamentalmente con la estabilidad de las formaciones de matorral. Los atributos actuales y la estabilidad del paisaje, evaluados a través de índices espectrales derivados de imágenes de satélite, explican de forma sustancial los patrones de conectividad funcional existentes entre las localidades estudiadas.

5. Existe un patrón geográfico de diferenciación morfológica en cuanto al tamaño de los individuos en función de la latitud, pero en sentido contrario a lo esperado de acuerdo con la regla de Bergmann. Esta discrepancia podría estar relacionada con procesos de deriva génica y adaptación local en el sistema Central, el extremo más meridional de distribución de la especie, donde nidifica a mayor altitud.

6. Los caracteres morfológicos (biométricos y morfométricos) de los pechiazules nidificantes ibéricos varían significativamente entre localidades y grupos genéticos. La mayor divergencia corresponde a las localidades del sistema Central, donde se sitúa el grupo genético más aislado geográfica y funcionalmente.

7. La diferenciación de las variables sobre las que existe una mayor presión selectiva, como la longitud del tarso y el ala, no muestra correlación con la distancia genética, sino que podría relacionarse con adaptaciones locales por diferencias en el hábitat y las estrategias migratorias.

8. La población de pechiazules nidificantes ibéricos presenta importantes variaciones espaciales en cuanto a la complejidad y estructura del canto. Una multitud de fuerzas determinísticas y estocásticas están involucradas en la diferenciación en el canto, pero los factores con un efecto más significativo son el tamaño corporal, el tipo de vegetación, la calidad del hábitat y la distancia geográfica.

9.   El marco teórico de la Ecología del Paisaje y la Genética de Poblaciones, así como la aplicación combinada de técnicas de análisis genético, evaluación fenotípica (morfología y canto), Teledetección y modelado espacialmente explícito, permiten generar el conocimiento científico necesario para el diseño de estrategias de conservación fundamentadas y eficientes a diferentes escalas geográficas.

10. Las políticas dirigidas a la preservación de las poblaciones nidificantes Ibéricas de pechiazul deberían tener en cuenta la importancia del manejo del matorral a través de actividades agroganaderas sostenibles ambientalmente y la prevención de incendios forestales.

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