Genética de la Conservación del Cernícalo Primilla:

Variación Neutral y Adaptativa

Memoria presentada por **MIGUEL ALCAIDE TORRES** para optar al grado de **DOCTOR EN BIOLOGÍA**

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INTRODUCCIÓN GENERAL

Los numerosos avances en biología molecular han contribuido de manera notable a mejorar nuestra manera de entender y gestionar la naturaleza y sus recursos. El descubrimiento de marcadores genéticos polimórficos ha permitido abordar cuestiones de gran importancia en ecología y evolución a distintos niveles taxonómicos. Una de las primeras variaciones genéticas entre individuos y especies fue puesta de manifiesto durante la década de los 50 a través del uso de isoenzimas, variantes proteicas codificadas por un mismo gen que mostraban diferencias en su velocidad de migración en el interior de una matriz sometida a un campo electromagnético. Posteriormente, el descubrimiento de las enzimas de restricción en la década de los 60 permitió detectar de forma indirecta polimorfismos en las secuencias de los ácidos nucleicos al generar fragmentos de ADN de distinto tamaño que podían ser separados mediante técnicas electroforéticas. Los patrones de bandas obtenidos a partir de la digestión de los ácidos nucleicos servían a modo de huellas diagnósticas para la detección de diferencias a nivel genético. El desarrollo posterior de las técnicas de clonación de ácidos nucleicos hizo factible la obtención de librerías genómicas y de ADN complementario en un abanico muy diverso de organismos. El desciframiento del orden de nucleótidos en la totalidad o parcialidad de los genomas de las especies facilitó el aislamiento de numerosos marcadores moleculares polimórficos que proporcionaban los cimientos necesarios para aumentar la aplicabilidad y el grado de discriminación individual proporcionado por las técnicas anteriores. La invención de la reacción en cadena de la polimerasa o PCR, descrita por Kary Mullis en 1986, supuso el salto cualitativo definitivo al permitir el estudio específico de ciertas regiones variables del genoma en grupos de especies relacionadas. Toda la información acumulada durante todos estos años propulsó entonces la aparición de nuevas disciplinas científicas que pusieran orden y concierto para la aplicación de los nuevos métodos moleculares desarrollados en los ya tradicionales campos de la

ecología, evolución y conservación de la biodiversidad. De esta manera, y con un impacto cada más importante en el panorama científico actual, la ecología molecular y la genética de la conservación han generado nuevos nodos que soportan las interacciones entre diversas disciplinas científicas (ver por ejemplo Frankham y cols. 2002, Beebe & Rowe 2003).

Uno de los casos más ilustrativos en los que el desarrollo de técnicas genéticas ha cambiado nuestra antigua percepción de la biología de muchas especies afecta a los sistemas reproductivos. Como uno de los ejemplos más sobresalientes, las aves fueron largamente consideradas iconos de la monogamia estricta (Lack 1968). Años más tarde, estudios de paternidad realizados con marcadores genéticos polimórficos demostraron la existencia de paternidades extra-pareja en torno al 90% de las especies investigadas. En algunos grupos de aves como los paseriformes, la monogamia estricta ha demostrado ser realmente la excepción más que la regla (revisado por Griffiths y cols. 2002). El almacén de esperma de varios machos en el oviducto de las hembras de tortuga y la paternidad múltiple no podría tampoco imaginarse sin la aportación de la genética (Ej. Pearse y cols. 2002), por citar algún otro ejemplo. Los métodos genético-moleculares se han convertido también en un gran punto de apoyo para el seguimiento y monitorización de fauna amenazada. Una de las ventajas más importantes en relación a los tradicionales métodos de captura-marca-recaptura radica en la posibilidad de poder aprovechar material no invasivo tales como pelos, plumas, cáscaras de huevos o heces. Todo ello ha permitido la determinación de presencia/ausencia de especies esquivas y la inferencia de estimas mínimas de tamaños poblacionales (revisado por Schwartz y cols. 2007). Por otra parte, el análisis de las frecuencias alélicas en varios marcadores genéticos permite inferir el grado de conectividad entre distintas poblaciones, un fenómeno que ha sido de gran utilidad y complementariedad a los tradicionales métodos de captura-recaptura y seguimiento por satélite (Ej. Paetkau et al. 1995, Vilá et al. 2002). Por

consiguiente, los numerosos avances en el campo de la ecología molecular han contribuido de forma muy notable a un desarrollo más efectivo de estrategias encaminadas a la preservación de la biodiversidad.

El objetivo de la presente tesis es la aplicación de técnicas genético-moleculares encauzadas a potenciar nuestro entendimiento sobre los distintos factores y procesos que tienen un efecto directo sobre la conservación de la biodiversidad. Para ello, hemos utilizado al cernícalo primilla *Falco naumanni* como especie modelo. Este pequeño halcón colonial, catalogado como vulnerable en el conjunto de su distribución mundial (BirdLife Internacional 2008), es sin duda una de las aves mejor estudiadas del planeta. El cernícalo primilla ha sido el protagonista de varias tesis doctorales en el ámbito de la ecología y la conservación de la biodiversidad durante las últimas dos décadas (Bustamante 1990, Negro 1991, Tella 1996, Olea 2001, Serrano 2003, Rodríguez 2004, Ursúa 2006). Sólo una tesis doctoral ha utilizado sin embargo esta especie como modelo en genética evolutiva y de la conservación (Ortego 2007). No obstante, el área estudiada no superó los 1,000 km^2, y los análisis genéticos se basaron exclusivamente en patrones de variación en una decena de marcadores genéticos neutrales, es decir, regiones del genoma no codificantes que no se ven sometidas a presiones selectivas a no ser que se demuestre su ligamiento a regiones del genoma bajo selección. Esta tesis supone un paso más allá en este sentido al extender el área de estudio a prácticamente la totalidad del área de distribución de la especie en el Mediterráneo y al introducir variación genética en genes funcionalmente importantes como son los que se engloban dentro del complejo mayor de histocompatibilidad o MHC. En este sentido, las reuniones de los investigadores más prestigiosos han subrayado la necesidad de incluir estimaciones de diversidad genética adaptativa en estudios relacionados con la conservación de la biodiversidad (ver Vernesi y cols. 2008). Una de las causas principales para estimular dichos esfuerzos radica en la baja correlación entre diversidad genética

neutral y adaptativa que algunos estudios científicos han puesto de manifiesto (Ej. Aguilar y cols. 2004, Jarvi y cols. 2004). En particular, los genes del MHC codifican proteínas que juegan un papel crucial en el desarrollo de la respuesta inmune en vertebrados, y por tanto, están expuestos a fuertes presiones selectivas mediadas por patógenos y parásitos (Klein 1986). Los patrones de variación genética en los genes del MHC han focalizado la atención de los investigadores en el campo de la ecología evolutiva debido a su posible implicación en numerosos procesos biológicos tales como los mecanismos de reconocimiento celular, susceptibilidad a enfermedades infecciosas, olores individuales, reconocimiento de parientes, patrones de elección de pareja con la que reproducirse y desarrollo del embarazo (ver revisiones por Sommer 2005, Piertney y Oliver 2006). Los objetivos de la presente tesis doctoral se dividen consiguientemente en dos grandes bloques. Un primer bloque examina las aplicaciones en conservación derivadas del empleo de marcadores supuestamente neutrales de microsatélite, mientras que el segundo bloque introduce los primeros datos publicados en aves de presa en relación a patrones de variación genética en loci evolutivamente relevantes y sometidos a fuerte selección natural.

El primer bloque ahonda en las relaciones de parentesco entre individuos y en el equilibrio migración-deriva génica subyacente a la diversidad genética y conectividad en poblaciones fragmentadas. De una forma dramática en muchos casos, la fragmentación del hábitat asociada a actividades humanas se ha convertido en un obstáculo importante para la conservación de muchas especies (Young & Clarke 2000). La fragmentación no sólo tiene consecuencias demográficas notables que aceleran las tasas de extinción local, sino que también provocan disminuciones en el flujo genético traducidas en pérdidas de diversidad genética y aumento de la consanguinidad en poblaciones aisladas. Las restricciones en el intercambio de individuos disparan las tasas de fijación de alelos deletéreos que provocan una disminución en la eficacia biológica de los individuos, aumentado

asimismo las probabilidades de extinción (Frankham y cols. 2002). El primer capítulo de esta tesis testa la utilidad de marcadores de microsatélite, previamente aislados en el halcón peregrino *Falco peregrinus* (Nesje y cols. 2000), como indicadores de variación genética en el cernícalo primilla. En este sentido, una segregación alélica acorde a las típicas proporciones Mendelianas asegura la idoneidad de dichos marcadores para futuros estudios basados en la distribución de las frecuencias alélicas entre distintas poblaciones. Por otra parte, las tasas de paternidad extra-pareja son relevantes a la hora de determinar la contribución genética de los individuos adultos que crían los pollos, ya que muchos de nuestros análisis genéticos estarán basados en individuos de pocos días de edad muestreados a pie de nido.

Una vez seleccionado un conjunto de marcadores de microsatélite apropiado para llevar a cabo trabajos de ecología molecular y genética de la conservación en el cernícalo primilla, se procederá a testar diversas hipótesis y predicciones derivadas del seguimiento y estatus demográfico actual de las poblaciones de la especie en su área de distribución a distintas escalas geográficas. La monitorización de individuos marcados mediante anillas de PVC en varias poblaciones españolas ha documentado alta filopatría y una dispersión sesgada hacia distancias geográficas por debajo de los 10 km (Negro 1991, Serrano 2003). El capítulo 2 de la presente tesis testa si dichas restricciones en el flujo de individuos entre distintas colonias de cría se ha traducido en la generación de estructuras genéticas locales a nivel de la progenie. Esta aproximación permite por tanto analizar la congruencia entre las predicciones derivadas del seguimiento demográfico de individuos (métodos directos) con técnicas más modernas basadas en marcadores genéticos (métodos indirectos) (ver por ejemplo, Koenig 1986). En el capítulo 3, se investiga el grado de diferenciación genética y la existencia de posibles disminuciones en la diversidad genética que pudieran asociarse a la pérdida de hábitat en el cernícalo primilla. Para ello hemos comparado esta especie filopátrica, que ha experimentado un

acusado declive desde la segunda mitad del siglo XX (Biber 1990), con una especie simpátrica y genéticamente cercana, el cernícalo vulgar. Mientras el cernícalo primilla puede considerarse una rapaz especialista de ecosistemas esteparios y pseudoesteparios, el cernícalo vulgar es un halcón tremendamente cosmopolita y mucho menos filopátrico que ha amortiguado mucho mejor las alteraciones de hábitat y cuyo rango de distribución se mantiene relativamente continuo en el Paleártico Occidental. En el último capítulo de este bloque hemos realizado la primera evaluación genética de los programas de cría en cautividad y reintroducción del cernícalo primilla. Los objetivos en este punto fueron encaminados a la optimización de la transmisión de la diversidad genética durante la fase de cría en cautividad y posterior reintroducción en medio salvaje. La base teórica subyacente a esta aproximación experimental radica en las correlaciones positivas generalmente asumidas entre variación genética, productividad y potencial adaptativo (ver Frankham y cols. 2002).

Los dos primeros capítulos del segundo bloque comienzan con la aportación que consideramos más novedosa de la presente tesis doctoral. En ambos capítulos, se describen técnicas moleculares para el aislamiento y caracterización de genes de MHC de clase II y de clase I en aves de presa. Los genes de MHC de clase II codifican para glicoproteínas de membrana que presentan pequeños péptidos (antígenos) derivados del procesamiento de patógenos extracelulares tales como bacterias o parásitos (ver revisiones por Sommer 2005, Piertney y Oliver 2006). Por su parte, los genes de MHC de clase I presentan antígenos de patógenos que cubren una parte de su ciclo vital dentro de las células, incluyendo virus y algunos protozoos como el causante de la malaria. El reconocimiento de péptidos exógenos por parte de células especializadas del sistema inmune inicia el desarrollo de una respuesta inmune adaptativa, abarcando desde la síntesis de anticuerpos hasta la destrucción de la célula presentadora de antígenos. Las regiones analizadas en el presente estudio se focalizan en el exón 2 de genes de MHC de clase II B y en el

exón 3 de genes clásicos de MHC de clase I. Ambos exones codifican para las regiones de unión de péptidos o PBR, que son las que determinan la especificidad de las moléculas MHC por un grupo de antígenos determinado. En este sentido, el aislamiento de loci polimórficos y funcionales permite testar una gran variedad de hipótesis derivadas de las múltiples interacciones evolutivas entre los patógenos y sus hospedadores.

Una vez más, el cernícalo primilla pondrá de manifiesto su idoneidad como especie modelo. La presente tesis reportará el primer caso documentado de un ave silvestre en el que se ha conseguido estudiar específicamente genes de MHC de clase I y clase II con altos niveles de polimorfismo y pronunciadas evidencias de selección natural. Este hallazgo se opone a lo generalmente documentado en aves salvajes, especialmente paseriformes, donde una alta incidencia de duplicaciones génicas, pseudogenes y loci poco polimórficos han constituido un obstáculo considerable en estudios encaminados a elucidar las implicaciones evolutivas y de conservación de esta familia multigénica. La simplicidad del MHC dentro del género *Falco* en general aceleró la suma de información genética en genes relacionados con la resistencia a enfermedades infecciosas, y por tanto, directamente relacionados con parámetros indicativos de calidad individual. Así, el capítulo 7 investiga la aparición de adaptaciones locales en genes funcionalmente relevantes en una especie con poblaciones fragmentadas y flujo genético limitado. Los patrones de diversidad genética en el MHC son comparados con patrones de diversidad en marcadores neutrales (ADN mitocondrial y microsatélites) y se testa el papel de las variaciones espaciales en las presiones selectivas ejercidas por patógenos como fuente de polimorfismo genético (ver Hills 1991).

Por otra parte, la diversidad genética en el MHC se cree actualmente vinculada a la diversidad de especies de patógenos a los cuales los hospedadores han de enfrentarse. Dentro de este contexto, el capítulo 8 correlaciona la diversidad

genética en secuencias de genes de MHC de clase I y clase II en especies y subespecies de cernícalos que difieren en distintos aspectos ecológicos. De nuevo, el cernícalo primilla ejemplariza el caso de una especie especialista tanto en dieta como en patrones de ocupación de hábitat con respecto a una especie cosmopolita y con una dieta más amplia como el cernícalo vulgar. Como ejemplo más extremo, este estudio también analiza dos subespecies sedentarias del cernícalo vulgar procedentes de las Islas Canarias, *Falco tinnunculus dacotiae* y *Falco tinnunculus canariensis*. El interés de la insularidad radica en las comunidades de patógenos típicamente empobrecidas que deben tener reflejo a la hora de moldear la diversidad genética en los genes del MHC. Por último, el capítulo 9 investiga correlaciones directas entre la composición alélica de genes de MHC de clase II y parámetros de calidad individual tales como el éxito reproductor. Debido a la alta homogeneidad de condiciones medioambientales y de manejo que existen en los centros de cría en cautividad, analizamos la genética de los individuos más exitosos desde un punto de vista de la productividad durante al menos 3 años consecutivos. Finalmente, la selección natural es esperable que genere desviaciones en las proporciones mendelianas de segregación de alelos a través de procesos de fertilización selectiva y/o afectando las tasas de eclosión de huevos y supervivencia de individuos recién nacidos. La última hipótesis que testaremos en la presente tesis investiga pues la herencia genética de ciertos alelos de MHC de padres a hijos en hasta 40 familias de cernícalos primilla con un mínimo de 4 pollos.

El CERNÍCALO PRIMILLA

El cernícalo primilla es un pequeño halcón fundamentalmente insectívoro cuyo rango de distribución reproductiva se extiende desde la península Ibérica hasta China, siempre en latitudes medias y bajas. Se trata de un ave migradora de larga distancia que pasa los inviernos en el África subsahariana, habiéndose documentado las máximas concentraciones de individuos en Sudáfrica y Senegal. El cernícalo primilla es un ave generalmente asociada a ecosistemas esteparios y pseudoestaparios, siendo habitual su presencia en entornos urbanos y agrícolas. En Europa occidental, colonias de hasta más de 100 parejas pueden situarse en edificaciones humanas tales como iglesias, castillos, granjas abandonadas o silos. Desde un punto de vista reproductivo, este pequeño halcón es una especie generalmente monógama y exhibe un pronunciado dimorfismo sexual. Suele poner entre 1 y 7 huevos, siendo el tamaño de puesta más habitual de entre 3 y 4. Ambos sexos incuban los huevos, aunque los machos juegan un papel predominante en la ceba de las hembras así como en la defensa del nido. El seguimiento intensivo de esta especie mediante marcaje con anillas de PVC ha documentado altas tasas de filopatría, siendo la dispersión natal el factor más influyente en la dinámica de las poblaciones pero mostrando tendencias dispersivas hacia distancias geográficas cortas. El cernícalo primilla ha sufrido un marcado declive poblacional desde la segunda mitad del siglo XX que se traducido en una merma considerable de sus poblaciones e incluso en su desaparición total de varias regiones en toda su área de distribución (Biber 1990). A día de hoy, alteraciones de hábitat asociadas a cambios en las políticas agrarias son señaladas como las principales causas del declive poblacional, con una menor incidencia de perturbaciones en las colonias durante el periodo de cría (Tella y cols. 1998). Su estatus de conservación actual cataloga esta pequeña rapaz como globalmente vulnerable (BirdLife Internacional 2008). Para más información sobre la especie ver Cramp & Simmons 1980, Negro 1997, Fergusson-Lee & Chirstie 2001.

METODOLOGÍA GENERAL

La base de todos los análisis genéticos que se llevan a cabo en la presente tesis doctoral radica en la amplificación de pequeños fragmentos de ADN a través de la reacción en cadena de la polimerasa o PCR. Para ello, muestras de sangre preservadas en etanol al 96% así como plumas arrancadas de la región dorsal de las aves fueron utilizadas como fuente de material genético. Las plumas en crecimiento se encuentran muy irrigadas por capilares sanguíneos en individuos jóvenes, lo que las convierte en fuentes generosas de ácidos nucleicos. Las plumas arrancadas de individuos adultos contienen células epiteliales en la base del cálamo así como un pequeño coágulo sanguíneo atrapado en la zona del cálamo próxima al ombligo superior donde comienzan las barbas de la pluma (ver Hörvarth y cols. 2005). El protocolo de extracción de ácidos nucleicos se llevó a cabo siguiendo el protocolo descrito por Gemmel & Akiyama (1996).

Se han amplificado 3 tipos de marcadores moleculares. Los marcadores de microsatélite o SSR (Simple Sequence Repeats) consisten en pequeños motivos nucleotídicos repetidos en tándem en los que las distintas variantes alélicas se diferencian en el número de unidades repetidas, y por tanto, en el tamaño del fragmento amplificado. El marcaje con fluorescencia de los fragmentos amplificados permite la resolución de los alelos mediante electroforesis capilares en secuenciadotes automatizados como los comercializados por la compañía biotecnológica Applied Biosystems. Estos marcadores codominantes y altamente polimórficos se ubican en zonas no codificantes del genoma nuclear, y salvo que no se demuestre su cosegregación con algún gen funcional, son considerados como neutrales desde un punto de vista de la selección natural (ver revisión por Li y cols. 2002 para más información). La caracterización tanto de ADN mitocondrial como de exones polimórficos de genes del MHC se lleva a cabo mediante técnicas de secuenciación directa basadas en el método de Sanger. Esta

técnica incorpora di-desoxinucleótidos marcados con fluorescencia (terminadores) a una concentración tal que se favorece la detención de la cadena en síntesis en cada una de las bases. La posterior electroforesis de los fragmentos de ADN sintetizados permite la obtención de cromatogramas que nos permiten leer la secuencia nucleotídica del fragmento amplificado. La secuenciación de ADN mitocondrial se puede realizar de forma directa al ser la molécula de ADN mitocondrial exclusivamente de origen materno y por tanto haploide. Sin embargo, los marcadores nucleares son diploides, es decir, existe herencia biparental siendo uno de los alelos de origen materno y el otro de procedencia paterna. Por tanto, el aislamiento de alelos individuales en loci extensamente polimórficos como los pertenecientes a genes del MHC requiere la separación física de los alelos previa a su secuenciación. Ello se lleva a cabo mediante la clonación de los productos de PCR amplificados a través de bacterias competentes. Cada alelo se inserta dentro de un plásmido que es introducido dentro de una bacteria que a continuación forma una colonia. Es esperable que cada clon bacteriano contenga uno de los alelos amplificados, y por tanto, se procede a la secuenciación del inserto. Los marcadores de MHC caracterizados en la presente tesis abarcan la región de unión de antígenos tanto en genes de MHC de clase II como en genes de MHC de clase I.

El análisis de los datos genéticos ha sido llevado a cabo mediante el empleo de una diversa batería de paquetes informáticos de última generación. La asignación de alelos en los marcadores de microsatélite ser realizó mediante los programas de análisis GENOTYPER y GENEMAPPER (Applied Biosystems). El alineamiento y edición de secuencias de ADN tuvo lugar en el programa BIOEDIT (Hall 1999). Los análisis de parentesco se llevaron a cabo mediante el programas CERVUS (Marshall y cols. 1998) La probabilidad de encontrar 2 individuos en la población con genotipos idénticos fue calculada por medio del programa IDENTITY 1.0 (Wagner & Sefc 1999). El grado de diferenciación genética para microsatélites

fue calculado acorde con el tradicional parámetro F_{ST} propuesto por Weir y Cockerham en 1984 y asumiendo el modelo de alelos infinitos. Tanto los valores de F_{ST} entre poblaciones como el testado de patrones de aislamiento por distancia mediante tests de Mantel fueron calculados a través del programa GENETIX 4.04 (Belkhir et al. 1996-2004). La existencia de estructuras genéticas locales fue testada utilizando modernos análisis de autocorrelación espacial implementados dentro del paquete informático GENALEX 6.0 (Smouse & Peakall 2006). A mayor escala, el programa STRUCTURE (Pritchard et al. 2000) investiga la existencia de grupos de individuos más genéticamente emparentados que lo esperable al azar sin tener en cuenta el origen geográfico de los individuos introducidos en los análisis. Estimas de diversidad genética tales como el número de alelos por locus, riqueza alélica, heterocigosidad observada o coeficientes de consanguinidad para marcadores neutrales fueron calculados mediante los programas GENETIX 4.04 y FSTAT ver. 2.9.3 (Goudet 2001).

Los patrones de selección positiva en regiones codificantes del MHC fueron investigados comparando las tasas de sustituciones nucleotídicas sinónimas (aquellas que no implican sustituciones de aminoácidos) frente a las tasas de sustituciones nucleotídicas no sinónimas (aquellas que implican variaciones en la composición aminoacídica). Esta tesis recoge al menos dos tipos de análisis distintos: uno basado en los métodos de máxima verosimilitud (PAML, Yang y cols. 2000) y un nuevo método Bayesiano basado en la teoría de la coalescencia que coestima las tasas de recombinación en las secuencias de MHC (OMEGAMAP, Wilson y McVean 2006), y que por tanto, es menos propenso a la detección de falsos positivos (ver por ejemplo Anisimova y cols. 2003). Las estadísticas de polimorfismo en secuencias de ADN fueron calculadas a través del programa DNAsp (Rozas y cols. 2003). La divergencia genética en las secuencias de ADN entre poblaciones distintas fue calculada en base al parámetro K_{ST} (Hudson 1982), también calculado en DNAsp. Las relaciones filogenéticos entre secuencias

de MHC fueron inferidas mediante el empleo del recientemente desarrollado software SPLITSTREE 4.0 (Huson & Bryant 2006). Este software permite la construcción de redes moleculares que son sugeridas como más eficaces a la hora de representar las relaciones filogenéticas entre secuencias cuando hay evidencias de recombinación, en comparación con los tradicionales árboles filogenéticos. Finalmente, la diversidad aminoacídica en los exones codificantes del MHC fueron analizados mediante el programa DIVAA (Rodi y cols. 2004). Todos los análisis estadísticos restantes fueron realizados en el paquete SSPP 13.0.

BLOQUE 1

VARIACIÓN

GENÉTICA NEUTRAL

Capítulo 1

Extra-pair paternity in the Lesser Kestrel *Falco naumanni*: a re-evaluation using microsatellite markers

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INTRODUCTION

Modern molecular techniques based on DNA typing have revolutionized our view of avian mating systems. Birds were considered to be essentially monogamous only a few years ago (Lack 1968). In contrast, Griffith *et al*. (2002) have recently summarized how molecular techniques have revealed that birds are only rarely monogamous, with extra-pair offspring found in approximately 90% of the species studied to date. It has been known for some time that individuals in supposedly monogamous species often adopt a mixed reproductive strategy (Trivers 1972). Both males and females may seek extra-pair copulations (EPC), which could provide them with some genetic benefits (for a review of benefits and drawbacks of engaging in EPC, see Birkhead & Møller 1992; Wink & Dyrcz 1999).

Although there is great interest in the reasons for differences in extra-pair paternity (EPP) rates in birds, few studies have compared the results obtained by different molecular techniques. Among available markers, DNA fingerprinting in the 1990s and, more recently, microsatellites, are the most popular in elucidating genetic relationships in birds (Burke & Bruford 1987, Ellegren 1992), but, to our knowledge, comparisons of the performance of these two techniques using the same sample sets have not been reported.

In this study, we reappraised EPP in the Lesser Kestrel *Falco naumanni*. Our aim was to recalculate levels of EPP in this species using microsatellite markers instead of DNA fingerprinting, and to compare the results obtained with both techniques. Earlier studies of the copulatory behaviour of Lesser Kestrels (Negro *et al*. 1992) have already shown that some males pursue a mixed reproductive strategy, with a 6.7% incidence of EPC attempts, and DNA fingerprintings of 26 families revealed that 3.4% of the nestlings resulted from extra-pair fertilizations (Negro *et al*. 1996). We reanalysed 23 families from which we still had usable DNA from the fingerprinting study of Negro *et al*. (1996)

together with DNA from a further eight new families from a different population sampled during the 2003 breeding season.

MATERIALS AND METHODS

Study species and sample collection

The Lesser Kestrel is a small migratory falcon which breeds in the Western Palearctic, from the Iberian Peninsula to China, and winters in Africa (Cramp & Simmons 1980). Lesser Kestrels typically breed in urban colonies of up to 100 pairs, usually in buildings (Cramp & Simmons 1980). They are socially monogamous, although polygynous males have been reported (Hiraldo *et al*. 1991, Tella *et al*. 1996).

Twenty-three Lesser Kestrel families were sampled at breeding colonies located in Los Monegros (Aragón, northeast Spain, 41°21'N, 0°11'W) during the 1993 breeding season. Eight families were sampled at two colonies in Huelva (Andalusia, southwest Spain, 37°10'N, 6°21'W) during the 2003 breeding season. Blood or feather samples were collected for both the putative parents and all nestlings. In both populations, the putative parents were captured at the nest when incubating or brooding small chicks to ensure that they were providing parental care and were not visitors unrelated to the nests. During their first week, young from selected nests were marked on the leg with a cloth strap. The purpose of this early banding was to detect cases of nest switching by nestlings and their subsequent adoption, a phenomenon frequently observed in the Los Monegros population (Tella *et al*. 1997). Early banding was not carried out in Andalusian colonies because movements between nests could be made only by flying. Adults and young were colour-banded. Banded adults were observed through spotting scopes to confirm that they were attending the nests where they were caught and were feeding their putative offspring.

In 1993, approximately 0.4 mL of blood from the brachial veins of both adults and nestlings was taken using 1-mL syringes and 30-gauge needles. Blood was preserved in lysis buffer consisting of 0.01 M NaCl, 0.01 M EDTA and 1% *n*-lauroylsarcosine, pH 7.5 (Seutin *et al*. 1991). Samples were stored at 4 °C until processing. In 2003, blood was collected from adults in the same way as in 1993. Blood was preserved in absolute ethanol and stored at 4 °C until processing. In the case of nestlings, one or two feathers were pulled from the back and preserved in a paper envelope. Feathers were stored at room temperature until processing.

DNA extractions

DNA extracts from the samples collected in 1993 (Negro *et al*. 1996) were preserved in TE buffer (5 mM Tris / HCl, 0.1 mM EDTA, pH 7.4) and stored at −80 °C until required. The extraction protocol we used for the additional samples collected in 2003 follows that described by Gemmell and Akiyama (1996). Aliquots of blood and feather shafts were suspended in 1.5-mL microfuge tubes with 300 µL of digestion buffer (100 mM NaCl, 50 mM Tris /HCl, 1% SDS, 50 mM EDTA) and 3 units[a] of proteinase K. Digestion of the samples was carried out over a period of 2 h or more at 55 °C. Once the digestion was complete, an equal volume (300 µL) of 5 MLiCl was added to each tube. The sample was mixed thoroughly by inversion for 1 min and 600 µL of chloroform-isoamylic alcohol (24 : 1) was added. After vortexing, samples were spun for 15 min at 13 000 rev/min and the supernatant carefully removed to a new tube. Then, 1 mL of absolute ethanol was added until DNA precipitated. DNA was recovered by centrifuging at 13 000 rev/min for 15 min. The pellet was dried and washed twice with 70% ethanol, and later placed in 0.1–0.2 mL of TE buffer and stored at −20 °C.

Microsatellite genotyping

A microsatellite-based genotyping system was employed to enable the

assignment of paternity to chicks (Ellegren 1992, Primmer *et al*. 1995). We used seven markers initially developed for the Peregrine Falcon *Falco peregrinus* by Nesje *et al*. (2000), which were also known to be polymorphic in Lesser Kestrels and other *Falco* species (Groombridge *et al*. 2000). The number of alleles, observed heterozygosity and parentage exclusion probability for first and second parents were estimated with Cervus 2.0 software (Marshall *et al*. 1998). The probability that any two individuals shared the same genotype was calculated with Identity 1.0 software (Wagner & Sefc 1999).

For each locus, the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following PCR profile: 35 cycles of 40 s at 94 °C, 40 s at 55 °C, 40 s at 72 °C and finally 4 min at 72 °C. Each reaction was carried in 11 µL of a mix containing 0.2 units of Taq polymerase (Bioline), 1× PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.02% gelatin, 0.12 mM of each dNTP, 5 pmol of each primer and approximately 10 ng of genomic DNA. Primer sequences are available in the GenBank Database. F-primers were 5'-end labelled with HEX, TET or 6-FAM. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyser (Applied Biosystems).

Parentage testing

All adults and chicks were genotyped. Mendelian inheritance was checked at every locus in every family. Nestlings sharing alleles from their putative mother and father at all loci were considered actual offspring of the couple. Those chicks sharing one allele from the mother in all loci but failing to match any of the two alleles of the putative father at some loci were considered the product of extra-pair fertilization. Nestlings that did not share alleles with either the putative mother or father were considered the result of intraspecific brood parasitism (IBP). Our sampling protocol (see above) precluded the possibility that these nestlings had moved from other nests.

Mendelian inheritance failed at locus fp107 in several families. As analysis with Cervus 2.0 software (Marshall *et al*. 1998) showed a significant ($P < 0.01$) heterozygote deficit in this locus, it was excluded for further analysis as the presence of null alleles was confirmed (see Pemberton *et al*. 1995).

RESULTS

We genotyped 96 nestlings and 62 adults from 31 broods. We found 72 different alleles, with an observed heterozygosity of 0.65. The combined probability of exclusion for the marker set was greater than 0.99. The likelihood of two individuals carrying an identical genotype was estimated at 3.13×10^{-6}. Seven of the 96 (7.25%) chicks mismatched with the 'social' male in several loci. All of them were considered to be extra-pair offspring.

Overall, extra-pair young appeared in three out of 31 nests sampled (9.67%). The level of EPP using microsatellites was low for the two populations that we studied: six nestlings out of 72 (8.3%) in Los Monegros vs. one nestling out of 24 (4.2%) in Huelva. The difference between the two populations was not significant (Yates corrected chi-squared = 0.45, $P = 0.19$). We detected two nests in the Los Monegros population where the attendant males could not be assigned as the actual fathers. Therefore, their respective broods of three nestlings each arose from extra-pair fertilizations as microsatellite analysis confirmed the females as the actual mothers. The remaining extra-pair chick came from a brood in the Huelva population and had three nest-mates sired by the male attending the nest. This single extra-pair nestling shared the alleles of the attending mother but not those of the putative father at some loci. This was interpreted as a case of mixed fertilization, the first ever detected in the Lesser Kestrel. In addition, two nestlings in two other broods from Los Monegros shared alleles from neither the attending male nor the attending female at several loci. They were considered the

result of IBP, as suggested by Negro *et al*. (1996).

In the Los Monegros subset of families, all previous results from our DNA fingerprinting study (Negro *et al*. 1996) matched those obtained using microsatellites, except in one family. In this case, we detected unambiguously that the male attending the nest was not the genetic father of the nestlings, contrary to our previous DNA fingerprinting results.

DISCUSSION

This microsatellite analysis showed a slightly higher incidence of EPP than that reported in our previous study based on multilocus radioactive probes (7.25% vs. 3.4%). The difference is unrelated to the inclusion of samples from a different population but it is due to a misinterpretation of the fingerprinting band pattern in just one of the families. The microsatellite analysis for this family has been repeated in triplicate, and always yielded the same results. Despite the fact that a mean (± sd) of 10.9 ± 2.6 scorable bands was analysed in the fingerprintings of the families (see Negro et al. 1996), the re-examination of the band profile in the controversial family raised some doubts about its interpretation. In fact, the number of diagnostic bands was very low in this family, and some bands taken as identical by descent could actually be different given that a further re-examination revealed that some of them could not be exactly the same band in size, and their intensity on the Southern hybridization was different. The band patterns obtained with multilocus radioactive probes can be misleading and difficult to interpret in some cases, especially when the potential fathers are relatives, the population is highly inbred or when additional bands appear in the offspring due to mutation (see for instance Lubjuhn et al. 2002). However, working with microsatellite markers provides better resolution and a better background for detecting locus polymorphism, thus reducing the chance of human error.

The level of EPP reported in this study (9.67%), although higher than previously estimated (3.8%), still remains relatively low. It seems to be typical of raptors that they show low rates of EPP compared with other birds, and particularly with short-lived passerines (Birkhead & Møller 1992, Griffith *et al*. 2002); low rates of EPP have been reported in close relatives of the Lesser Kestrel, including the solitary-breeding Eurasian Kestrel *Falco tinnunculus* (1.9%, Korpimäki *et al*. 1996) and the American Kestrel *Falco sparverius* (11.2%, Villaroel *et al*. 1998).

To conclude, our reappraisal of EPP in Lesser Kestrels confirms a low incidence in this species, and identifies microsatellite markers rather than multilocus DNA fingerprinting as a better choice for this kind of study. This is because paternity assignments are straightforward and there is no need to consider, as is necessary with DNA fingerprinting, whether two bands of the same molecular weight correspond to the same allele.

Capítulo 2

Strong philopatry derived from capture-recapture records does not lead to fine-scale genetic differentiation in lesser kestrels

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SUMMARY

1. The integration of capture-recapture and molecular approaches can improve our understanding of the consequences of habitat fragmentation on population connectivity. Here, we employed microsatellites to test dispersal hypotheses derived from intense and long-term ringing programs of lesser kestrels *Falco naumanni* in Western Europe.

2. Reencounters of 1308 marked individuals in Spain have revealed that most first-breeders settled within 10 km from their natal colony, with a negative association between dispersal and geographic distance. Although these findings would predict fine-scale spatial patterns of genetic differentiation, the genetic impact of rarely reported events concerning long-distance effective dispersal (>100 km) is unknown.

3. We firstly investigated a spatially structured and geographically isolated population located in North-eastern Spain, where capture-recapture records and genetic data could be appropriately compared over similar spatial and temporal scales. Spatial autocorrelation analyses (N = 174 nestlings from different broods) did not reveal either significant differences in average relatedness at any distance class nor decreased relatedness as a function of distance. At a broader spatial scale, Bayesian analysis of population structure (N = 432 nestlings) indicated panmixia across Western Europe. However, F_{ST} comparisons between four geographically distinct populations indicated low but significant genetic differentiation.

4. Our genetic data would therefore challenge traditional assumptions associating philopatry with the emergence of fine-scale genetic structuring. This could be due to the fact that even low levels of gene flow are enough to

preclude the development of local genetic structure. Nevertheless, the analysis of a geographically isolated and small population from Southern France exemplifies a situation where restricted dispersal has translated into weak but consistently significant genetic differentiation.

5. Relevant to conservation genetics and evolutionary biology, our results may soften the genetic concerns derived from population fragmentation at relatively small geographical scales in species with apparently limited dispersal abilities, but warns about increased genetic divergence in small and isolated demes. In this respect, the integration of direct and indirect estimates of dispersal may decisively improve our knowledge of the possible effects of habitat alteration.

INTRODUCTION

Dispersal of individuals can influence the genetic structure, demography and long-term persistence of populations (Young & Clarke 2000, Clobert et al. 2001). The use of molecular approaches and the development of powerful statistical methods have revolutionised the study of dispersal by providing an alternative that solves many methodological limitations of traditionally laborious capture-recapture studies (see Koenig et al. 1996,). For example, long-distance dispersal events are difficult to document with capture-recapture techniques, but genetic methods have proved efficient in this task (e.g. Paetkau et al. 1995; Vilá et al. 2003). Genetic approaches, however, may fail to reflect current gene flow as well as non-effective dispersal movements of crucial importance, for instance, in spreading diseases. In addition, indirect measures of dispersal are often difficult to interpret because different populational processes may result in similar genetic patterns (e.g. Bossart & Prowell 1998, Whitlock & McCauley 1999, but see Manel et al. 2005). Thus, it is not surprising that ecological and genetic methods have sometimes yielded conflicting results (e.g. Van Bekkum et al. 2006, Senar et al. 2006). Combining capture-recapture and genetic inferences in the same study models can help explain these discrepancies, and such integration may be critical to deal with basic ecological and evolutionary questions (e.g. Gompper et al. 1998, Peacock & Ray 2001, Berry et al. 2004, Hansson et al. 2004b, Double et. al. 2005, Temple et al. 2006).

The majority of species suffers from the effects of habitat loss and reduction because of human activity. The demographic and genetic consequences of habitat fragmentation depend on the interaction between the dispersal ability of the species and the number, size and spatial distribution of local populations, as well as on time since fragmentation (Young & Clarke 2000, Frankham et al. 2002). While restricted gene flow typically leads to genetic differentiation among fragments, a spatially

structured population will behave just like a single large panmictic population if sufficient dispersal and associated gene flow rates are occurring (see Mills & Allendorf 1996, Vucetich & Waite 2000, Martínez-Cruz et al. 2004). In this respect, natal and breeding philopatry, i.e. the tendency of individuals to breed close to their birthplace or their previous breeding territory, are relevant life-history traits expected to generate genetic population differentiation because of limited gene flow (Greenwood 1980, Greenwood & Harvey 1982, Sugg et al. 1996).

Intense and long-term monitoring of marked individuals in the globally vulnerable (BirdLife International 2008) and facultatively colonial lesser kestrel *Falco naumanni* in Spain revealed that the frequency distribution of movements is distance-dependent, with a majority of birds breeding for the first time within 10 km from their natal colony. This pattern of natal dispersal was independently documented for both a population located at the Guadalquivir valley (69%, N=321 individuals; Negro et al. 1997a, see Fig. 1), and a population located at the Ebro valley (66%, N=751, Serrano et al. 2003, Fig. 1). Apparently, only a few birds (~ 1%) settled at distances greater than 100 km from their natal colony (1% and 0.6 % at the Guadalquivir and Ebro valley respectively, Negro et al. 1997a, Serrano et al. 2003).

Contrarily to most avian studies, natal dispersal showed to be not or only slightly sex-biased (Negro et al. 1997a, Serrano et al. 2003), and the potential differences between sexes have been preferentially attributed to their different role in nest acquisition and defence, rather than to the development of effective mechanisms of inbreeding avoidance (see more details in Negro et al. 1997a, Serrano et al. 2003). Natal dispersal have also been shown to be linked to density-dependent factors (Negro et al. 1997a, Serrano et al. 2003, 2004), with settlement decisions of first-breeding birds being constrained in the largest colonies by agonistic interactions with previous residents (Serrano and Tella 2007). After

monitoring 486 consecutive breeding attempts at the Ebro valley, high philopatry was also documented in adult birds, with most kestrels remaining faithful to the colony where they bred the year before (71.6%, Serrano et al. 2001). Adult females seemed to disperse more often than males (34% vs 19%), and both sexes apparently dispersed less with age and experience (Serrano et al. 2001). Moreover, most of the dispersing adults settled within 2 Km of their previous colony, and just 6% of them dispersed > 10 Km (Serrano et al. 2001).

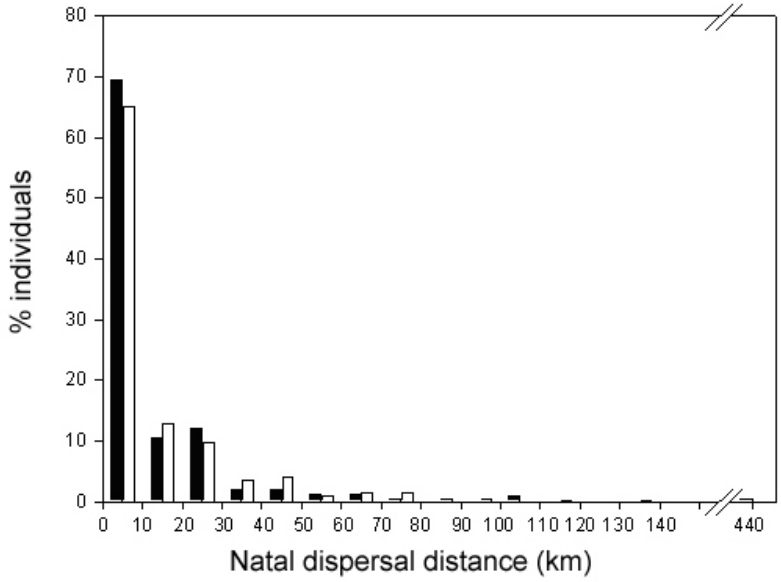

Fig. 1. Frequency distribution of natal dispersal distances of lesser kestrels in the Guadalquivir Valley (SW Spain, N = 321 individuals, black bars; Negro et al. 1997) and in the Ebro Valley (NE Spain, N = 961, white bars; Serrano et al. 2003).

Although high philopatry and restricted dispersal over short distances are expected to generate fine-scale non-random spatial patterns of genetic differentiation (Greenwood 1980, Greenwood & Harvey 1982, Sugg et al. 1996), the extent and genetic impact of long-distance dispersal in our study model is poorly understood. Thus, the key question that this article will address is whether local estimates of dispersal relying on capture-recapture data are good predictors of spatial patterns of genetic differentiation at two spatial scales, using microsatellite markers. In order to cover this aim, we firstly employed an individual-based spatial autocorrelation analysis to investigate fine-scale genetic structuring in the

demographically monitored and spatially structured population located at the Ebro Valley (see e.g. Serrano & Tella 2003, Serrano et al. 2005). This population is also geographically isolated (see Fig. 2), and both immigration from and emigration to other populations have been rarely documented by direct observations. Based on this evidence of limited gene flow, we then employed population-based analyses to test for genetically distinct clusters at a wider geographic scale covering the entire distribution range of the species in Western Europe.

METHODS

Study Species and Populations

Lesser kestrels are small migratory falcons that breed in Eurasia and winter in Africa (Cramp & Simmons 1980). Mostly monogamous, first breeding of lesser kestrels takes place at 1-2 years of age (Serrano et al. 2003). Levels of extra-pair paternity are in the low range, typical of raptors (7.25%, Alcaide et al 2005; see also Körpimaki et al. 1996, Arsenault et al. 2002). This fact increases the probability for adult males to raise their own offspring and reduces the probability of sampling half-sibs from different broods. Average life span for lesser kestrels is 3-4 years, but some individuals are known to have lived more than 10 years (see Negro 1997b for more information on the species).

Our first spatial scale of analysis covers 10,000 km^2 in an 8-year (1993-2000) demographically monitored population located at the Ebro Valley, North-eastern Spain (Fig. 2). This region contains a recently founded (circa 1960), fast-growing and spatially structured population of lesser kestrels, in which breeding pairs have increased from 224 in 1993, when monitoring began, to 787 in 2000 (Serrano & Tella 2007). Lesser kestrels breed there exclusively in farmhouses containing a variable number of pairs (1-43), and these colonies aggregate into different subpopulations (see Serrano & Tella 2003, Fig. 2). An extensive ringed-

based data set of dispersal, a considerable sampling effort at different spatial scales including 180 colonies with a maximum distance of about 210 km between them, and a known and recent demographic history (see Tella et al. 1998, Serrano et al. 2001, Serrano et al. 2003, Serrano & Tella 2003, Serrano et al. in press) makes this geographically isolated population an optimal candidate to compare capture-recapture records and genetic data over similar spatial and temporal scales. Our second spatial scale covers the species' distribution range in Western Europe, where four distinct populations can be defined on the basis of geographic criteria: Ebro Valley, Spanish core area, Portugal, and France (Figure 2). In the main Spanish core area, samples were obtained from different localities comprising the distribution borders (NES, CS, SES, and SWS,) and one central region (CWS, see Table 1, Fig. 2). In spite of the population decline experienced by the species during the second half of the 20th century (Biber 1990), this population still remains as the largest population in Western Europe. The number of breeding pairs is estimated at 12,000-20,000 (BirdLife International 2007), and the population has maintained a relatively continuous and stable distributional range. Part of this population in the Guadalquivir Valley (South-western Spain) was also the subject of demographic studies which continue, in part, today (Negro 1991, Negro et al. 1997, Rodriguez and Bustamante 2003). The current Portuguese population, estimated to be less than 300 breeding pairs, is concentrated in the South of the country and constitutes the South-western border distribution of the species in Eurasia (Fig. 2). This population is currently recovering from a population bottleneck (see Alcázar & Henriques 2006 for details). Finally, the most geographically isolated breeding population of our study model is located in Southern France (Fig. 2). This population was near extinction at the end of the 1970's (Cheylan 1991) but it has undergone a geographic and demographic expansion during the last two decades, reaching around 180 breeding pairs in 2007 (see Biber 1990, Pilard & Brunn 1998, http://crecerellette.lpo.fr/population/population.html).

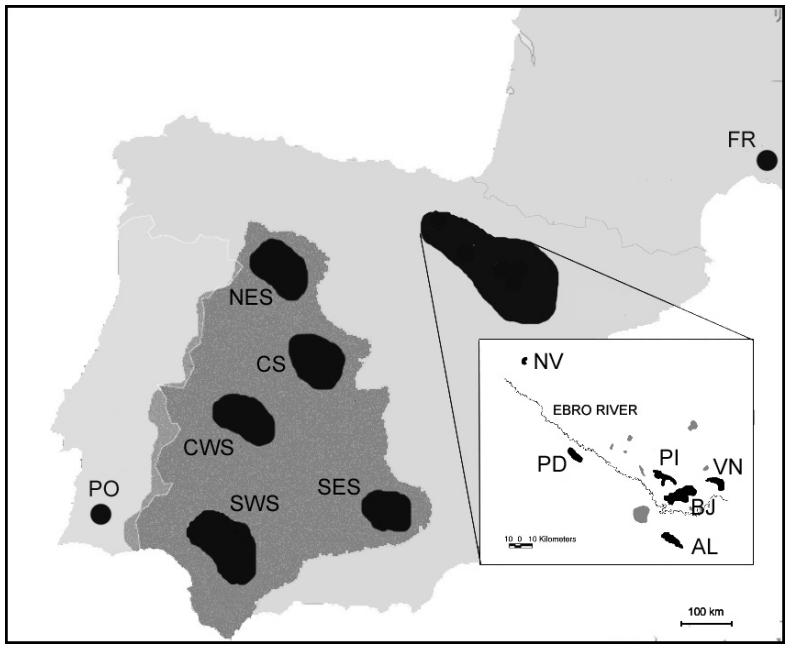

Fig2. Breeding distribution of the Lesser Kestrel in Western Europe. Dark grey areas represent the distributional range of the species. Black areas include sampled locations. The Ebro Valley population (North-eastern Spain) has been enlarged. Reintroduced populations are not indicated in this map. See Table 1 for location codes.

Genetic Sampling and DNA Extraction

During the 2002 and 2003 breeding seasons, blood or feathers were taken from 432 nestlings, each individual belonging to a different brood and presumably unrelated to all other sampled individuals. All individuals sampled from the same colonies belonged to the same cohort. Thus, we sampled 432 nests from 95 breeding colonies located in Spain, France and Portugal (see Table 1). Blood samples were preserved in absolute ethanol and feathers pulled from the nestlings' back were stored in paper. High concentrations of DNA can be obtained from growing feathers, given that tips are irrigated with plenty of blood vessels. Both types of samples were placed at 4º C until processing. The extraction protocol follows that described by Gemmell and Akiyama (1996). Blood and feathers tips were digested by incubation with proteinase K for at least 3 hours. DNA purification was carried out using 5M LiCl, organic extraction with chloroform-isoamylic alcohol (24:1) and DNA precipitation with absolute ethanol. Pellets obtained were dried and washed twice with 70% ethanol and later stored at –20º C in 0.1ml of TE buffer.

Table 1. Summary of the origin of Lesser Kestrel nestlings sampled for genetic analyses in Western Europe. Location code includes within brackets whether the sampling colonies were at the Ebro valley (EB), the Spanish core area (CA), the french (FR) or the portuguese population (PO). See Figure 1 for geographical locations.

Location	Location code	Number of sampled colonies	Number of sampled nestlings
Navarra	NV (EB)	2	21
Pedrola	PD (EB)	1	16
Pina	PI (EB)	6	25
Bujaraloz	BJ (EB)	6	48
Ventas	VN (EB)	7	20
Alcañiz	AL (EB)	13	44
North-western Spain	NWS (CA)	11	34
Central Spain	CS (CA)	6	27
Central-western Spain	CWS (CA)	14	53
South-western Spain	SWS (CA)	14	64
South-eastern Spain	SES (CA)	11	29
Portugal	PO (PO)	2	25
France	FR (FR)	1	26
Total		95	432

Microsatellite genotyping

We amplified nine microsatellite markers originally isolated in the peregrine falcon *Falco peregrinus.* Loci Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89 and Fp107, developed by Nesje and co-workers (2000), have previously shown to be suitable for genetic studies in other *Falco* species (e.g. Groombridge et al. 2000). We also designed two set of primers flanking two microsatellite sequences available in GenBank (AF448412 and AF448411, respectively). Locus Cl347 was amplified using primers Cl347Fw: tgtgtgtgtaaggttgccaaa and Cl347Rv: cgttctcaacatgccagttt. Locus Cl58 was amplified using primers Cl58Fw: tgtgtctcagtggggaaaaa and Cl58Rv:

tgctttggtgctgaagaaac. For each locus, the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following PCR profile: 35 cycles of 40s at 94ºC, 40s at 55ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 11 µl reaction contained 0.2 units of Taq polymerase (Bioline), 1x-manufacter supplied PCR buffer, 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP, 5 pmol of each primer and, approximately, 10 ng of genomic DNA. F-Primers were 5'-end labelled with HEX, TET or 6-FAM. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyser (Applied Biosystems).

Genetic analyses

Polymorphism statistics (i.e. number of alleles and observed heterozygosities) at each microsatellite marker were calculated with the software GENETIX 4.04 (Belkhir et al. 1996-2004). Conformity to Hardy-Weinberg expectations was analysed through GENEPOP (Raymond & Rousset 1995), using a single locus and a global multi-locus test for heterozygosity deficit or excess by the Markov Chain Method (Raymond & Rousset 1995). Linkage disequilibrium was also tested with GENEPOP.

Genetic structuring in the Ebro Valley population (N =174 individuals, Table 1) was investigated with a spatial autocorrelation analysis, which employs randomization procedures to test the null hypothesis of no spatial genetic structure. Spatial autocorrelation analyses are individual-based rather than population-based, and therefore they are not influenced by the subjective pooling of samples. Analysis was performed in Excel using the macro of the GenAlEx package version 6 (Peakall and Smouse 2005). GenAlEx uses pairwise geographic and pairwise squared genetic distance matrices to calculate an autocorrelation coefficient *r* for a set of distance classes specified by the user (Smouse and Peakall 1999, Peakall et al. 2003). The autocorrelation coefficient provides a measure of the genetic similarity between

pairs of individuals whose geographic separation falls within the specified distance class. We used the total pairwise genetic distance matrix (i.e. the matrix obtained from the sum of the matrixes obtained for each locus) as long as no evidence of linkage disequilibrium between each pair of loci was detected. The linear pairwise geographic distance matrix was calculated from x- and y-coordinates of each of the 35 colonies sampled in the Ebro Valley. Since most re-sights concerning dispersal occurred within a radius of 10 km (Fig. 1), we chose a set of variable distance classes for the analysis with a minimum distance class of 10 km. Consequently, we may expect the highest genetic similarity at this level. The calculated autocorrelation coefficients *r* were then plotted as a function of distance to produce spatial genetic autocorrelograms. Following Peakall et al. (2003), tests for statistical significance were performed using two methods: random permutations and bootstrap estimates of *r*, with the number of permutations and bootstraps set to 999.

The software STRUCTURE 2.2 (Pritchard et al. 2000) was used to test for the presence of genetically distinct cluster in Western Europe (N = 432 individuals). We did not use any prior information about the origin of the individuals and we assumed correlated allele frequencies and the admixture model. Ten simulations were performed for each of the K values ranging from 1 to 6 (i.e. number of putatively different genetic clusters) and probability values of the data, i.e. lnPr(X/K), were plotted. Analyses were carried out with 100,000 iterations, following a burn-in period of 10,000 iterations. We also calculated the traditional estimate of genetic differentiation F_{ST} to investigate population differentiation in Western Europe. The distribution of allele frequencies between the four geographically distinct populations (i.e. Ebro valley, Spanish core area, Portugal and France, Fig. 2) were compared using the software GENETIX 4.04 (Belkhir et al. 1996-2004). The significance of F_{ST} pair-wise comparisons was given by a P-value calculated using 10,000 random permutations tests that was further adjusted

according to sequential Bonferroni corrections for multiple tests (Rice 1989). Previously, we tested whether the Ebro Valley and the Spanish core area could be considered as large random breeding units attending to their conformity to Hardy-Weinberg equilibrium. In addition, the spatial autocorrelation analysis will check for local genetic structuring in the Ebro Valley and pairwise F_{ST} values between peripheral and central sampled localities will test for population differentiation within the Spanish core area. Even though STRUCTURE results suggest a genetically uniform population (i.e. K=1), testing for differences in allele frequencies between geographically distinct populations can be more powerful than STRUCTURE analyses when dealing with low levels of genetic differentiation (see software documentation in http://pritch.bsd.uchicago.edu/software/structure22/readme.pdf).

RESULTS

Loci properties

Overall, 105 alleles were detected across nine microsatellite markers and 432 genotyped birds. Feather samples were successfully genotyped at each microsatellite, and no evidence of allele dropout, i.e. systematic failure in the detection of some alleles, was detected compared with blood samples. Loci properties (i.e. number of alleles per locus, range size and average heterozygosities) are summarized in Table 2. No significant evidence of linkage disequilibrium was observed in any pair of loci analysed. Only locus Fp107 departed significantly from Hardy-Weinberg expectations. This locus consistently showed heterozygosity deficits that must be related to the presence of null alleles (see Alcaide et al. 2005). Since null alleles may violate several assumptions of the genetic methods we intended to apply, locus Fp107 was removed from further analysis.

Table 2. Microsatellite diversity at each of the four geographically distinct populations investigated in this study. Observed (Ho) versus expected heterozygosities (He) and estimates of allele richness (Ar) based on 25 individuals are indicated.

Population	He	Ho	Ar (Number of alleles per locus)
Ebro Valley	0.65	0.64	6.99
Spanish Core Area	0.65	0.65	7.5
Portugal	0.66	0.65	7
France	0.60	0.60	6.22

Genetic Structure in the Ebro Valley

The spatial autocorrelation analysis within the Ebro Valley population revealed a lack of fine-scale spatial patterns of genetic differentiation. The autocorrelogram plotted by GenAlex 6.0 (Fig. 3) showed that no genetic autocorrelation coefficient was significantly different from zero at any distance class. In addition, there is no statistically significant evidence of decreased genetic similarity in nestlings as a function of geographical distance.

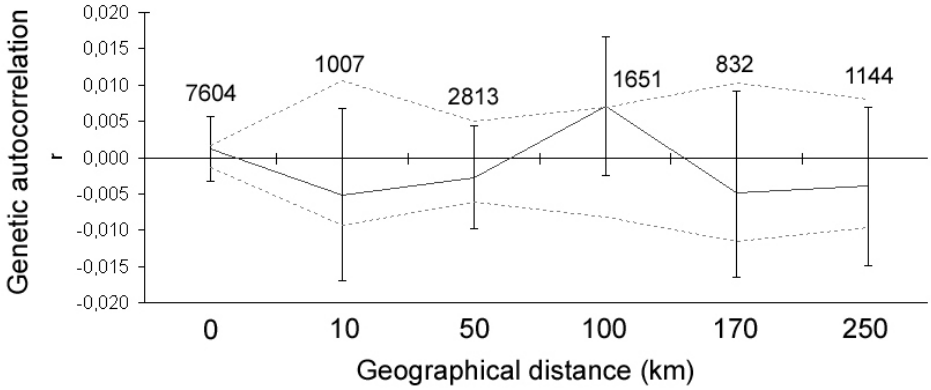

Fig 3. Correlogram plot of the degree of genetic similarity between Lesser Kestrel nestlings as a function of geographical distance in the Ebro Valley population (N = 174 individuals). The permuted 95% confidence interval (dashed lines) and the bootstrapped 95% confidence error (bars) are also shown. The number of pair-wise combinations within each distance class is presented above the plotted values.

Genetic Structure in Western Europe

The Bayesian model-based clustering method implemented in STRUCTURE suggested panmixia (i.e. K=1) as the most likely scenario in Western Europe (see Fig. 4).

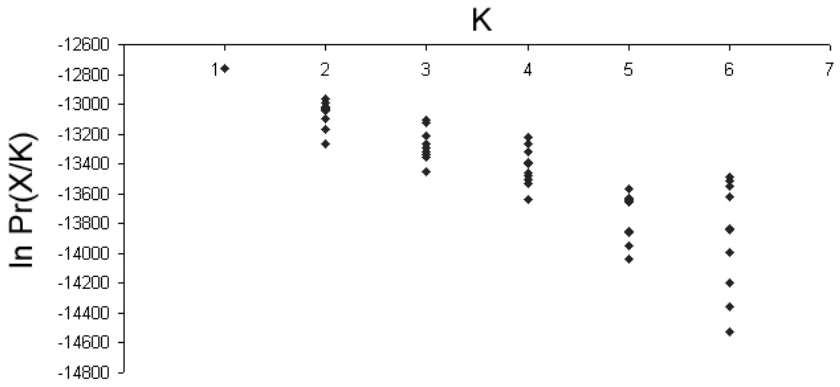

Fig 4. Bayesian clustering analysis of 432 Lesser Kestrels sampled in the Iberian Peninsula. For each value of K (i.e. number of putatively different genetic clusters tested), ten simulations were carried out to obtain the probability of the data (y-axis)

In addition, we did not find genetic differentiation within the Spanish core area as revealed by the lack of statistically significant F_{ST} values between peripheral and central localities (All F_{ST} values < 0.006, Bonferroni corrected P-values > 0.05). Conformity to Hardy-Weinberg expectations in the Spanish core area (He: 0.65, Ho: 0.65, Bonferroni corrected P-value > 0.05) also supports its consideration as large random breeding unit. Both the sampled populations from France and Portugal fitted to Hardy-Weinberg equilibrium as well (France, He: 0.60 vs Ho: 0.60, Bonferroni corrected P-value > 0.05; Portugal, He: 0.66 vs. Ho: 0.65, Bonferroni corrected P-value > 0.05). Even though we did not find local genetic structure in the Ebro Valley population and STRUCTURE results suggested a genetically uniform population of Lesser Kestrels in Western Europe, our F_{ST} analysis between the four geographically distinct populations revealed weak but statistically significant population differentiation (Table 3). Genetic divergence seemed to be stronger and biologically relevant in the case of the geographically isolated breeding population of Southern France and genetic differentiation with

respect to France appeared to increase as a function of geographical distance. According to these arguments, the highest F_{ST} value was found between the most distant populations of France and Portugal (Table 3).

Table 3. F_{ST}-pairwise values (above diagonal) between the four geographically distinct populations of Western Europe (see Fig. 2). Significant values after Bonferroni corrections for multiple tests are outlined in bold. Non-Bonferroni corrected P-values are given below the diagonal.

	Ebro Valley	Spanish main distribution	Portugal	France
Ebro Valley (N = 174)		**0.003**	0.005	**0.012**
Spanish core area (N = 207)	0.002		0.004	**0.016**
Portugal (N = 25)	0.08	0.011		**0.027**
France (N = 26)	0.002	<0.001	<0.001	

DISCUSSION

The main finding of this study is the lack of local genetic structure among lesser kestrel nestlings in spite of the high philopatry rates and restricted dispersal previously documented by capture-recapture analyses (Negro et al. 1997, Serrano et al. 2001, 2003, Serrano and Tella 2003, Ortego et al. 2008, Serrano et al. in press). We employed a highly polymorphic microsatellite set that has been suitable for detecting genetic differentiation at a very large geographical scale in lesser kestrels (i.e., covering its Eurasian distribution, Alcaide et al. 2008) and other *Falco* species (e.g. Hille et al. 2003, Nittinger et al. 2007). However, our genetic analyses failed to detect any evidence of increased relatedness at relatively small geographical scales. Even though spatial autocorrelation analyses have already demonstrated to be effective methods to detect fine-scale patterns of genetic structure in bird populations when analysing similar genetic data (e.g. Double et al. 2005, Temple et al. 2006), our results rather support previous genetic patterns found in other philopatric, socially monogamous and colonially breeding seabirds such as albatrosses or shearwaters, where genetic structure was not found at

relatively small spatial scales (Austin et al. 1994, Abbot & Double 2003, Burg & Croxall 2004, Van Bekkum et al. 2006, Huyvaert & Parker 2006). These findings would to some extent challenge traditional assumptions associating philopatry with the emergence of genetic structuring (Greenwood 1980, Sugg et al. 1996). In this respect, our results could seem contradictory with a recent study of a population of lesser kestrels from Central Spain, which found that relatedness between individuals decreased with geographical distance between breeding colonies (Ortego et al. 2008). However, two remarkable methodological differences could explain this potential discrepancy. First, these authors sampled breeding individuals, and their analyses probably included close relatives recruited in the natal colonies. We deliberately avoided this circumstance by sampling one fledgling per nest, and therefore, our results suggest that although the fine-scale genetic structure of breeding adults found by Ortego and coauthors could be extrapolated to other populations, patterns of relatedness seemed to disappear to some extent in the offspring, maybe because of the existence of behavioural mechanisms precluding mating between close relatives. Second, these authors admittedly used some microsatellite markers with null alleles, which could have inflated the statistical relationship between relatedness and distance, especially for close relatives, by artificially increasing the proportion of (false) homozygotes. We did not employ information provided by a locus with heterozygosity deficit in our statistical analyses, thus avoiding this potential bias.

At relatively small geographical scales, patterns of genetic structure in avian populations have been associated with habitat fragmentation, often linked to human perturbations (e.g. Caizergues et al. 2003, Martínez-Cruz et el. 2004). In this sense, our data suggest that population subdivision at the Ebro valley has not been sufficient enough to restrict dispersal capabilities of this species (Serrano & Tella 2003). We are confident that patterns of dispersal derived from capture-recapture data are scarcely biased by long-distance dispersers in this intensively

monitored population, given that (1) annual survival probabilities between fledgling and first-breeding as estimated with capture-recapture models were about 50-60% (D. Serrano unpublished data), similar to the highest estimates described for the species (Prugnolle et al. 2003), and hence hardly unbiased by permanent dispersal; (2) maximum dispersal distance within our population (136 Km) was much shorter than maximum distance between colonies (210 Km) (Serrano et al. 2003), and (3) only one local bird was known to recruit in the surrounding populations (see below), which were subject to thorough monitoring programs. Thus, our combined approach seems to indicate that a few long-distance dispersal events are enough to connect genetically distant patches, dilute genetic signatures, and homogenise allele frequencies, as previously suggested in the literature (Mills & Allendorf 1996, Vucetich & Waite 2000). Further, this population could have maintained or reached effective population sizes large enough to prevent the development of local genetic structure through genetic drift in spite of an initially low number of breeding pairs (see Serrano & Tella 2007).

Apart from spatial population fragmentation, the emergence of genetic structure in avian populations at relatively small geographical scales have been related to complex mating systems such as those displayed by lekking (e.g. Höglund & Shorey 2003, Bouzat & Johnson 2004) or cooperatively breeding species (e.g. Woxvold et al. 2007), in which one sex is much more philopatric than the other. In fact, several avian studies have related fine-scale spatial patterns of genetic differentiation to pronounced sex-biased dispersal (e.g. Fowler 2005, Double et al. 2005, McKinnon et al. 2006, Temple et al. 2006). Although our sampling protocol did not allowed to detect such a sex-specific genetic structuring, lesser kestrels are socially monogamous (see Tella et al. 1996 for a rare exception) and do not exhibit cooperative breeding strategies. Hence, we did not expect local genetic structuring linked to a complex reproductive biology. Strong sex-biased dispersal patterns, on the other hand, seem to have also evolved to avoid

inbreeding among close relatives (Greenwood 1980). In lesser kestrels, however, both males and females have shown to be highly philopatric, with natal dispersal distances greatly overlapping for the two sexes (Negro et al. 1997, Serrano et al, 2003). Moreover, capture-recapture studies have shown that the presence of the parent or a sibling of the opposite sex had no effect on whether or not first breeders returned to breed to the natal colony, neither at the Guadalquivir (Negro et al. 1997) nor at the Ebro Valley population (Serrano et al. 2003).

Although philopatry does not seem to have generated local genetic structure, restricted dispersal could have enhanced the effect of population fragmentation at a larger geographical scale. The Bayesian clustering method implemented in STRUCTURE, however, did not provide evidence for the existence of genetically distinct clusters once we scaled-up our study area to the entire distribution of the species in Western Europe. In the well-studied Ebro valley population, reencounters of ringed birds suggest that both immigration and emigration are anecdotal, with three immigrant birds banded elsewhere (two birds banded in central Spain and one bird banded in France, Tella, Serrano & Ursúa, unpublish. data), and one male banded as a nestling at the Ebro Valley that recruited as a breeding adult in the reintroduced population of eastern Spain, 300 km away (M.Alberdi, pers. comm.). As mentioned above, these few long-distance dispersal events may be sufficient to result in the development of low genetic subdivision, although at this scale the importance of long-distance dispersal events may have been much more underestimated by capture-recapture methods (see for instance Koenig & Dickinson 2004). Nonetheless, F_{ST} comparisons revealed weak but statistically significant population differentiation among the four geographically distinct populations of Western Europe. At the very least, genetic divergence showed to be consistent and biologically relevant in the case of the isolated French population. This finding would underscore the limitations of STRUCTURE to detect genetic differentiation when F_{ST} values are low (see also Latch et al. 2006).

Moreover, genetic data affected by isolation by distance is not well suited to its underlying model (see software documentation in http://pritch.bsd.uchicago.edu/software/structure22/readme.pdf), and the French population seems to show increased genetic divergence as a function of geographical distance (see Table 3). Restricted gene flow in the case of this population could be also a consequence of limited immigration resulting from the low conspecific attraction exerted by small breeding populations (see Serrano & Tella 2003, Serrano et al. 2004). Further, some evidence suggests increased genetic divergence as a function of geographical distance in Western Europe (Table 3), an isolation-by-distance pattern which emerges when studying the whole species' distribution in the Western Palearctic (see Alcaide et al. 2008). Previous genetic studies in birds (e.g. Caizergues et al. 2003, Martinez-Cruz et al. 2004), have postulated that population fragmentation limits gene flow following isolation-by-distance patterns, a fact that is consistent with the distance-dependent model of dispersal derived from capture-recapture analyses in lesser kestrels (Negro et al. 1997, Serrano et al. 2003, Serrano & Tella 2003). In any case, increased genetic divergence as a function of distance may be reflecting not only contemporaneous but also historical gene flow across the study area. Our genetic data would therefore provide supplementary evidence of population connectivity at a larger geographical scale than recoveries of banded birds and a recent genetic study of lesser kestrels at a small geographic scale (see Ortego et al. 2007a).

In conclusion, our genetic data suggest a lack of fine-scale genetic structuring in lesser kestrel populations, with low genetic differentiation at a larger geographical scale. Ringing records, in turn, indicate that most individuals were philopatric to the natal colony or dispersed short distances along their life. In this respect, our integrating approach suggests that rare long-distance dispersal events seem sufficient to override the predictions derived from high philopatry rates in birds. This fact highlights the benefits of combining traditional capture-recapture

with modern genetic methods in order to improve our understanding of dispersal and connectivity in animal (meta)populations. One could argue that the straightforward genetic approach shows enough gene flow to maintain panmitic populations that the more laborious capture-recapture methodology is unable to detect, thus making doubts about the utility of the second approach. However, the first approach overlooks the actual limited dispersal ability of the species, which might compromise the viability of small and isolated local populations even at small spatial scales (Serrano & Tella 2003). Whereas our results may therefore soften the genetic concerns derived from habitat fragmentation in species with apparently limited dispersal abilities, this study also warns about increased genetic divergence in small and geographically distant populations. Determining minimum thresholds above which dispersal capabilities cannot counteract the harmful genetic and demographic effects of habitat fragmentation is a laborious species- and even population-specific task, but of crucial importance to effectively manage spatially structured populations.

Capítulo 3

Population fragmentation leads to isolation by distance but not genetic impoverishment in the philopatric Lesser Kestrel: a comparison with the widespread and sympatric Eurasian Kestrel

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Manuscrito en 2ª Revisión: *Heredity*

ABSTRACT

Population fragmentation is a widespread phenomenon usually associated with human activity. As a result of habitat transformations, the philopatric and steppe-specialist Lesser Kestrel *Falco naumanni* underwent a severe population decline during the last century which increased population fragmentation throughout its breeding range. In contrast, the ubiquitous Eurasian Kestrel *Falco tinnunculus* did not suffer such adverse effects, its breeding range still remaining rather continuous. Using microsatellites, we tested the effects of population fragmentation on large-scale spatial patterns of genetic differentiation and diversity by comparing these two sympatric and phylogenetically related species. Our results suggest that habitat fragmentation has increased genetic differentiation between Lesser Kestrel populations, following an isolation-by-distance pattern, whilst the covered population of Eurasian Kestrels is panmictic. Contrarily to expectations, we did not detect significant evidence of reduced genetic variation or increased inbreeding in Lesser Kestrels. Two island subspecies of the Eurasian Kestrel, *Falco t. canariensis and Falco t. dacotiae*, however, did exhibit significant signs of inbreeding and lower microsatellite diversity. These findings suggest that gene flow as well as large enough effective population sizes may have mitigated genetic depauperation in the Lesser Kestrel. Relevant to conservation genetics and evolutionary biology, this study reports genetic differentiation due to habitat alteration in a species that has potential for long-distance dispersal but philopatry-limited gene flow. Nonetheless, genetic diversity in Lesser Kestrels only seems to become seriously reduced after severe population bottlenecks following extreme habitat fragmentation.

INTRODUCTION

Human activities transform the natural habitats of many species. Population fragmentation often leads to overall reductions in population sizes and diminishes connectivity among habitat patches. While population fragmentation increases extinction risks because of deterministic and stochastic factors acting on demographic parameters, restricted gene flow may jeopardize long-term persistence of populations due to inbreeding depression and loss of genetic diversity. Both demographic and genetic impacts of population fragmentation are believed to depend on the number, size and spatial distribution of populations as well as on time since fragmentation. In this regard, dispersal and associated gene flow become one of the most critical factors influencing the genetic structure and demography of fragmented populations (e.g. Young & Clarke 2000, Frankham et al. 2002). However, restricted gene flow and the subsequent emergence of genetic structuring is not only the result of physical or anatomical barriers to achieve long-distance displacements. Natal and breeding philopatry (i.e. the tendency of individuals to breed close to their birthplace or their previous breeding territory) are relevant life-history traits expected to enhance the effects of habitat fragmentation as well (e.g. Greenwood 1980). Genetic differentiation among fragments is hence expected to be inversely correlated with the dispersal ability of the species.

In spite of all the factors mentioned above, there is not necessarily a direct association between the spatial distribution of populations and the spatial distribution of genetic diversity (e.g. Dannewitz et al. 2005, Koopman et al. 2007, Jones et al. 2007). Independent demographic and genetic approaches are therefore being encouraged to rigorously evaluate the consequences of population fragmentation (e.g. Koenig & Dickinson 2004). In this respect, elucidating the demographic and ecological factors that determine the distribution of genetic variation in populations of the same or different species and subspecies at different scales has become a crucial issue in conservation and evolutionary biology.

Polymorphic molecular markers and powerful statistical methods have allowed the investigation of the spatial distribution of genetic variation in fragmented populations, also providing a measure of population connectivity. Such kind of approaches, combined with life-history and demographic information, have consistently provided relevant clues later considered for appropriate conservation and management initiatives aimed at preserving genetic diversity of endangered species. (e.g. Caizergues et al. 2003, Martínez-Cruz et al. 2004, Hansson & Richardson 2005, Koopman et al. 2007).

Studies on genetic structure and diversity in birds of prey are accumulating due to an emerging concern about the threats derived from population fragmentation and habitat alteration in this charismatic avian group (e.g. Martínez-Cruz et al. 2004, Godoy et al. 2004; Helbig et al. 2005; Nittinger et al. 2007; Hailer et al. 2007, Brown et al. 2007, Cadahía et al. 2007). Birds of prey typically have small populations with extended distributional ranges, but usually long-distance dispersal capabilities. Although raptor populations tend to be poorly structured (see references above), habitat fragmentation potentially increases genetic divergence and provokes a loss of genetic variation. In this study, we employed polymorphic microsatellites to assess the influence of population fragmentation on genetic diversity and large-scale (continental) spatial patterns of genetic differentiation in two phylogenetically related and sympatric birds of prey, the Lesser Kestrel *Falco naumanni* and the Eurasian Kestrel *Falco tinnunculus*. Both species breed in Eurasia, a continental mass with a broad tradition of human-induced landscape transformations which have generated serious threats for the conservation of many species (Goriup & Batten 1990, McNeely 1994). While the Lesser Kestrel is a specialist falcon inhabiting steppe and pseudosteppe ecosystems (Cramp & Simmons 1980), the Eurasian Kestrel is considered a truly cosmopolitan falcon that can live in most open-country environments (Village 1990). Open habitats in Europe have increased due to agriculture and clear-cutting of forests, a fact that

may explain why the breeding range of the Eurasian Kestrel has not decisively been affected by human activities. In contrast, Lesser Kestrels have experienced a well-documented population decline during the 20th century that is mostly explained by human perturbations, such as the substitution of traditional agricultural practices by intensive agriculture and irrigated crops that reduce foraging habitats (Tella et al. 1998, Ursúa et al. 2005). Such dramatic population regression led to the extirpation or disappearance of the Lesser Kestrel from several European countries (Biber 1990). This is to a great extent responsible for a patchier distributional breeding range as compared to its generalist counterpart (Fig.1). In addition, long-term and extensive ringing studies of Lesser Kestrels in Spain have documented high natal and breeding philopatry as well as a negative association between effective dispersal and geographical distance (Negro et al. 1997, Serrano et al. 2001, Serrano et al. 2003, Serrano et al. 2008). On the contrary, Eurasian Kestrels have shown low philopatry and frequent effective long-distance dispersal in populations from Northern and Western Europe (Korpimäki 1988, Village 1990, Korpimäki et al. 2006, Vasko 2007), although preliminary data from a Spanish population suggest higher philopatry rates in Southern Europe (J.A. Fargallo, pers. comm.).

Hence, the main question that this article will address is whether habitat alteration has resulted in population differentiation and loss of genetic diversity in the highly philopatric Lesser kestrel compared with the widely distributed and highly dispersive Eurasian kestrel. The suitability of the genetic methods we used here was tested by means of additional analyses of two insular subspecies of the Eurasian Kestrel inhabiting the Canary Islands. We expected the populations of these subspecies to hold comparably lower levels of genetic variation because of the well documented effects of insularity on demography and genetic diversity (e.g. Bollmer et al. 2005).

MATERIALS AND METHODS

Study Species and Populations

The Lesser Kestrel is a small trans-saharian migratory falcon whose breeding range covers mid-latitude and low elevations of Eurasia (Cramp & Simmons 1980). This colonial falcon originally occupied small cliffs surrounded by natural steppes (Tella et al. 2004), but most pairs breed nowadays in human structures surrounded by traditional agricultural land. The Eurasian Kestrel is a sedentary or partially migratory falcon of slightly larger size that is widespread in Eurasia, normally showing a territorial breeding behaviour (Cramp & Simmons 1980). In Europe, the estimated population size of Lesser Kestrels is about 25,000-42,000 breeding pairs, whilst that of Eurasian Kestrels is about 300,000-500,000 breeding pairs. We analysed breeding populations of the Lesser Kestrel in south-western Spain, central-western Spain, north-eastern Spain, France, Italy, Greece and Israel (see Fig. 1, Panel A). The continental subspecies of the Eurasian Kestrel (*Falco tinnunculus tinnunculus*) was sampled in south-western Spain, central-western Spain, north-eastern Spain, Switzerland, Finland and Israel (see Fig.1, Panel B). Two insular subspecies of the Eurasian Kestrel inhabiting the Canary Islands, *Falco tinnunculus canariensis* and *Falco tinnunculus dacotiae*, (see Fig. 1, Panel B) were also investigated to provide comparative data. Estimated population sizes are about 400-500 breeding pairs for *F.t. dacotiae* and less than 4,000 breeding pairs for *F.t. canariensis* (Madroño et al. 2004)

The majority of sampled individuals (>90%) were nestlings, and we only analysed one individual per brood to minimize problems associated with close relatedness. Extra-pair paternity in Lesser and Eurasian Kestrels has shown to be rare (below 7.5% of nestlings, see Alcaide et al. 2005 and Korpimäki et al. 1996 for details), and thus, the probability for adult males to raise their own offspring is high. Estimated population sizes of the geographically distinct populations of Lesser

Kestrels investigated in this study are shown in Table 1. The number of Lesser and Eurasian Kestrels sampled at each location is shown in Tables 3 and 4, respectively.

Table 1. Estimated population sizes of Lesser Kestrels during the period of time when samples were collected. Data were taken from BirdLife International (2004), Serrano & Tella 2007, Liven-Schulman et al. (2004). See Fig. 1 for geographic locations

Location	Code	Population size (breeding pairs)
Spanish core area	SWS and CWS	12,000-19,000
Ebro Valley	NES	< 1,000
France	FRA	< 100
Italy	ITA	3,640-3,840
Greece	GRE	2,000-3480
Israel	ISR	< 1,000

DNA isolation and microsatellite genotyping

About 100 µl of blood preserved in 96% ethanol or growing feathers that were pulled from the birds' dorsal plumage were digested by incubation with proteinase K for at least 3 hours. DNA purification was carried out by using 5M LiCl organic extraction method with chloroformisoamylic alcohol (24:1) and further DNA precipitation using absolute ethanol. Pellets obtained were dried and washed twice with 70% ethanol, and later stored at –20º C in 0.1ml of TE buffer. We amplified seven microsatellites that were isolated originally in the peregrine falcon *Falco peregrinus* by Nesje and co-workers (2000) (Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89, Fp107). In addition, we designed two set of primers flanking two microsatellite sequences also isolated in the peregrine falcon that were available in GenBank (AF448412 and AF448411, respectively). Locus Cl347 was amplified using primers Cl347Fw: tgtgtgtgtaaggttgccaaa and Cl347Rv: cgttctcaacatgccagttt. Locus Cl58 was amplified using primers Cl58Fw: tgtgtctcagtggggaaaaa and Cl58Rv: tgctttggtgctgaagaaac. For each locus, the polymerase chain reaction (PCR) was

carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following PCR profile: 35 cycles of 40s at 94ºC, 40s at 55ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 11 µl reaction contained 0.2 units of Taq polymerase (Bioline), 1x PCR manufacturer supplied buffer, 1.5 mM MgCl2 , 0.02% gelatine (Amersham Life Sciences), 0.12 mM of each dNTP, 5 pmol of each primer and, approximately, 10 ng of genomic DNA. Forward primers were 5'-end labelled with HEX, NED or 6-FAM. Amplified fragments were resolved on an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

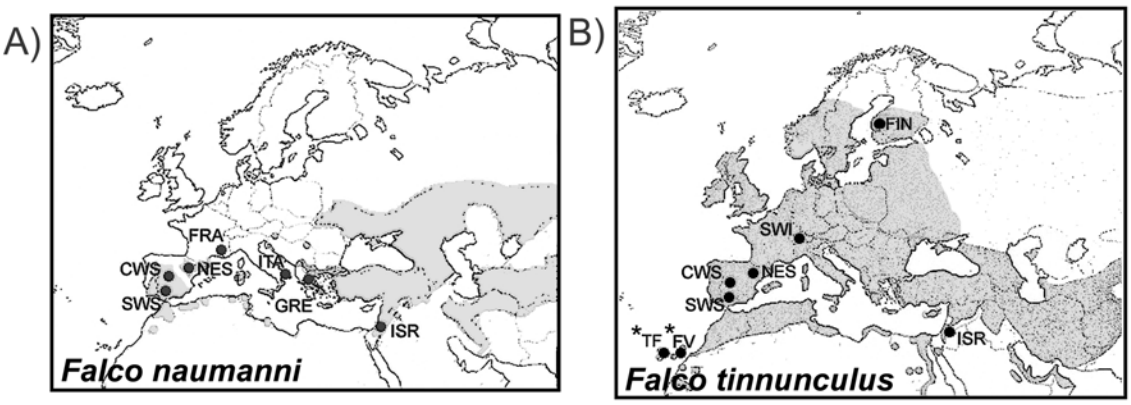


FIG 1. Breeding distributional ranges (grey areas) of Lesser (panel A) and Eurasian (panel B) Kestrels across the Western Paleartic. Populations analysed in this study are indicated by black dots. Lesser Kestrels were sampled from south-western Spain (SWS), central-western Spain (CWS), north-eastern Spain (NES), France (FRA), Italy (ITA), Israel (ISR) and Kazakhstan (KAZ). The continental subspecies of the Eurasian Kestrel was sampled from south-western Spain (SWS), central-western Spain (CWS), north-eastern Spain (NES), Switzerland (SWI), Finland (FIN) and Israel (ISR). In addition, two subspecies of the Eurasian Kestrel inhabiting the Canary Islands (indicated by asterisks) were sampled (FV for *Falco t. dacotiae* and TF for *Falco t. canariensis*).

Genetic analyses

Polymorphism statistics at each microsatellite marker (i.e. number of alleles and range size of the amplified fragment) were calculated using the programme Genetix 4.04 (Belkhir et al. 1996-2004). Conformity to Hardy-Weinberg equilibrium was analysed through GENEPOP (Raymond & Rousset 1995), using a single locus and a global multi-locus test for heterozygosity deficit or excess by the Markov Chain Method (Raymond & Rousset 1995).

We employed the software STRUCTURE 2.2 (Pritchard et al. 2000) to test for the presence of genetically distinct clusters within our study system. We did not use any prior information about the geographic origin of the individuals, and we assumed correlated allele frequencies and the admixture model. Ten simulations were performed for each of the K values ranging from 1 to 6 (i.e. number of putatively different genetic clusters), and probability values of the data, i.e. lnPr(X/K), were plotted. Values of K=1 indicate a genetically uniform population, whilst values of K=2 and so on indicate the existence of genetically different arrays of individuals. Analyses were carried out with 100,000 iterations, following a burn-in period of 10,000 iterations. Nonetheless, testing for differences in allele frequencies between geographically distinct populations may be more useful than clustering analyses performed in STRUCTURE when genetic differentiation is weak (e.g. Latch et al. 2006) or affected by isolation-by-distance (see software documentation in http://pritch.bsd.uchicago.edu/software/structure22/readme.pdf). Thus, we employed the programme GENETIX 4.04 to calculate F_{ST} values between groups of individuals sampled from different locations of the Lesser Kestrel breeding distribution. Although the distribution range of the Eurasian Kestrel is relatively continuous, we also calculated F_{ST} values between distant sampled locations in order to contrast F_{ST} pair-wise values with STRUCTURE results. The significance of F_{ST} pair-wise comparisons was given by a P-value calculated using 10,000 random permutations tests that was further adjusted according to sequential Bonferroni corrections for multiple tests (Rice 1989). Isolation by distance was investigated through Mantel tests based on the traditional F_{ST} / 1-F_{ST} approach. We introduced in the programme GENETIX a matrix containing values of genetic differentiation between each pair of sampled populations (i.e. F_{ST} / 1-F_{ST} values represented in the Y axis) plus a matrix containing the geographical distance in kilometres between each pair of sampled locations (represented in the X axis). Geographic distances

were calculated according to a straight line connecting the geometrical centre of each pair of sampled populations. The significance of the correlation between genetic differentiation and geographical distance was tested in GENETIX 4.04 through a P-value calculated using 10,000 permutations.

Allelic richness, average observed heterozygosities and the inbreeding coefficient F_{IS} among groups of samples encompassing individuals from different species or subspecies were compared using the permutation test (N = 10,000) implemented in FSTAT (Goudet 2001). The allelic richness estimate, which is calculated from random permutations of a minimum shared number of individuals between groups, is especially useful in this study since highly polymorphic loci such as Fp79-4 may decisively bias estimates of genetic diversity in relation to sample size. The non-parametric Wilcoxon-test was also employed to detect significant differences between sampled locations in polymorphism statistics obtained at each locus (i.e. allelic richness and average observed heterozygosities). Finally, microsatellite diversity at each pair of locations, measured as the mean number of alleles per individual, was compared using Student-t-tests.

RESULTS

Loci properties and Hardy-Weinberg equilibrium

Overall, 103 alleles were found in 320 Lesser Kestrels, 75 alleles in 128 mainland Eurasian Kestrels and 46 alleles in 28 island Eurasian Kestrels (see Table 2). Locus Fp107 departed significantly from Hardy-Weinberg expectations, showing heterozygosity deficits in most populations that are probably related to the presence of null alleles (see Alcaide et al. 2005). Since null alleles may violate several assumptions of the genetic methods we intended to apply, locus Fp107 was removed from further analysis.

Table 2. Number of alleles across 9 microsatellite markers in the Lesser Kestrel *(Falco naumanni)*, the European subspecies of the Eurasian kestrel *(Falco tinnunculus tinnunculus)* and the two subspecies of the Eurasian kestrel inhabiting the Canary Islands *(Falco tinnunculus canariensis and Falco tinnunculus dacotiae)*. The number of individuals analysed for each species or subspecie is shown in brackets.

Locus	*Falco naumanni*	*Falco t. tinnunculus*	*Falco t. canariensis*	*Falco t. dacotiae*
Range Size (bp)	(n=320)	(n=128)	(n=12)	(n=16)
Fp5	7	8	7	7
	99-111	101-115	101-113	101-113
Fp13	5	4	2	4
	86-106	92-98	92-94	92-98
Fp31	8	7	3	2
	124-142	128-142	134-138	134-138
Fp46-1	10	6	4	6
	115-139	117-127	119-125	115-125
Fp79-4	35	19	6	8
	125-192	129-154	137-149	137-152
Fp89	4	5	2	4
	116-122	116-124	118-120	116-122
Fp107	17	17	5	5
	185-231	195-233	193-221	193-221
Cl347	11	9	5	5
	96-116	100-116	100-112	100-112
Cl58	6	-	-	-
	118-123	-	-	-

Mainland populations from both kestrel species fitted to Hardy-Weinberg expectations after excluding this locus. We found, in contrast, statistically significant heterozygosity deficits, even after Bonferroni corrections for multiple tests, in the smallest insular population corresponding to *Falco t. dacotiae*.

Population differentiation

In Lesser Kestrels, the Bayesian analysis of population structure excluding any a priori information about the origin of individuals indicated panmixia (i.e. K=1, see

Fig. 2) as the most likely scenario.

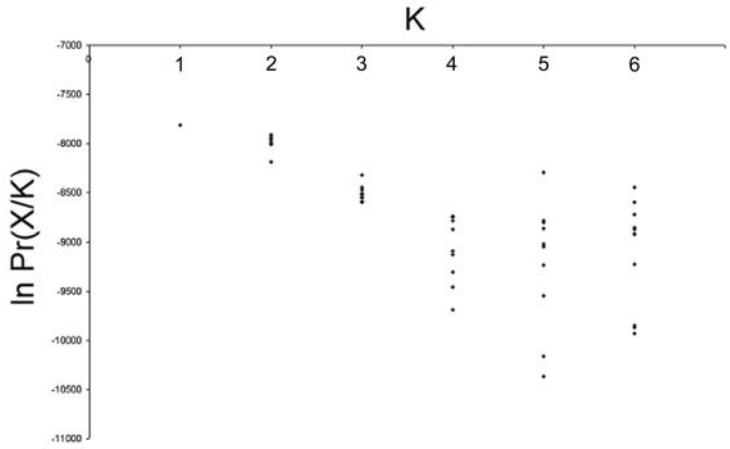


Fig 2. Bayesian clustering analysis of 320 Lesser Kestrels sampled in different regions of the Western Palearctic. For each value of K (i.e. number of putatively different genetic clusters tested), ten simulations were carried out to obtain the probability of the data (y-axis)

Nevertheless, traditional estimates of population differentiation relying on differences in allele frequencies revealed weak (F_{ST}<0.055) but significant patterns of genetic differentiation, even after Bonferroni corrections for multiple tests, when we compared geographically distinct populations (Table 3). In fact, genetic divergence across the study area adjusted significantly to an isolation-by-distance pattern (Fig. 3). On the other hand, the clustering analysis implemented in STRUCTURE only detected two genetically distinct clusters within Eurasian Kestrels (i.e. K=2) that distinguished the mainland subspecies against the two insular subspecies. This finding agrees with the comparably high and statistically significant pairwise F_{ST} values reported between Eurasia and the Canary Islands (F_{ST}>0.075, all Bonferroni-corrected P-values<0.05; Table 4). Conversely, there was no evidence for genetic subdivision within Eurasia, as none of the pairwise F_{ST} values were significantly different from zero (F_{ST}<0.015, all non-Bonferroni corrected P-values>0.05), or within the Canarian Archipelago (F_{ST}=-0.018, P=0.87) (see Table 5). Contrary to Lesser Kestrels, our set of genetic markers did not reveal significant evidence of isolation-by-distance in the mainland subspecies of the Eurasian Kestrel (Fig. 3). To compare data from both species, we performed a generalized linear model with F_{ST} as the response variable and species identity and Euclidean distance between populations as independent variables. After conservatively adjusting the denominator degrees of freedom to

avoid non-independence between sampling locations (see Bailey et al. 2007), the interaction term remained significant ($F_{1,9}$ = 9.11, P = 0.015).

Table 3 Pairwise F_{ST} values (above diagonal) between Lesser Kestrel populations from the Western Paleartic (see Fig.1 for geographical locations). Sample sizes at each location are indicated in brackets. Significant values after Bonferroni corrections for multiple tests are outlined in bold. Non-Bonferroni corrected P-values are given below the diagonal.

	NES	CWS	SWS	FRA	ITA	GRE	ISR
NES (68)		**0.008**	**0.008**	**0.014**	0	**0.009**	**0.035**
CWS (76)	<0.001		0.001	**0.019**	**0.016**	**0.014**	**0.041**
SWS (69)	0.0012	0.19		**0.023**	**0.013**	**0.013**	**0.038**
FRA (26)	0.0021	<0.001	<0.001		0.009	**0.041**	**0.034**
ITA (26)	0.56	<0.001	0.0048	0.0664		**0.017**	**0.021**
GRE (21)	0.002	0.0026	0.002	0.001	0.005		**0.054**
ISR (34)	<0.001	<0.001	<0.001	0.001	0.006	<0.001	

Genetic diversity

The permutation test performed in FSTAT did not reveal statistically significant differences in genetic diversity (allelic richness and average observed heterozygosity) or increased inbreeding (FIS) when comparing the Lesser Kestrel and the mainland subspecies of the Eurasian kestrel (all two-sided P-Values > 0.05, Table 5). In contrast, average observed heterozygosity was significantly lower in island than in the continental subspecies of the Eurasian kestrel (0.46 vs 0.66, two-sided P-value = 0.009; Table 5), and allelic richness was marginally significant in the same direction (4.24 vs 5.28, two sided P-value = 0.08; Table 5). Furthermore, we found statistically significant evidence of increased inbreeding (FIS) in the kestrel genotypes from the Canary Islands (0.265 vs 0.084, two sided P-value = 0.02; Table 5).

Table 4. Pairwise F_{ST} values (above diagonal) between Eurasian Kestrel populations from the Western Paleartic and the Canary Islands (see Fig.1 for geographical locations). Sample sizes at each location are indicated in brackets. Significant values after Bonferroni corrections for multiple tests are outlined in bold. Non-Bonferroni corrected P-values are given below the diagonal.

	NES	CWS	SWS	SWI	FIN	ISR	TF	FV
NES (18)		0.009	0.002	0.009	0.006	0.008	**0.066**	**0.083**
CWS (18)	0.34		0.010	0	0.004	0	**0.103**	**0.121**
SWS (19)	0.14	0.35		0.014	0	0.006	**0.078**	**0.107**
SZ (26)	0.19	0.53	0.09		0	0.003	**0.077**	**0.099**
FIN (23)	0.23	0.29	0.49	0.60		0.001	**0.078**	**0.105**
ISR (24)	0.18	0.42	0.22	0.31	0.39		**0.077**	**0.105**
TF (12)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-0.018
FV (16)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.87

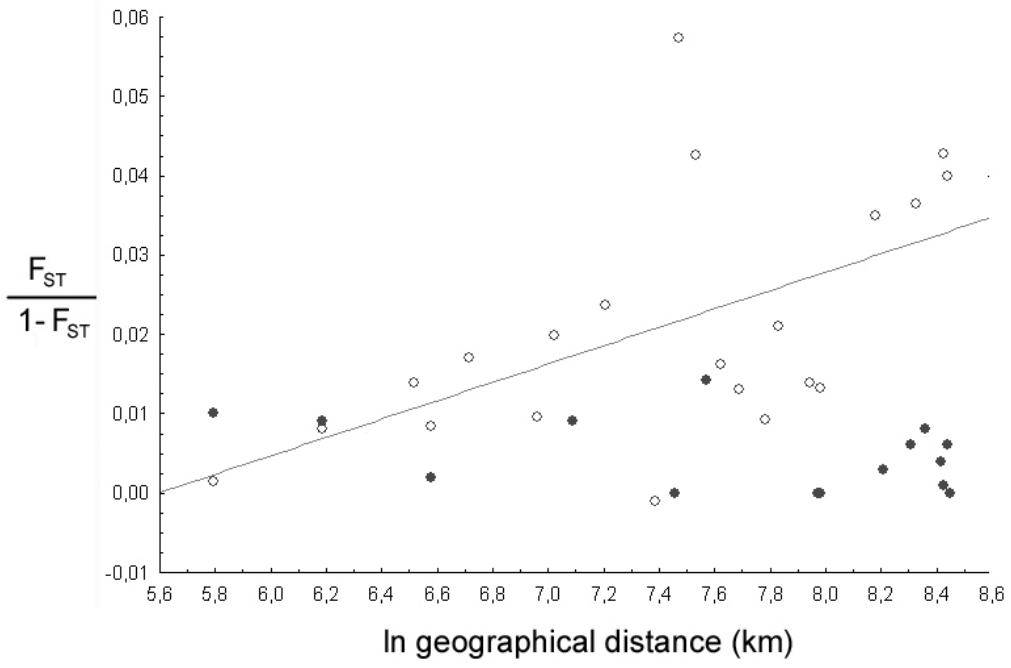


Fig 3. Relationships between the extent of genetic differentiation and geographical distance in lesser kestrel (open dots, r = 0.50, P = 0.04) and European kestrel (black dots, r = -0.44, P = 0.84) populations sampled across the Western Paleartic.

Finally, pairwise analyses comparing locus by locus failed to detect statistically significant decreased genetic diversity between any of the geographically distinct populations of Lesser Kestrels investigated (Non-parametric Wilcoxon-test, all P-values > 0.05; see Table 6). Average microsatellite diversity per individual was not statistically different among populations either (t-tests, all P-Values > 0.05), except for a couple of comparisons involving the smallest and geographically isolated population from Southern France. Such comparisons involved the less genetically diverse population (France) and two of the most genetically diverse (Italy and Israel, see Table 6).

Table 5. Comparison of average genetic estimates among groups of kestrel populations that was performed using the permutation test (N = 10,000) implemented in the programme FSTAT. Allelic richness was calculated over a minimum number of 12 individuals.

	Allelic Richness	Observed heterozygosity	Inbreeding coefficient (F_{IS})
Lesser kestrel	5.82	0.66	0.024
Eurasian kestrel (Mainland)	5.28	0.66	0.084
Eurasian kestrel (Canary Islands)	4.24	0.46	0.265

Table 6. Genetic diversity across eight microsatellite markers in six geographically distinct populations of Lesser Kestrels. Allelic richness estimates were adjusted to a minimum sample size of 21 individuals. See Fig. 1 for geographical locations.

	Allelic Richness	Average Observed Heterozygosity	Inbreeding Coefficient (F_{IS})
NES	6,6	0.63	0.07
CWS+SWS	7,06	0.65	0.05
FRA	6,02	0.60	0.04
ITA	6,89	0.67	-0.06
GRE	6,88	0.64	0.01
ISR	7,42	0.66	0.03

DISCUSSION

We studied the genetic implications of habitat fragmentation by comparing the generalist, continuously distributed mainland subspecies of the Eurasian Kestrel and the steppe-specialist, patchily distributed Lesser Kestrel. Our findings indicate similar levels of genetic variation in both species, but lower levels of genetic diversity in two island subspecies of Eurasian Kestrels. With respect to population differentiation, the Bayesian clustering method separated the mainland population of Eurasian Kestrels from their island counterparts. Coherently, F_{ST} analyses showed significant genetic differentiation between but not within both sampled clusters. In Lesser Kestrels, STRUCTURE assigned all individuals to a unique putative population. Nonetheless, estimates of population differentiation relying on the distribution of allele frequencies revealed low but significant levels of genetic differentiation following an isolation-by-distance model.

It is currently assumed that species thriving within a range of environmental conditions are more sensible to habitat transformations, their distributional ranges becoming patchier and the risk for genetic drift within fragments increasing (e.g. Ferrer & Negro 2004). Our empirical approach exemplifies a situation whereby genetic differentiation reflects the spatial distribution of populations, which, in turn, is delimited by habitat requirements. Thus, genetic differentiation between Lesser Kestrel populations increases with geographical distance (see also Alcaide et al. 2008 for data on MHC genes). Even though the Lesser Kestrel is a long-distance migratory species, gene flow is restricted over short distances due to high natal and breeding philopatry (Negro et al. 1997, Serrano et al. 2001, Serrano & Tella 2003). Elsewhere, we found, however, a lack of fine-scale patterns of genetic

differentiation in a spatially structured population of Lesser Kestrels located in north-eastern Spain (Alcaide et al. in third revision, Journal of Animal Ecology). This finding was attributed to the fact that population subdivision at the geographical scale studied (about 10,000 km^2) could have not been sufficiently important with respect to the dispersal capabilities commonly displayed by the species, and enough gene flow rates had homogenised allele frequencies. Nonetheless, long-distance effective dispersal in Lesser Kestrels (> 100 km) have been rarely documented by direct observations (Serrano et al. 2003, Prugnolle et al. 2003, P. Pilard and F. Martín, pers. comm., D.Serrano, E. Ursúa and J.L. Tella unpublish. data, M. Alberdi, pers. comm.), a fact that would be in agreement with the emergence of genetic structuring at large geographical scales. In contrast, it has been shown in several European populations of Eurasian Kestrels that natal dispersal regularly occurs over large distances (e.g. Snow 1968). Such amplitude of dispersal movements (see also Korpimäki 1988, Village 1990, Korpimäki et al. 2006, Vasko 2007) as well as a low incidence of habitat fragmentation in the Eurasian Kestrel would therefore explain a genetically uniform population.

Population genetics theory predicts that reductions in population size as well as limited migration decrease genetic variation, triggering negative genetic processes such as inbreeding depression and loss of adaptive potential (Frankham et al. 2002). Following these predictions, recent studies in the Lesser Kestrel have repeatedly looked at positive correlations between fitness component-traits and individual genetic diversity at 11 polymorphic microsatellite markers (Ortego et al. 2007b, 2007c). However, our genetic analyses, relying on at least six microsatellites previously amplified by Ortego and co-workers (Fp5, Fp13, Fp31,

Fp46-1, Fp79-4 and Fp89), have not revealed comparably low levels of microsatellite diversity or increased inbreeding in Lesser Kestrels in relation to the putatively outbreed subspecies of the Eurasian Kestrel. Genetic variation at functionally and evolutionary relevant MHC loci have also been shown extraordinary levels of polymorphism (> 100 alleles at a single locus) and heterozygosities above 95% in Lesser Kestrels (Alcaide et al. 2008). Although even normally outbred populations are expected to show inter-individual differences in the levels of inbreeding, this fact may explain the extremely weak (e.g. Ortego et al. 2007b,c) or even the lack of significant relationships (Ortego et al. 2007d) found by Ortego and co-workers. In addition, a short array of supposedly neutral markers is currently considered a poor predictor of fitness in open populations (reviewed by Coltman and Slate 2003) with the exception of those cases in which a strong linkage between certain neutral markers and some polymorphic fitness-related loci is demonstrated (e.g. Hansson et al. 2004a).

We believe that additional analyses of the pre-bottlenecked population are however needed to evaluate the degree of genetic depauperation in the Lesser Kestrel. In any case, this study recommends caution when assuming that the population decline experienced by this species has likely translated into contemporary reduced levels of genetic variation and increased inbreeding. For instance, Brown and co-workers (2007) have recently failed to detect signatures of a genetic bottleneck in peregrine falcons after a devastating decline in the mid-20th century due to organochlorine contaminants. In a similar way to this peregrine falcon study, some Lesser Kestrel populations have been known to experience demographic growth, either through a natural way (e.g. Tella et al. 1998, Ortego et

al. 2007a) or by means of reintroduction or supplementation programs (e.g. Pomarol 1993). Yet even in the bottlenecked and geographically isolated population from Southern France, from where we report the lowest levels of microsatellite polymorphism (Table 7), there is no documented evidence of a relationship between inbreeding depression and fitness. Conversely, local first-year survival in Southern France was similar or even higher than in Spain (Hiraldo et al. 1996, Prugnolle et al. 2003, D. Serrano unpublished data), which suggests that ecological constraints may play nowadays a more prominent role in individual fitness than genetic diversity.

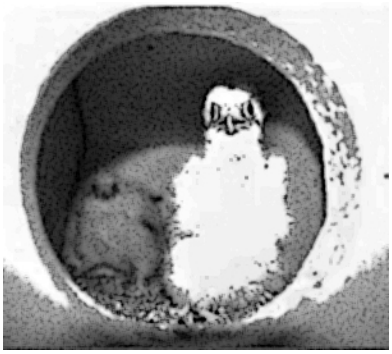
Our genetic analyses also indicate that genetic drift has provoked weak but significant fluctuations in allele frequencies (F_{ST} < 0.05) in Lesser Kestrels, but enough migration rates may have mitigated allele fixation (see for instance Mills & Allendorf 1996). In fact, it has been theoretically concluded that the rule of one migrant per generation is sometimes sufficient to maintain genetic diversity while allowing some divergence between fragmented populations (reviewed by Keyghobadi 2007). Moreover, interpopulation differentiation is though to proceed faster than loss of genetic variation after habitat fragmentation (e.g. Keyghobadi 2005). Although anecdotal, long distance dispersal events connecting adjacent populations of Lesser Kestrels have been recorded. For instance, several birds ringed as nestlings in the Iberian Peninsula have been resighted as breeding birds in Southern France, covering dispersal distances of up to 1,000 km (Prugnolle et al. 2003, P. Pilard, pers. comm.). Such effective dispersal displacements provide opportunities for genetic rescue (e.g. Vilá et al. 2003), probably explaining why lesser kestrels do not show reduced genetic diversity when compared to the

continental subspecies of the Eurasian kestrel. The comparison between continental and insular subspecies of the Eurasian kestrel, using the same genetic methods, provides a valuable supporting reference in this respect. Speciation processes in islands may require the lack of gene flow after colonization (see for instance Coyne & Orr 2004). Restricted gene flow is therefore expected to accelerate genetic divergence (Table 4), loss of genetic variation and increased inbreeding. These predictions are in accordance with our estimates of genetic diversity (Table 5) and also with other comparisons between mainland and insular populations of kestrels (e.g. Nichols et al. 2001).

In conclusion, this study illuminates about the genetic consequences of habitat fragmentation in open populations of birds of prey. Even though habitat loss, population decline and restricted gene flow over short distances may increase genetic divergence, low rates of long-distance effective dispersal may provide enough opportunities to counteract the loss of genetic variation through genetic drift.

Capítulo 4

Captive breeding and reintroduction of the Lesser Kestrel *Falco naumanni*: a genetic analysis using microsatellites



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ABSTRACT

Molecular markers may provide valuable information for the management of wildlife which is often unattainable via other approaches. Here we use microsatellites to assess captive breeding and reintroduction programs of the lesser kestrel. We demonstrate that the high rates of hatching failure occasionally documented in captivity can not be attributed to depressed genetic variation. Nevertheless, reintroduced populations showed significant decreased average heterozygosities and increased inbreeding coefficients compared to the captive demes from which released birds come from. Monitoring of reproductive parameters during single-pairing breeding and paternity inference within colonial facilities suggest that large variations in breeding success are responsible for low effective population sizes. The relative number of breeding pairs that contribute to a large part of captive-born birds (50-56%) was similar in both cases (28.6 and 26.9%, respectively). Thus, the chances for polygyny (rarely in this study), extra-pair paternity (not found in this study) and/or social stimulation of breeding parameters do not seem to greatly affect the effective population size. Independently of breeding strategies, we encourage a set of management measures to counteract the extent of founder effects. Firstly, the release of unrelated fledglings into the same area should be mandatory. Secondly, the investigation of genes involved in mate choice and genetic compatibility, such as those belonging to the MHC, may provide relevant clues to increase overall productivity. Finally, immigration is expected to increase heterozygosity, and therefore, kestrels irrecoverable for the wild and even decoys can be useful given the well documented effect of conspecifc attraction in settlement decisions.

INTRODUCTION

Captive breeding of endangered species has become a widespread practice to provide individuals for reintroduction or supplementation programs of extinct or declining populations. Although traditional approaches have identified ecological and behavioural constraints conditioning the short-term success of these initiatives (e.g. Hirzel et al. 2004, Martínez-Meyer et al. 2006), most monitoring programs do not take full advantage of the potential afforded by molecular markers. Monitoring population genetic metrics can provide insights into relevant processes that are difficult or impossible to obtain via traditional approaches (e.g. Schwartz et al. 2006). For example, captive breeding and reintroduction programs could potentially be counterproductive if the genetic consequences of the various management options are not fully considered (Woodworth et al. 2002, Gilligan and Frankham 2004). In this respect, loss of genetic variation linked to founder effects and inbreeding may have serious fitness consequences and can jeopardize the evolutionary and adaptive potential of populations and species (Frankham et al. 2002).

The lesser kestrel *Falco naumanni* was considered as one of the most abundant raptors in Europe before a sharp population decline which began in the late 1960's (Bijleveld 1974). As a result, this small migratory and facultatively colonial falcon totally or partially disappeared from several locations of its former breeding range (Biber 1990), now patchily distributed from Portugal to China (Cramp & Simmons 1980). To date, numerous captive breeding programs have successfully contributed to the reinforcement and re-establishment of decimated or extinct populations in Western Europe (e.g. Pomarol et al. 1993) by using the method of hacking (Sherrod et al. 1981).

In this study, we have performed the first genetic assessment of ongoing captive breeding and reintroduction programs of the globally vulnerable lesser kestrel (BirdLife International 2008). Our goals can be summarized as follows: i) to investigate levels of genetic diversity in captive populations. Hatching failure, one of the most cited fitness consequences of inbreeding in birds (e.g. Keller 1998), has been unusually high in captivity (> 50% of fertile eggs; Colás et al. 2002), contrasting with the normal values of this parameter in the wild (<10% of fertile eggs, e.g. Serrano et al. 2005). ii) to compare single-pairing (one male and one female) and colonial captive breeding (multiple males and females) strategies. We focused on variations in breeding success as primary determinants of genetically effective population size (e.g. Hedrick 2005). To this aim, we calculated the minimum number of highly succesful pairs that produced at least the 50% of fledglings at two captive centres working on single-pairing into individual pens. However, paternity of fledglings within colonial enclosures can only be confirmed through genetic inference, and therefore, we employed polymorphic microsatellites to infer kinship relationships. Two hypotheses can be made in this respect. The first hypothesis would predict an increase of the variance in male breeding success because of mixed reproductive strategies such as those observed, although at low rates, in wild colonies (see exceptional polygynous mating systems in Tella et al. 1996 and low extra-pair paternity rates <7.5% in Alcaide et al. 2005). Second, the simulation of colonial environments may stimulate the breeding behaviour of individuals which could otherwise remain sexually inactive (see for instance Waas et al. 2005), with the subsequent increase in overall productivity compared to single-breeding pairs iii) to evaluate the extent of genetic variation that has been successfully transmitted from captive stocks to the wild as a means of providing clues that can help to optimize the main genetic goal of a reintroduction program. In this regard, it is currently assumed that high leveles of genetic diversity maximizes the possibilities of re-establishing a self-sustaining population in the long term (e.g. Ballou and Lacy 1995, Frankham et al. 2002).

MATERIALS AND METHODS

Captive, reintroduced and wild populations

In Spain, three captive (GREFA in Madrid, DEMA in Extremadura and TORREFERRUSA in Catalonia) and three reintroduced populations of lesser kestrels (Lleida and Gerona in Catalonia plus La Rioja) were investigated. Four geographically distinct natural populations (Southern France, Ebro Valley, Spanish core area and Portugal) were also analysed in order to provide comparative support (see Table 1, Fig. 1). Founder individuals of captive demes, usually injured birds which could not be rehabilitated and returned into the wild, were obtained from the main Spanish population or other captive populations (Fig. 1). Different captive stocks have contributed to several reintroduction and reinforcement programs in Spain and France (e.g. Pomarol et al. 2001, http://crecerellette.lpo.fr/life/life.html)

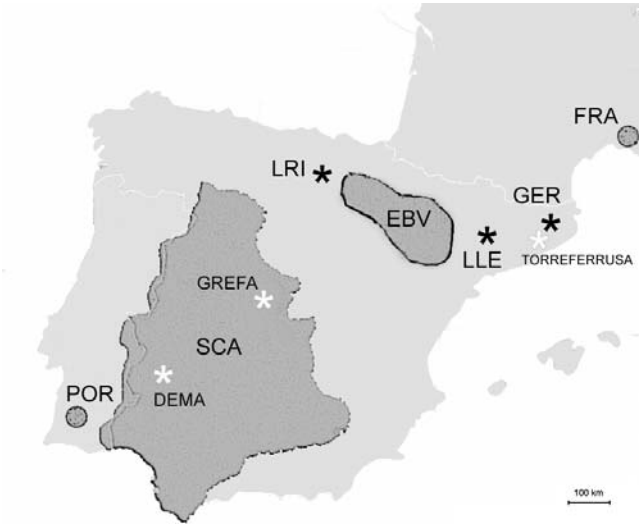


FIG 1. Breeding distribution of the lesser kestrel in Western Europe. Reintroduced (black asterisks) and captive (white asterisks) populations investigated in this study are indicated. See Table 1 for codes.

Sampling and DNA extraction

Biological samples for genetic analyses were obtained from wild and reintroduced populations during the 2002 and 2003 breeding seasons. Only one nestling per brood was analysed in order to avoid kinship relationships. In 2004, we sampled the breeding stocks of DEMA and GREFA (see Table 1). This included the captive-born progeny raised at the two largest colonial pens of DEMA (N=96 nestlings),

containing 36 and 16 adult birds respectively. Plastic rings were used for individual identification within colonial pens. Patterns of nest occupancy and within-pair copulations were also registered to assign potential breeding pairs.

The DNA extraction protocol we used follows that described by Gemmell and Akiyama (1996). Blood and feathers tips were digested by incubating with proteinase K for at least 3 hours. DNA purification was carried out with a 5M LiCl organic extraction method with chloroform-isoamylic alcohol (24:1) and DNA precipitation using absolute ethanol. Pellets thus obtained were dried and washed twice with 70% ethanol, and later stored at –20º C in 0.1ml of TE buffer.

Microsatellite genotyping

We amplified nine microsatellite markers originally isolated in the peregrine falcon *Falco peregrinus* (Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89, Fp107; Nesje et al. 2000, Cl347 and Cl58; see appendix) For each locus, the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) using the following PCR profile: 35 cycles of 40s at 94ºC, 40s at 55ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 11 µl reaction contained 0.2 units of Taq polymerase (Bioline, London, UK), 1x manufacturer-supplied buffer, 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP, 5 pmol of each primer and, approximately, 10 ng of genomic DNA. Forward primers were 5'-end labelled with HEX, NED or 6-FAM fluorocroms. Amplified fragments were resolved on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Genetic analyses

Conformity to Hardy-Weinberg equilibrium was analysed through GENEPOP (Raymond & Rousset 1995), using a single locus and a global multi-locus test for heterozygosity deficit or excess by the Markov Chain Method (Raymond & Rousset 1995). Linkage disequilibrium was also tested with GENEPOP. We employed the

permutation test (N=10,000) implemented in the program FSTAT ver 2.9.3 (Goudet 2001) to test for significant differences in genetic diversity among populations. In order to avoid bias caused by uneven sampling, the software FSTAT calculates a standardised estimate of allelic richness (R_S) independent of sample size. The extent of population differentiation was calculated according to the traditional F_{ST} estimate using the software GENETIX 4.04 (Belkhir et al. 1996-2004). The significance of F_{ST} pair-wise comparisons was given by a P-value calculated using 10,000 random permutations tests that was further adjusted according to sequential Bonferroni corrections for multiple tests (Rice 1989). Paternity within DEMA colonial pens and the parentage exclusion probability for first and second parents were inferred with Cervus 2.0 (Marshall et al. 1998). The probability that any two individuals shared the same genotype was calculated with Identity 1.0 (Wagner & Sefc 1999).

Calculation of variances in breeding success of captive birds during single-pairing breeding strategies

From 1996 to 2007, breeding parameters of 35 reproductive adults kept in TORREFERRUSA were registered. Breeding estimates of 70 reproductive adults kept in GREFA were available from 2005-2007 breeeding seasons. In both cases, we focused exclusively in those kestrels that raised offspring. Hence, non-reproductive birds were not included in the number of individuals given above. We calculated the minimum number of breeding pairs that yielded at least the 50% of the fledglings during the period of time investigated in each particular case.

RESULTS

Genetic Diversity in captive, reintroduced and wild populations

We found 103 alleles across 9 microsatellite markers (Table 1). All populations fitted to Hardy-Weinberg expectations after excluding Locus Fp107, which showed

significant heterozygosity deficits in most population and was subsequently removed from further analyses (see also Alcaide et al. 2005).

Table 1. Polymorphism statistics of wild (W), captive (C) and reintroduced (R) populations of lesser kestrels across 8 microsatellites. The number of alleles detected at each marker in each population is indicated in its corresponding column. The number of individuals sampled at each population (N), average observed heterozygosities (Ho) and allelic richness (Rs) estimates are showed. Estimated population sizes in breeding pairs (BP) when the samples were taken are also given. See Fig. 1 for geographical locations.

			Locus									
			Fp	Fp	Fp	Fp	Fp	Fp	Cl	Cl		
Population			5	13	31	46	79	89	347	58		
Pop. Size	Code	N									Ho	Rs
Southern France (W)	FRA	26	5	3	6	6	17	3	6	3	0.60	4.59
< 100 BP												
Ebro Valley (W)	EBV	174	6	4	7	10	33	4	10	5	0.64	4.92
<1,000 BP												
Spanish core area (W)	SCA	207	6	4	7	9	38	4	11	5	0.65	5.12
12,000-19,000 BP												
Portugal (W)	POR	25	6	3	6	7	19	3	8	3	0.65	5.06
< 300 BP												
GREFA (C)		32	6	3	7	9	25	4	9	3	0.67	5.33
< 100 BP												
DEMA (C)		59	6	4	7	7	28	4	8	4	0.68	5.04
< 100 BP												
Gerona (R)	GER	14	5	4	6	4	16	3	5	3	0.62	4.93
< 50 BP												
Lleida (R)	LLE	25	5	3	4	7	21	4	8	4	0.61	4.95
<100 BP												
La Rioja (R)	LRI	16	4	4	5	7	14	3	8	3	0.63	5.02
< 50 BP												

Population sizes were obtained from BirdLife International (2004) and Serrano & Tella (2007)

No significant evidence of linkage disequilibrium between any pair of loci was detected.

Polymorphism statistics at each population are summarized in Table 1. The permutation test performed in FSTAT did not report statistically significant differences in allelic richness (5.04 vs 5.18), average observed heterozygosities (0.64 vs 0.68) or the inbreeding coefficient F_{IS} (0.021 vs 0.006) between wild and captive populations (all two-tailed P-values > 0.05). Similarly, we did not find statistically significant differences in allelic richness (5.04 vs 4.97), average observed heterozygosities (0.64 vs 0.62) or the inbreeding coefficient F_{IS} (0.021 vs 0.049) between natural and reintroduced populations (all two-tailed P-values > 0.05). On the contrary, reintroduced populations showed statistically significant decreased average heterozygosities (0.62 vs 0.68) and increased inbreeding coefficients F_{IS} (0.049 vs 0.006) in relation to the captive demes from which released birds come from (two-tailed P-values = 0.012 and 0.031, respectively).

Patterns of genetic differentiation between captive, reintroduced and natural populations

Both captive populations of DEMA and GREFA were genetically differentiated from the Ebro Valley and the French population. Nonetheless, pair-wise F_{ST} comparisons revealed that both captive populations did not differ from the wild populations of Southern-western Iberia (Table 2). In fact, such breeding range of the species represents the source population of the founder individuals assigned to captive breeding initiatives (Fig. 1). On the other hand, reintroduced populations only showed statistically significant evidence of genetic differentiation when compared to the geographically isolated population from Southern France (Fig. 1). Unsurprisingly, genetic divergence in relation to the French population is

comparably high in spite of the geographic proximity of reintroduced populations (Fig. 1, Table 2). Thus, reintroduced populations somewhat depart from the isolation-by-distance patterns documented for free-ranging populations of lesser kestrels in Eurasia (see Alcaide et al. 2008 for details).

Table 2. F_{ST}-pairwise values (above diagonal) between four geographically distinct natural populations of lesser kestrels (W), captive (C) and reintroduced populations (R). Significant values after Bonferroni corrections for multiple tests are outlined in bold. See Fig. 1 for geographic locations.

	EBV (W)	SCA (W)	POR (W)	FRA (W)	GER (R)	LLE (R)	LRI (R)	GREFA (C)	DEMA (C)
EBV (W)		**0.003**	0.005	**0.012**	0.008	0.006	0.013	**0.010**	**0.008**
SCA (W)			0.004	**0.016**	0.010	0.006	0.009	0.006	0.006
POR (W)				**0.027**	0.007	0	0.010	0.003	0.008
FRA (W)					**0.019**	**0.028**	**0.032**	**0.033**	**0.025**
GER (R)						0.001	**0.030**	0.017	0.013
LLE (R)							0.012	0.010	0.010
LRI (R)								0.013	0.014
									0.005

Paternity inference within colonial breeding enclosures

The combined probability of exclusion for a marker set composed by Fp5, Fp31, Fp46, Fp79, Fp89 and Cl347 was estimated at 0.95. The likelihood of two individuals carrying an identical genotype was estimated at 6.21×10^{-6}. Paternity inference revealed that only seven breeding pairs (i.e. 27% of reproductive birds) contributed to the 56% of fledglings during the 2004 breedig season. Concerning mixed-reproductive strategies, we detected two cases of sequential polygyny, i.e. males raising two broods with successive females, in the largest colonial pen in DEMA. On the contrary, no genetic evidence of extra-pair paternity was found.

Variances in breeding success of captive birds during single-pairing breeding strategies

The analysis of breeding performance in the captive stocks of GREFA and DEMA revealed that, in both cases, the 28.6 % of reproductive birds contributed with the 50% and 56% of fledglings, respectively.

DISCUSSION

Undoubtedly, genetic monitoring is a desirable practice to ensure high reproductive fitness and ample genetic variation in captive-born individuals which will be subsequently released into the wild or used to supplement the captive stocks (Frankham et al. 2002; see examples in Gautschi et al. 2003, Ralls & Ballou 2004, Hedrick and Fredrickson 2008). Genetic monitoring can however become costly and time-consuming, especially if specific molecular markers for the target species are not available. Although some conservation initiatives can not simply afford it, the experiences summed from other captive breeding and reintroduction programs can become of high assistance.

To date, only a recent study by Lenz and co-workers (2007) have proposed manipulating sex ratios as a means of increasing the effective population size during captive breeding and reintroduction programs of lesser kestrels. Ours is the first study that has genetically evaluated both conservation initiatives for this small bird of prey usually associated with human and rural environments (Cramp & Simmons 1980). Polymorphisms statistics at 9 microsatellite markers reject inbreeding as a cause of the high rates of hatching failure (reviewed by Morrow et al. 2002) occasionally documented in captivity. Hence, current practices of breeders such as avoiding crosses between relatives via the registration of proper stud books and the frequent introduction of new blood into the genetic pools have proven to be satisfactory without the need of previous genetic assessment. Given that the incorporation of new individuals into the captive stocks is not constrained by the

number of lesser kestrels available in the study area (see Table 1), the proportion of birds which annually die (about 5%) can become easily replaced (see Pomarol et al. 2004a). Nevertheless, that is not the case of other endangered birds of prey such as the bearded vulture *Gypaetus barbatus* or the California condor *Gymnogyps californianus*. In these highly endangered species, the number of founders remain below the recommended minimum (20-30 individuals), and the incorporation rates of new birds to refresh the genetic pools is comparably low (see Gautschi et al. 2003, Ralls & Ballou 2004).

Our analyses indicate that the extent of genetic variability within reintroduced populations is a factor that can be considerably improved despite the lack of statistically significant reduced genetic variation with respect to natural populations (Table 1). Uneven contributions of breeding individuals to the captive-born progenies emerge as one of the main reasons underlying loss of genetic diversity (see for instance Hedrick 2005). The case of the lesser kestrel may receive special attention in this regard since many of the most prolific breeding pairs are forced to produce a second and even a third clutch during the same breeding season (Pomarol et al. 2004a, J.L. Antolín, M. Martín and I Gámez pers. comm.). As this study demonstrates, large variations in reproductive success of individuals are occurring at similar levels for both single-pairing and colonial breeding facilities. Hence, the occurrence of polygynous behaviours at low rates does not seem to significantly decrease the effective population size. Interestingly, both polygynous behaviours were achieved after the removal of the first clutch and not before. In this sense, the lack of extra-pair fertilizations also suggests that an increase in mate guarding investments and within-pair copulation rates might have overridden the effects of large breeding densities or female promiscuity in colonial breeding systems with *ad-libitum* feeding. On the other hand, our results do not seem to support that social stimulation of breeding parameters and and a broader availabity of potential mates translante into smaller variances in individual breeding success.

Certainly, loss of genetic diversity linked to founder effects could have been more pronounced without the contribution of different captive populations to reintroduction (Pomarol et al. 2004b, I. Gámez, personal communication) and/or without the continuous recruitment of wild birds. As Pomarol and co-workers (2004b) have previously indicated, immigration from the close Ebro Valley population may have decisively contributed to population growth in the reintroduced populations from Catalonia. Immigration has even involved long-distance dispersal, as we know for sure one bird hatched in the Ebro Valley (North Eastern Spain) which established itself as a breeder in the reintroduced population of Villena (Middle Eastern Spain), 300 km away (M. Alberdi, personal communication). Such a kind of gene flow events may also explain the lack of significant patterns of genetic differentiation between natural and reintroduced populations, with the exception of the geographically isolated French population (Table 2).

In conclusion, the main genetic consequences of the present study encourages minimizing the extent of founder effects from captive to reintroduced populations. To this aim, several management inititives should help to maintain the high levels of diversity found within the captive genetic pools. Firstly, the spatial and temporal diversification of fledglings should be aimed at minimizing the release of relatives during the same or consecutive years into the same geographical area. Releasing fledglings from different broods and different captive populations should therefore be mandatory. Secondly, improving our understanding about the clues that rule patterns of genetic compatibility is necessary to assist human-induced husbandry practices. In the case of lesser kestrels, breeders maintain the most prolific breeding pairs together, whilst non-successful birds are alternatively moved to pair with new mates. Genes at the major histocompatibility complex (MHC) are optimal candidates taking into account that they have been widely related to mate choice and breeding output in vertebrates (reviewed by Piertney & Oliver 2006). To

this aim, molecular markers for birds of prey are already available in the literature (see Alcaide et al. 2007 for MHC class II genes and Alcaide et al. CG for MHC class I genes). Finally, immigration is expected to increase overall heterozygosity (e.g. Ortego et al. 2007a). The effect of conspecific attraction for the recruitment of individuals is well documented (e.g. Serrano et al. 2004), and thus, birds kept in pens or even plaster models should be regularly used in newly established colonies to promote immigration.

BLOQUE 2

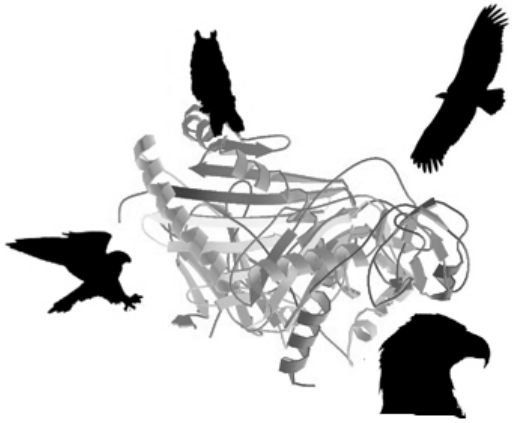
VARIACIÓN

GENÉTICA

ADAPTATIVA

Capítulo 5

Characterization, Polymorphism, and Evolution of MHC Class II B genes in birds of prey



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ABSTRACT

During the last decade, the major histocompatibility complex (MHC) has received much attention in the fields of evolutionary and conservation biology because of its potential implications in many biological processes. New insights into the gene structure and evolution of MHC genes can be gained through study of additional lineages of birds not yet investigated at the genomic level. In this study, we characterized MHC class II B genes in five families of birds of prey (Accipitridae, Pandionidae, Strigidae, Tytonidae, and Falconidae). Using PCR approaches, we isolated genomic MHC sequences up to 1300 bp spanning exons 1 to 3 in 26 representatives of each raptor lineage, finding no stop codons or frameshift mutations in any coding region. A survey of diversity across the entirety of exon 2 in the Lesser Kestrel Falco naumanni reported 26 alleles in 21 individuals. Bayesian analysis revealed 21 positively selected amino acid sites, which suggests that the MHC genes described here are functional and probably expressed. Finally, through interlocus comparisons and phylogenetic analysis, we also discuss genetic evidence for concerted and transspecies evolution in the raptor MHC.

INTRODUCTION

The major histocompatibility complex (MHC) is a multi-gene family that plays a central role in the vertebrate immune system. MHC genes encode protein receptors that recognise and bind foreign peptides for presentation to specialised immune cells and subsequent initiation of an immune response (Klein 1986). MHC genes are the most highly polymorphic genes described in vertebrates with several hundred of different alleles at some loci, for instance, in humans (Robinson et al. 2000). Two main types of balancing selection, heterozygote advantage and frequency-dependent selection, have been suggested to be important in maintaining the high levels of MHC polymorphism needed to counteract the selection pressures imposed by pathogens (Bernatchez and Landry 2003; Hedrick 1999). Patterns of polymorphism in the MHC have been the focus in studies of evolutionary ecology and conservation as a consequence of their suggested implication in many relevant biological processes, including self versus non-self recognition, susceptibility to infectious diseases, individual odours, mating preferences, kin recognition or pregnancy outcome (Brown and Eklund 1994; Grimholt et al. 2003; Westerdahl et al. 2005; Zelano and Edwards 2002; Singh et al. 1987; Tregenza and Wedell 2000; Knapp et al. 1996). This widespread relevance for ecological processes has made MHC genes excellent models for the investigation of adaptive variation in vertebrates (see the recent reviews by Sommer 2005; Piertney and Oliver 2006).

There are two major classes of MHC molecules (class I and class II), which act in different ways. Class I molecules are heterodimers expressed in all nucleated cells that play an essential role in immune defence against intracellular pathogens by binding peptides mainly derived from viral proteins or cancer-infected cells. Class II molecules are primarily expressed on antigen-presenting cells of the immune system and bind peptides derived from the processing of extracellular pathogens such as bacteria or parasites. The MHC class II protein consists of two amino acid chains, called α and β, encoded by MHC class II A and MHC class II B

genes, respectively. While both amino acid chains shape the peptide-binding region (PBR), the second exon of the B gene is known to hold the majority of the polymorphism. Several studies have characterized the second exon of MHC class II B genes in a wide variety of non-model vertebrates including mammals (Otting et al. 2002; Musolf et al. 2004), reptiles (Miller et al. 2005;Shi et al. 2004), amphibians (Bos and DeWoody 2005), and fishes (Consuegra et al. 2005; Wegner et al. 2006).

Studies of the MHC in birds have been restricted mainly to galliform species or passerines (i.e. Wittzell et al. 1999a; Ye X et al. 1999; Edwards et al. 1998; Miller and Lambert 2004b; Jarvi et al. 2004; Bonneaud et al. 2004a), with few examples of other avian groups (Ekblom et al. 2003; Tsuda et al. 2001). These studies have shown that there is substantial variation in MHC gene structure and number between different species. Thus, this emerging picture encourages gathering of information from a wide array of taxa to broaden our understanding of the evolution of MHC genes (Edwards et al. 2000). By far the best-studied bird MHC is the B-complex of the chicken *(Gallus gallus)*, although integration of the updated chicken genome and of the less-well-characterised Rfp-Y complex may update even this picture (Hunt et al. 2006; Miller et al. 2003). Early studies soon revealed striking differences between the genomic organization of the MHC in chickens and mammals (Trowsdale 1995). The chicken MHC appears to be much smaller and compact, with shorter introns, a lower number of genes and rare occurrence of pseudo-genes (Bourlet et al. 1988; Guillemot et al. 1989; Kauffman 2000). For example, the mammalian MHC encodes multiple loci for both class I and class II genes whereas in the chicken the B-complex codes for only two class I and two class II genes. These findings led to the formulation of the minimal essential MHC hypothesis (Kaufman and Salomonsen 1997), which highlights that the chicken MHC is selected to be as small and compact as possible, containing only enough expressed genes to ensure resistance to common pathogens. While most

passerines have many copies of both class I and class II genes and pseudogenes are abundant (i.e. Sato et al. 2000; Hess et al. 2000), the genomic complexity of other non-passerine birds such as the great snipe *Gallinago media* seems to be intermediate between chicken and passerines, with at least two class II genes and intermediate gene lengths (Ekblom et al. 2003).

In this study, we have developed the molecular tools for the characterization of MHC class II B genes in birds of prey, a group of vertebrates including species of high conservation concern. We investigated 26 different species from the major raptor families (*Aves*: *Accipitridae*, *Pandionidae*, *Strigidae*, *Tytonidae and Falconidae*, Brooke and Birkhead 1991), making ours one of the largest phylogenetic surveys of MHC diversity in any avian group. We also conducted a wide survey of exon 2 diversity for the Lesser Kestrel *Falco naumani*. These data permit a preliminary investigation and testing of different mechanisms of molecular evolution already documented in the avian MHC, such as balancing selection (Hedrick 1999; Ekblom et al. 2003), concerted evolution (Edwards et al. 1995a; Wittzell et al. 1999a), and transspecies polymorphism (Klein 1987; Richardson and Westerdahl 2003).

MATERIALS AND METHODS

Study Species and DNA Isolation

The species we investigated and the numbers of individuals analysed per species are shown in Table 1. Blood or tissue samples were collected from different individuals in the field or at rehabilitation centres in Spain, Argentina and Namibia. The extraction protocol we used follows that described by Gemmell and Akiyama (1996). Blood or tissues were digested by incubating with proteinase K for at least 3 hours. DNA purification was carried out using 5M LiCl, organic extraction with chloroform-isoamylic alcohol (24:1) and DNA precipitation with absolute ethanol. Pellets hence obtained were dried and washed twice with 70% ethanol, and later

stored at –20C in 0.1–0.2 ml TE buffer.

Table 1. Birds of prey where MHC class II B genes have been characterized. The number of different exon 2 sequences isolated and the number of individuals analysed per species is also indicated. The codes here proposed will be employed for the naming of the sequences following the nomenclature recommended by Klein et al. (1990).

Family	Species	Exon 2 Seqs (No. of individuals)	GenBank Acc. No	Species Codes	Country of Origin
Falconidae Falcons and Kestrels	Lesser Kestrel *Falco naumanni*	21 (26)	EF370767-370820	Fana	Spain
	Eurasian Kestrel *Falco tinnuculus*	2 (1)	EF370821-370822	Fati	Spain
	Aplomado Falcon *Falco femoralis*	2 (1)	EF370951-370952	Fafe	Argentina
	Peregrine Falcon *Falco peregrinus*	2 (1)	EF370947-370948	Fape	Spain
	Lanner Falcon *Falco biarmicus*	2 (1)	EF370949-370950	Fabi	Italy
Tytonidae Barn owls	Barn owl *Tyto alba*	2 (1)	EF370927-370928	Tyal	Spain
Strigidae Owls	Eagle Owl *Bubo bubo*	4 (1)	EF370930-370932	Bubbu	Spain
	Common Scops Owl *Otus scops*	4 (1)	EF370937-370938	Otsc	Spain
	Little Owl *Athene noctua*	5(1)	EF370942-370946	Atno	Spain
	Tawny Owl *Strix aluco*	4(1)	EF370933-370936	Stal	Spain
	Long-eared Owl *Asio otus*	2(1)	EF370939-370941	Asot	Spain
Accipitridae Hawks and allies	Northern Goshawk *Accipiter gentilis*	2(1)	EF370917-370918	Acge	Spain
	Marsh Harrier *Circus aeruginosus*	3(1)	EF370919-370921	Ciae	Spain
	Golden Eagle *Aquila chrysaetos*	4(1)	EF370905-370908	Aqch	Spain
	Booted Eagle *Hieraaetus pennatus*	4(1)	EF370909-370912	Hipe	Spain
	Common Buzzard *Buteo buteo*	2(1)	EF370899-370900	Butbu	Spain
	Crowned Eagle *Harpyhaliaeetus coronatus*	4(1)	EF370901-370904	Haco	Argentina
	Red Kite *Milvus milvus*	2(1)	EF370897-370898	Mimil	Spain
	Short-toed Eagle *Circaetus gallicus*	4(1)	EF370913-370916	Ciga	Spain
Accipitridae Hawks and allies	Wild Cape Vulture *Gyps coprotheres*	11(3)	EF370879-370989	Gyco	Namibia
	White-backed Vulture *Gyps africanus*	12(3)	EF370867-370878	Gyaf	Namibia
	Eurasian Black Vulture *Aegypius monachus*	2(1)	EF370890-370891	Aemo	Spain
	Egyptian Vulture *Neophron percnopterus*	4(1)	EF370893-370896	Nepe	Spain
	Bearded Vulture *Gypaetus barbatus*	1(1)	EF370891	Gypa	Spain

	Black-shouldered Kite *Elanus caeruleus*	3(1)	EF370924-370926	Elca	Spain
Pandionidae Ospreys	Osprey *Pandion haliaetus*	2 (1)	EF370922-370923	Paha	Spain

Amplification, Sequencing and Alignment of MHC Fragments

Amplification strategies relying on the polymerase chain reaction (PCR) were performed over genomic DNA in a PTC-100 programmable thermal controller (MJ Research Inc.). The basic PCR profile for all amplifications was composed of 4 min at 94C following 35 cycles of 40 s at 94C, 40 s at 56–58C and 40–80 s at 72C, and finally 4 min at 72C. Each 25 ll reaction contained 0.2 units Taq polymerase (Bioline), 1·PCR buffer, 1–1.5 mM MgCl2, 0.02% gelatine, 5% DMSO, 0.12 mM of each dNTP, 10–20 pmol of each primer and approximately 10 ng of genomic DNA. Sequencing reactions were carried out using Big Dye 1.1 Terminator technology and labelled fragments were subsequently resolved in a 3100 automated sequencer (Applied Bio-systems). DNA sequences were aligned and edited using the software BioEdit (Hall 1999).

Amplification of Short and Long MHC Fragments

The degenerate primers 326 and 325 (Table 2; Edwards et al. 1995b) were employed to perform partial amplification of exon 2. We designed new degenerate primers (AlEx3F, AlEx3R; Table 2) across conserved regions emerging from an alignment of exon 3 sequences of different vertebrate taxa including birds (species names and GenBank accession number: *Homo sapiens* NM 002124, *Gallus gallus* DQ008586, *Coturnix japonica* AB110479, *Agelaius phoeniceus* U23971, *Gallinago media* AF485406, *Sphenodon punctatus* DQ124234). One individual of the following raptor species were sequenced: Lesser Kestrel *Falco naumanni*, peregrine falcon *Falco peregrinus*, Eurasian black vulture *Aegypius monachus*, booted eagle *Hieraaetus pennatus*, northern goshawk *Accipiter gentilis*, barn owl *Tyto alba*, little owl *Athene noctua* and eagle owl *Bubo bubo*. PCR reactions at this stage contained 20 pmol of each primer and 1 mM MgCl2. The annealing temperature was 56C. Uncloned PCR products were directly sequenced in order to confirm appropriate

amplification of MHC genes and to detect conserved regions among species.

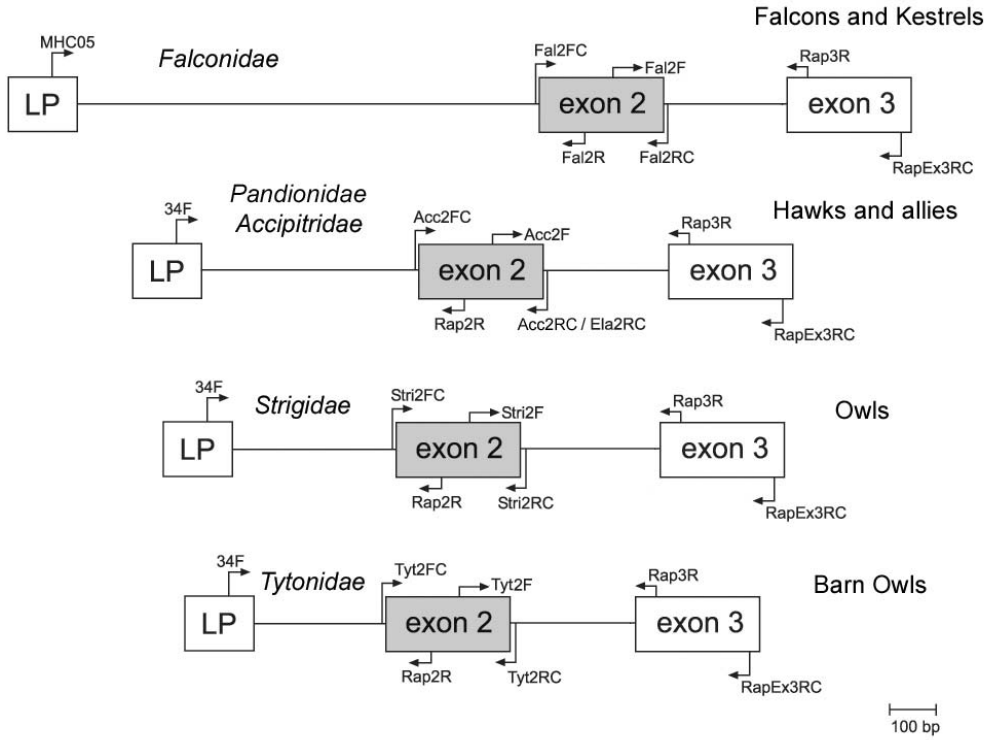


Fig. 1 Schematic illustration of MHC class II B genes in various families of birds of prey. The positions of the primers used in this study are indicated by arrows. Boxes represent exons and the shaded box, the highly polymorphic exon 2 that codes for the antigen binding sites. LP indicates the peptide leader sequence located in exon 1

Once obtaining partial exon 2 and exon 3 sequences, new primers will be designed across conserved regions in order to amplify intron 1 and intron 2. Primer design will be tested using Oligo 6.0 (Molecular Biology Insights). An additional forward primer in exon 1 needed to amplify intron 1 (MHC05 and 34F; Miller and Lambert 2004a, Ekblom et al. 2003) (Fig. 1) was also used. At this stage, our aim was obtaining the intron sequences flanking the highly polymorphic exon 2 to design new primers for the amplification of the whole exon in a single PCR. Furthermore, there are no available primers able to amplify long MHC class II fragments including exon 1, exon 2, introns 1 and 2, and exon 1 (MHC05 and 34F; Fig. 1, Table 2) and a newly designed reverse primer annealing to a distal conserved region of exon 3 that we identified after the alignment of different bird sequences deposited in the data bases (species names and GenBank accession

number: *Gallus gallus* DQ008586, *Coturnix japonica* AB110479, *Agelaius phoeniceus* U23971, *Gallinago media* AF485406).

Table 2. List of primers used for PCR and sequencing. Standard IUB codes are used for degenerate primers

Primer Name	Sequence 5′ – 3′	Reference
326	GAGTGYCAYTAYYTNAAYGGYAC	Edwards et al. 1995b
325	GTAGTTGTGNCKGCAGTANSTGTCCAC	Edwards et al. 1995b
AlEx3F	TGCTMCGTGMYGGRYTTCTACCC	This study
AlEx3R	CACCAGCASCTGGTASGTCCAGTC	This study
34F	CTGGTRGCACTGSTGGYGCTG	Ekblom et al. (2003)
BRMHC05	CGTRCTGGTGGCACTGGTGGYGCT	Miller and Lambert (2004a)
Rap2R	CCCACRTCRCTGTCRARGTG	This study
Fal2R	GTACWGCTGCCGGTTGTAGAT	This study
Fal2FC	CCTCCCTGTACAAACAGAG	This study
Acc2FC	GCACAAACAGGGTTYTTCC	This study
Stri2FC	CMCACACAGGGGTTTTCC	This study
Tyt2FC	CTATGCAAACAGAGGTTTTCC	This study
Fal2F	CGACSTGGGGTACTWCGTG	This study
Acc2F	TGYCRAGTACTGGAACAGCC	This study
Stri2F	GTGAGYMCCMAGCCMAGTAC	This study
Tyt2F	GTGTGCCCCAAGCCGAGTAC	This study
Fal2RC	GTGGCACTGGGAAACSTG	This study
Acc2RC	CAGGRAAAWRTTCTGGCAC	This study
Stri2RC	AACGYGYGGCCACGCGCTCA	This study
Tyt2RC	ACGCGGTGCCACGCACTCA	This study
Ela2RC	CGGGAAATGCTCCGGCAC	This study
Rap3R	ACCAYTTCACCTCRATCTSCG	This study
RapEx3CR	CAGGCTGRCGTGCTCCAC	This study

The amplification of long MHC fragments was checked in all raptor species investigated here (Table 1) using the basic PCR profile described above but

extending the extension time for the Taq to 80 s.

Molecular Cloning

Investigation of variation at single MHC loci requires separating the different PCR amplification products because of the possibility of amplifying more than one locus, and because individuals are likely to be heterozygous for these loci. We cloned PCR products resulting from the amplification of the complete second exon in all the species investigated here as well as long MHC fragments in which cloning was the only alternative to obtain unambiguous and complete sequences of the introns linked to both exons. After PCR clean-up in Microcon centrifuge tubes (Millipore), PCR products were cloned into bacterial plasmid using the PGEM-T easy vector system II (Promega). Clones were screened for the expected insert size in 1.5% agarose gels by running a second PCR with M13 primers. Positive clones (8–10 per individual) were selected for sequencing analysis when investigating polymorphisms in exon 2. Following Edwards and co-workers (1995b), rare exon 2 sequences found only once and differing by less than 3 bp from a redundant sequence of the same PCR product were considered artefacts of PCR errors and were discarded. Since recombination of cloned PCR products is an additional source of artefacts (Bradley and Hillis1996; Meyerhans et al. 1990), direct sequencing of uncloned PCR products was used to get agreement for polymorphic sites.

Analyses of Intraspecific Polymorphism

A wide survey of intraspecific polymorphism in exon 2 was conducted for 21 Lesser Kestrels hatched in Spain. Three white-backed vultures, *Gyps africanus*, and three cape vultures, *Gyps coprotheres*, from Namibia were also analysed. Polymorphism statistics were generated using the software DNAsp 4.0 (Rozas et al. 2003).

Test for Positive Selection in Exon 2 Sequences Using Maximum Likelihood Analyses

An excess of non-synonymous substitutions (dN) over synonymous substitutions

(dS) in functionally important amino acid sites indicates that positive selection is occurring. Then, ω = dN/dS>1. We used the programme CODEML of the PAML package ver. 3.15 (Yang 2000) to test for the presence of codon sites affected by positive selection and to identify those sites in exon 2 sequences of the Lesser Kestrel. This fact precludes assuming that codons comprising the PBR in birds are the same as in the human MHC class IIB genes (see Brown et al. 1993). The models considered in this study were M7 (beta) and M8 (beta and ω). Under the model M7 (beta), the ω ratio varies according to the beta distribution and does not allow for positively selected sites (0<ω<1). Model M8 provides an additional site class to account for sites under positive selection (ω>1). Models M7 and M8 were compared using likelihood ratio tests (LTR) (Nielsen and Yang 1998). The LRT statistics calculates twice the log-like-likelihood difference compared with a $\chi 2$ distribution with degrees of freedom equal to the difference in the number of parameters between the two compared models. The best tree by maximum likelihood search was in accordance with the one-ratio model (M0) used to provide phylogenetic information. Finally, we used a Bayesian approach implemented in CODEML to identify residues under positive selection in the Kestrel class II sequences.

Phylogenetic Relationships of MHC Class II B Genes in Birds of Prey

The phylogenetic relationships of MHC class II B sequences were visualized through Neighbour-Net networks based on Kimura's two parameter model that were built in the software Splits Tree 4 using maximum likelihood distances (Huson and Bryant 2006). Under complex models of evolution involving gene loss and duplication, hybridization, horizontal gene transfer or recombination, phylogenetic networks can provide a useful representation of the genetic relationships among sequences as compared to traditional phylogenetic trees. In this regard, gene loss and duplication in addition to recombination have been widely described in the MHC (i.e. Nei et al. 1997; Miller and Lambert 2004a; Hess and Edwards 2002; Schaschl et al. 2006).

Raptor exon 2 sequences jointly with exon 2 sequences obtained from galliform species (GenBank accession numbers: AM489776, AB282651, AJ224352, AY928104), passerines (GenBank accession numbers: L42335, AJ404376, AY437913, AF328737, U24411, U24426) and a tuatara Sphenodon punctatus sequence (GenBank accession number: DQ124237) as an outgroup were analysed. In addition, we built another network containing only intron 2 and exon 3 sequences from different species within the Accipitridae family. This network included 10 different sequences, from at least two different loci, that were isolated in three white-backed vultures and three different sequences from one cape vulture, respectively. Our aim at this point was to look for specific clusters that may reflect different loci within the same species or orthologus relationships among loci from different species.

Finally, the occurrence of gene conversion was assessed using the software GENECONV version 1.81 (Sawyer 1999). GENECONV analyses the distribution of nucleotide differences to detect gene conversion events by looking for stretches of nucleotides in a pair of sequences that are more similar to each other than would be expected by chance (Drouin et al. 1999). Putative gene conversion events were considered significant when the simulated global P value < 0.05. Such simulations were based on 10,000 permutations of the original data. The analysis was performed on a 1274 bp alignment of MHC class II B sequences from three white-backed vultures (n = 10), one cape vulture (n =3) and one Eurasian black vulture *Aegypius monachus* (n =1). Gscale values of 0, 1 and 2 were used, allowing for varying levels of mismatches (i.e. subsequent mutation) within the gene conversion event to take into account.

RESULTS

Amplification of Conserved MHC, Complete Exon 2 and Intronic Regions

A 159 bp fragment of exon 2 and an 81 bp fragment of exon 3, excluding primers sequences, were successfully obtained in multiple species using degenerate primers. GenBank accession numbers are not given here since PCR products were not cloned and because these sequences will overlap with longer MHC sequences we describe. Primers 34F and MHC05 (Miller and Lambert 2004a; Ekblom et al. 2003), in combination with new primers designed across conserved regions of exon 2, successfully amplified intron 1 in all raptor species tested so far. A novel battery of primers designed across conserved regions at the family level of exons 2 and 3 successfully amplified intron 2 (Fig. 1, Table 2).

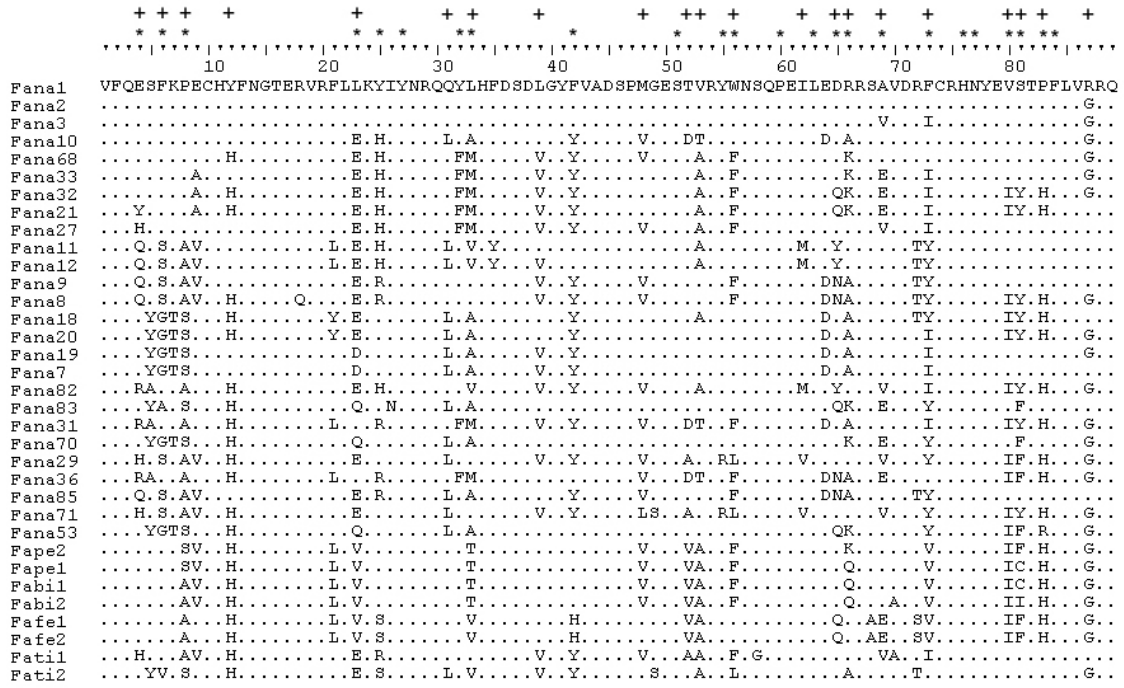


Fig. 2. Alignment of putative amino acid sequences of 26 MHC class II B exon 2 alleles of the Lesser Kestrel and other *Falco* species. *Dots* indicate identity with the top sequence. Asterisks on top indicate codons comprising the PBR in humans (Brown et al. 1993). *Crosses* indicate PBRs under strong positive selection in Lesser Kestrels (>0.95 posterior probabilities)

Newly designed primers targeting conserved regions at the familial level, which were located at the end of intron 1 and at the beginning of intron 2, cleanly

amplified the entire exon 2 in all species of birds of prey we investigated (Table 2, Fig. 1). These regions were preferentially chosen because of the high G + C content of both introns in the remainder sequence (Fig. 4). In addition, using primers 34F and RapEx3CR, we successfully amplified a long MHC class II fragment (> 1100 bp) in hawks and allies as well as in owls. Long MHC sequences for each species are deposited in GenBank (EF370953–EF370990). Whereas some species yielded a clean band of the expected size, other species required the excision of the band from the gel before cloning. Nonspecific bands exhibited low sizes (< 500 bp), and therefore were unlikely to include the entire region. The length of intron 1 (~1–1.5 kb), as estimated in 1.5% agarose gels, precluded the amplification of this fragment in falcons and Kestrels in a single PCR. For these species we utilized two different PCR reactions, one for the amplification of intron 1 using primers MHC05 and Fal2R, and the other for the amplification of the remaining downstream sequence using primers Fal2FC and RapEx3CR (Fig. 1, Table 2).

MHC Class II Polymorphism and Gene Duplications

The number of alleles per individual ranged from 1 to 5 and the number of potential MHC class II B loci per species ranged from 1 to 3 (Table 1). Analyses of intraspecific polymorphism in 21 Lesser Kestrels revealed 26 exon 2 alleles. (GenBank accession numbers: EF370767–370788; see Fig. 2 and Table 3 for polymorphism statistics). No more than two alleles were found in any Lesser Kestrel we investigated. The double peaks observed in the sequencing chromatograms of uncloned PCR products are congruent with the two cloned alleles obtained in each bird. The specific amplification of one single locus in this species is also supported by an ongoing study in which the segregation of the alleles in Lesser Kestrel families with at least four nestlings adjusts to a single model of biparental inheritance (Alcaide et al. unpublished data). On the other hand, we also found high intraspecific polymorphism in the white-backed vulture and in the cape vulture. Through the genotyping of three individuals from each species, we found 12 and 11

exon 2 alleles, respectively (Fig. 3). The finding of up to four alleles per individual in both species suggests the existence of at least two different MHC class II B loci; this also applies to other birds of prey. All exon 2 alleles differed in at least one nonsynonymous nucleotide substitution. A survey of interspecific polymorphism is shown in Fig 3. No identically shared alleles among any of the species we investigated were found.

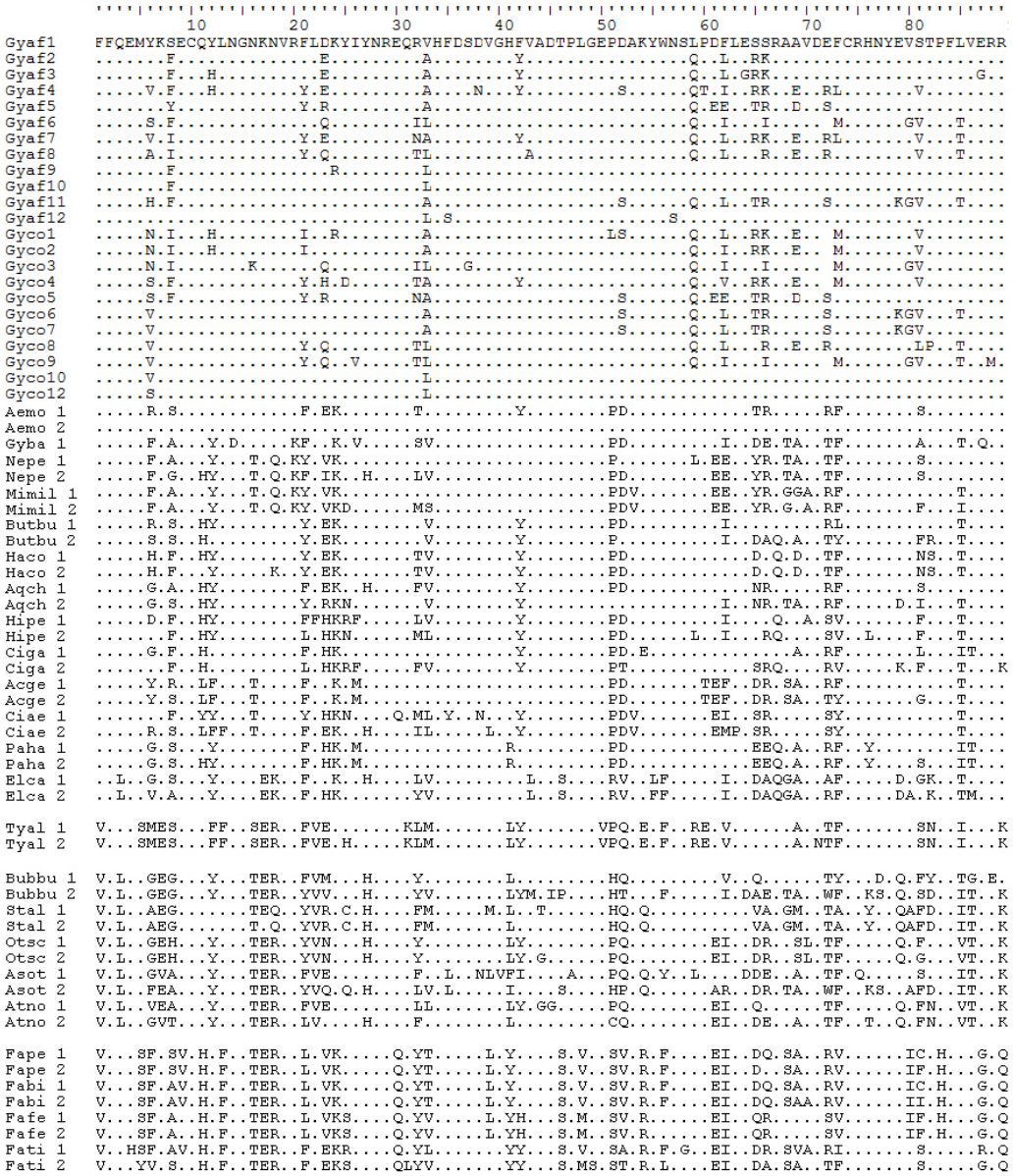


Fig. 3. Complete amino acid sequences of exon 2 in 25 raptor species. Two alleles per species are shown as well as the intraspecific polymorphism found in the white-backed vulture and the cape vulture. *Dots* indicate identity with the top sequence. See table 1 for species codes.

Table 3. Sequence statistics for five MHC Class II DR β exon 2 data sets. References for data sets analysed: Fana DAB, this paper; Game DRB, Ekblom et al. 2003, Pema EB, Richman et al. 2003; Mudo E, Edwards et al. 1997)

Locus	Species	Number of haplotypes	Base pairs sequenced	Base composition A/C G/T	Number of variable sites (S)	Nucleotide Diversity (π)	Waterson's θ per locus (per site)	Tajima's D
Fana DAB	Lesser Kestrel *Falco naumanni*	26	267	21,8 : 27,7 29,6 : 20,7	59	0,088	15,46 (0,26)	2,06 *
Game DRB	Great Snipe *Gallinago media*	20	270	24,8 : 26,3 30,7 : 18,1	33	0,034	9,3 (0,28)	0,16
Pema EB	Deer Mouse *Peromyscus maniculatus*	27	255	25,5 : 22,7 34,1 : 17,6	91	0,11	22,97 (0,25)	0,96
Mudo EB	House Mouse *Mus musculus domesticus*	15	270	23,3 : 25,2 32,9 : 18,5	63	0,084	18,63 (0,29)	0,94

Tests of selection

The LTR statistic comparing M7 and M8 model indicates that M8 fitted the data significantly ($P < 0.001$) better than M7. The estimates from M8 suggested that about 23% of the exon 2 amino acid sites were under strong positive selection in the Lesser Kestrel ($\omega = 8.216$, see Table 4). Bayesian identification of sites under positive selection is listed in Table 4. As it can be noticed in Fig.2, there are slightly differences regarding the human PBR-sites (HLA-DRB1 gene, Brown et al. 1993).

Table 4. Log-likelihood values and parameter estimates of MHC class II exon 2 alleles of the Lesser Kestrel. Lnl is the log-likelihood value, ω is the selection parameter and p_n is the proportion of sites that falls into ω_n site class. Sites inferred to be under positive selection at the 95% (*) and 99% (**) confidence interval level are also indicated.

	lnL	Estimates of parameters	Positively Selected Sites
M7 (beta)	-1321	p = 0.01391	Not allowed
		q = 0.02796	
M8 (beta and ω)	-1275	p_0 = 0.74222	4E** 6F**
		(p_1) = 0.25778	8P* 12Y*
		p = 0.005	23L** 31Q*
		q = 0.01178	33L ** 39L**
		ω = 8.216	48M** 52T**
			53V** 56W**
			62I** 65D**
			66R* 69A**
			73F** 80V**
			81S** 83P**
			87R**

Gene Structure and Evolution of MHC Class II B Genes in Birds of Prey

At about 1.1 kb, the most compact MHC class II B regions spanning exon 1 to exon

3 was found in the barn owl (Tyto alba) and other strigiformes. The length and sequence of introns were generally quite conserved within the same raptor family but not between families (Fig. 4). The length of intron 2 appears to be smaller in birds of prey (250–280 bp) than in passerines (380–950 bp or longer) (Edwards et al. 1998; Hess et al. 2000; Gasper et al. 2001). Nonetheless, the length of intron 1 in passerines (about 440 bp) is similar to the ones detected in the majority of raptors, except for the Falco species, where extremely long introns (1–1.5 kb) were documented (Fig. 1).

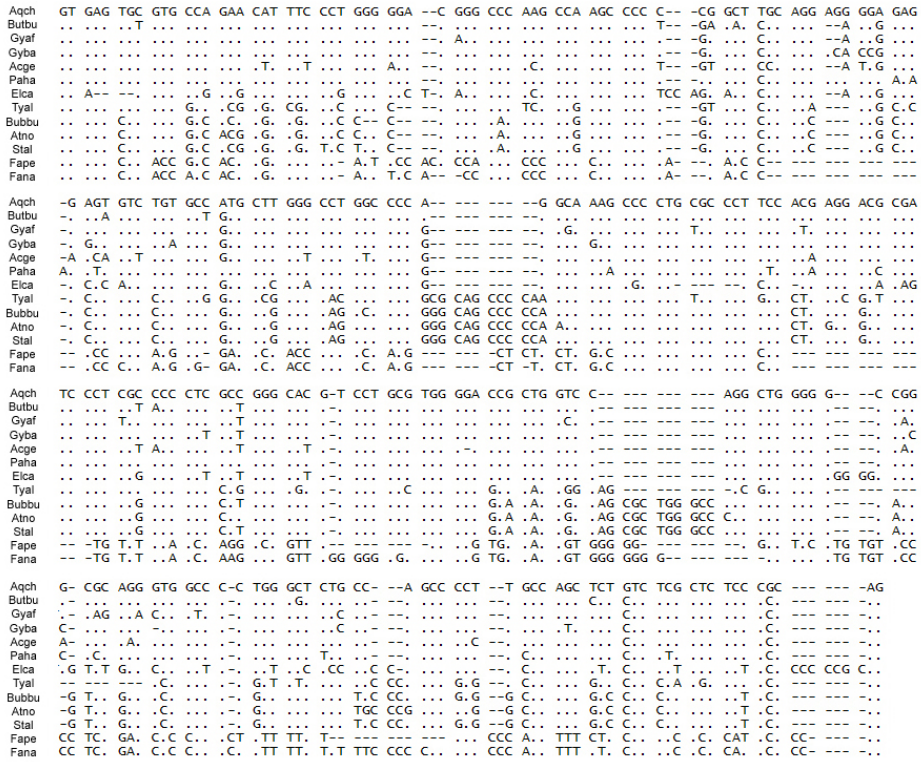


Fig. 4. Alignment of intron 2 sequences isolated in different raptorial genera. See table 1 for species codes.

The network relationships between different avian exon 2 sequences are presented in Fig. 5. This phylogeny shows that the major families of birds of prey are upheld but provides low resolution at the family level. Some sequences that were isolated in different species of the same family appeared to be more similar to each other than to sequences within species. These results are consistent with the transspecies evolution of the polymorphism typically found in MHC class II exon 2

alleles within particular loci (reviewed in Hedrick 2001).

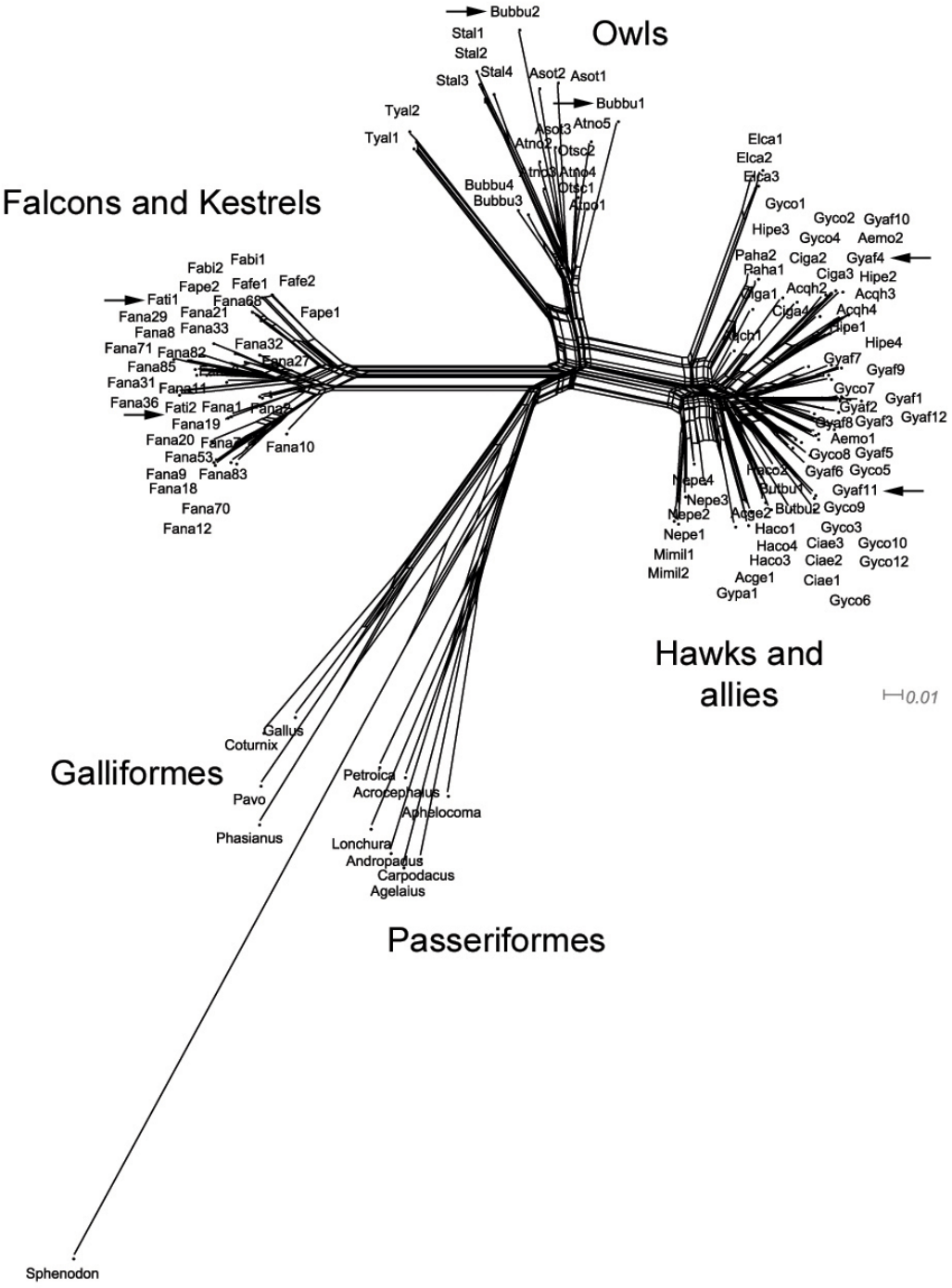


Fig. 5. A Neighbor-net constructed from 102 MHC class II B exon 2 sequences isolated in 37 avian species. The major clusters reflecting different avian groups are indicated. Evidence for transspecies allelism at the familial level is indicated by arrows. See table 1 for species codes.

In contrast, the phylogenetic relationships among intron 2 and exon 3 sequences from different Accipitridae species show that MHC sequences cluster together within species.

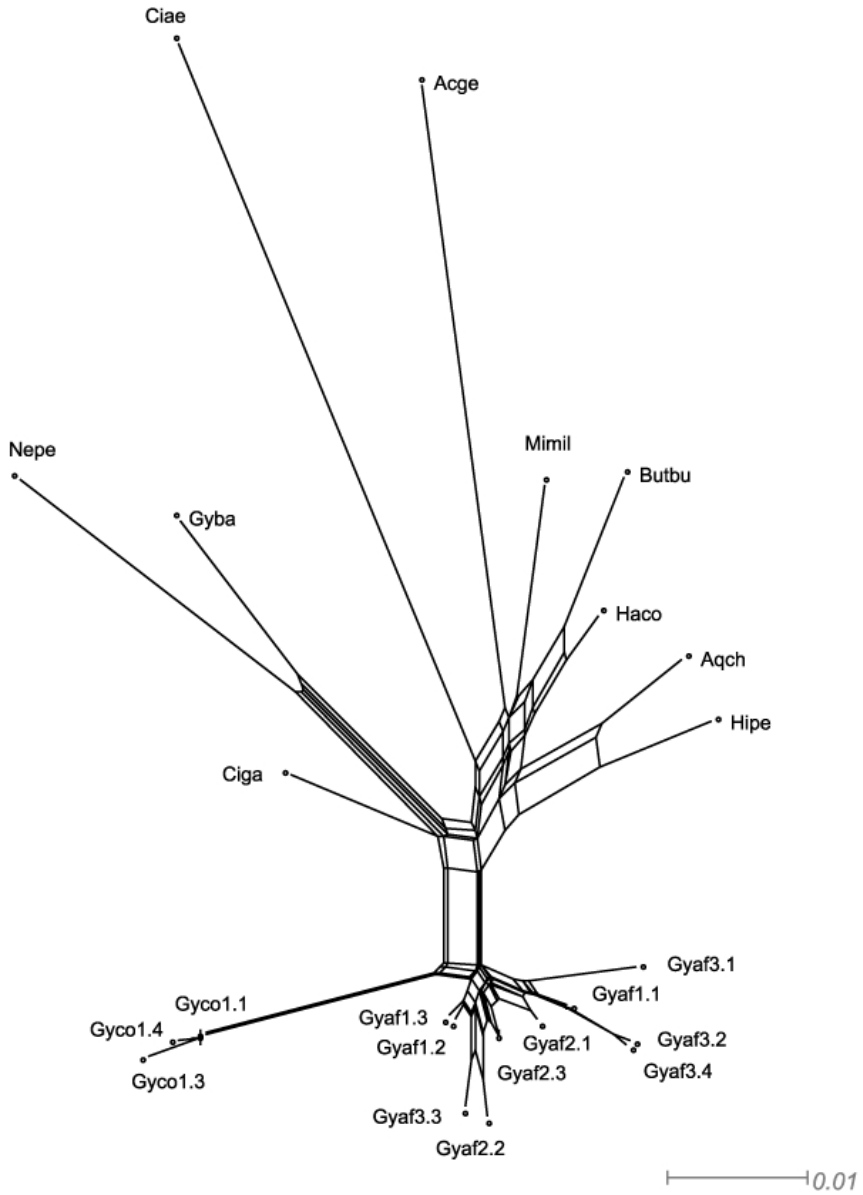


Fig.6. Phylogenetic network among intron 2 and exon 3 sequences from different *Accipitridae* species. Ten sequences from 3 white-backed vultures and 3 sequences from one cape vulture are included. Sequences that were isolated in the same individual are named as Gyaf1.1, 1.2 and further. Notice that there is no evidence of clusters within species reflecting different loci. See table 1 for species codes.

Even though this network includes ten different sequences from three white-backed vultures and three different sequences from one cape vulture, there is no specific clustering of sequences that might suggest the presence of different loci within species or orthologous loci between species (Fig. 6). Overall, the extent of homology in the whole sequences, excluding exon 2, from the same white-backed vulture and the same cape vulture is about 97%. The similarity still remains quite high (about 94%) when comparing these species. Recent gene duplication events or concerted evolution could explain this finding. In this regard, a total of three significant gene conversion events across long stretches of MHC sequences were detected using GENECONV in the white-backed vulture (Table 5).

Table 5. Gene conversion events between class II B sequences from the white-backed vulture, identified using GENECONV. Sequences that were isolated in the same individual are named as Gyaf1.1, 1.2 and further. Sim P = simulated P-values based on 10,000 permutation; Gscale indicates the mismatch penalty; NP is the number of polymorphic sites in the fragment; ND is the number of mismatches within the fragment; TD is the total number of mismatches between two sequences and MM is the penalty per mismatch for these two sequences.

Seq1	Seq2	SimP	Inner Fragments	Gscale	NP	ND	TD	MM
Gyaf2.2	Gyaf2.3	0.0101	Exon1 (62bp) + Intron1 (434bp) Total : 496 bp	0	34	0	35	0
Gyaf2.1	Gyaf3.4	0.0295	Intron 2 (205 bp)	0	22	0	48	0
Gyaf3.1	Gyaf3.3	0.0310	Exon 1 (62bp) + Intron 1 (436 bp) + Exon 2 (76 bp) Total: 574 bp	1	49	3	48	4

DISCUSSION

Genomic Architecture and Polymorphism of Raptor MHC Genes

Based on our PCR survey, the genomic structure of raptor MHC genes resembles non-passerine species in displaying the comparably lower genomic complexity documented in the chicken MHC versus passerines (Zoorob 1990, Ekblom et al. 2003). We have found evidence for a low number of MHC class II loci (1-3 genes) in comparison to passerine species where up to six different loci have been reported (Sato et al. 2000). Whereas we have commonly detected only two alleles per individual in some species such as the goshawk *Accipiter gentilis* or *Falco* species, up to 4 alleles per individual were frequently found in the majority of hawks and allies. Sequence evidence for 3 loci came from the finding of 5 exon 2 alleles in the little owl *Athene noctua* (see Table 1). Nonetheless, our PCR survey is likely to be biased downward since PCR might selectively amplify particular genes in multigene families (Wagner et al. 1994), and Southern blots would help resolve this issue further (Edwards et al. 2000; Westerdahl et al. 1999, Wittzell et al. 1999b). The lack of stop codons or frameshift mutation in any coding region here reported also suggests a low incidence of pseudogenes in the MHC of birds of prey. Pseudogenes have been commonly documented in passerines for both class I and II (Hess et al. 2000, Edwards et al. 2000; Westerdahl et al. 1999) but appear almost absent in other avian groups (see Kauffman et al.1999, Ekblom et al. 2003).

Our surveys of intraspecific polymorphism reveals high genetic diversity at the MHC genes here investigated (see Table 1). In addition, positive selection at several amino acid sites comprising the PBR indicates that balancing selection is operating. Although we have not performed gene expression analyses in this study, research in this topic has found high expression levels at the same loci we have characterized in the barn owl (R. Burri et al., pers. communication), and in general,

studies have observed a correlation between signatures for balancing selection and level of expression (Zoorob 1990).

Concerted Evolution Leads to Sequence Homogenization at Multiple Loci in the MHC of Birds of Prey

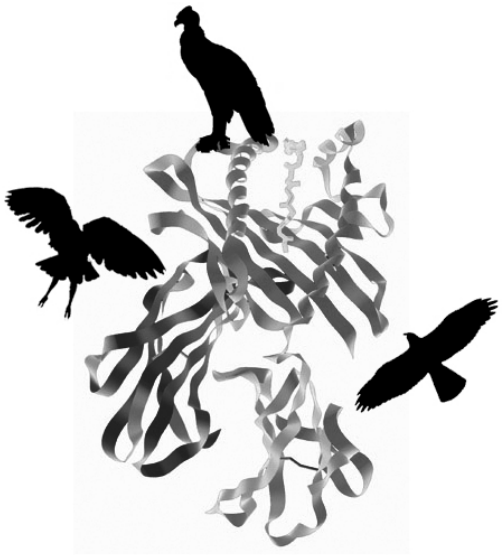
Concerted evolution is the tendency of different genes within multigene families to undergo genetic exchange. Without concerted evolution, genes are expected to evolve independently and therefore differences in the length of introns and the sequence of exons might be expected. The non-mutually exclusive birth-and-death model of molecular evolution typically documented in the mammalian MHC (Nei et al. 1997) allows the recognition of orthologous loci in such distant species as humans and mice (Trowsdale 1995). In contrast, revealing orthologous loci in birds has sometimes required the sequencing of the 3' untranslated (UT) region of the cDNA (Wittzell et al. 1999a; Miller & Lambert 2004a) because high homology among MHC sequences derived from putative different loci has typically been found (Edwards et al. 1995a). Gene conversion events across long regions of avian MHC genes cannot only explain the high diversity levels documented in the second exon of class II B genes but might also be responsible for the sequence homogenization of other parts of the gene within species, such as introns and exon 3 (Martinsohn et al. 1999). Indeed, our GENECONV analyses revealed significant gene conversion events in the white-backed vulture involving stretches of sequences of up to 574 bp (Table 5). Nonetheless, postspeciation duplication is a process that can produce patterns that mimic recent concerted evolution in birds (Edwards et al. 1999). In this regard, our phylogenetic analyses have revealed high homology in intron and exon 3 sequences not only within species but also among species belonging to the same family (Fig. 3). Furthermore, we only found two fixed nucleotide differences out of 238 bp sequenced among 14 different exon 3 sequences from three vulture species. Genetic data therefore suggest that gene duplications have taken place before a relatively recent split of the evolutionary lineages and not after.

CONCLUSIONS

The sequence data from this avian group presented in this paper, should contribute to a better understanding of the evolutionary significance and conservation implications of the MHC. In addition, our results suggest the occurrence of non-mutually exclusive concerted and transspecies evolutionary processes in the raptor MHC, and provide new insights into the structure and diversity of genetic processes in a diverse but phylogenetically problematic avian order. Because of their importance for conservation genetics (Edwards and Potts 1996; Hedrick 2001), our sequences may also aid in the conservation of genetic diversity in this globally threatened clade.

Capítulo 6

MHC Class I genes of birds of prey: isolation, polymorphism and diversifying selection



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ABSTRACT

The threat of emerging infectious diseases encourages the investigation of functional loci related to host resilience, such as those belonging to the major histocompatibility complex (MHC). Through careful primer design targeting to conserved regions of MHC class I sequences in birds, we successfully amplified a genomic fragment spanning exons 2 to 4 in three birds of prey. The identification of a highly conserved region within intron 2 allowed cross-amplifying complete exon 3 sequences in diurnal raptors, owls and New World vultures. We found evidence through PCR and cloning for 1-2 polymorphic class I loci, although this is almost certainly an underestimate. Inferences of diversifying selection in the kestrel MHC revealed that the two major regions of exon 3 exhibiting positive selection mostly agree with those described for the human HLA-A2 molecule. In contrast to passerines, where a high incidence of gene duplications and pseudogenes has been commonly documented, birds of prey emerge as nice model species for the investigation of the evolutionary significance and conservation implications of MHC diversity in vertebrates.

INTRODUCTION

During the last two decades, the major histocompatibility complex (MHC) has been the focus in studies of evolutionary ecology and conservation because of its implication in many relevant biological processes (reviewed by Sommer 2005, Piertney and Oliver 2006, but see Acevedo-Whitehouse and Cunningham 2006). Thus, several studies have emphasized the potential of MHC genes as valuable molecular markers to asses the evolutionary and adaptive potential of endangered populations and species in relation to the menace of changed and emerging diseases (e.g. Yuhki and O'Brien 1990, Hedrick and Parker 1998, Garrigan and Hedrick 2004, Wan et al. 2006, Bollmer et al. 2007). MHC genes encode cell surface glycoproteins that play an essential role in the immune response by presenting short peptides derived from the processing of pathogens and subsequent initiation of an adaptive immune response (e.g. antibody production or destruction of the antigen presenting cell). MHC class I glycoproteins are heterodimers expressed in all nucleated cells and are related to immune defence against intracellular pathogens such as virus and some protozoa. Critically for studies of MHC variation in non-model species, single class I α genes posses two polymorphic exons (α_1 and α_2) encoding the polymorphic peptide binding region (PBR) (Bjorkman et al. 1987).

As far as we know, MHC class I genes have only been investigated in detail in a handful of avian species, namely the chicken *Gallus gallus* (Kauffman et al. 1999), quail *Coturnix japonica* (Shiina et al. 1995), great reed warbler *Acrocephalus arundinaceus* (Westerdahl et al. 1999), Seychelles warbler *Acrocephalus sechellensis* (Richardson and Westerdahl 2003), Florida sandhill crane *Grus canadensis* (Jarvi et al. 1999), domestic goose *Anser anser* (Xia et al. 2005) and the mallard *Anas platyrhynchos* (Moon et al. 2005). Importantly, nearly all the above studies investigating class I diversity in non-model avian species have done so at the cDNA level. Thus, our knowledge of class I intron

sequences in birds is limited. In the present study, we aimed at developing molecular methods for the isolation of genomic MHC class I sequences in birds of prey, focusing on the α_2 domain encoded by exon 3.

MATERIALS AND METHODS

Amplification, sequencing and alignment of MHC class I fragments

Amplification strategies relying on the polymerase chain reaction (PCR) were performed on genomic DNA, extracted following the protocol described by Gemmell and Akiyama (1996), using a PTC-100 Programmable Thermal Controller (MJ Research Inc.). We designed degenerate primers (MHCI-ex2F: CGCTACAACCAGASCRRSG and MHCI-ex4R: GGGTAGAAGCCGTGAGCRC, see Fig. 1) across conserved regions of exon 2 and exon 4 emerging from an alignment of mRNA sequences of a few bird species deposited in GenBank (species names and GenBank accession numbers: Chicken *Gallus gallus* L28958, Domestic goose *Anser anser* AM114924, Duck *Anas platyrhynchos* AB115246 and Sandhill Crane *Grus canadensis* AF033106). Our aim at this stage was obtaining intronic sequences flanking exon 3 in order to design specific primers for the amplification of this polymorphic region in raptors. After sequencing the target MHC fragment in at least three raptor species, we created an alignment including genomic class I sequences of the chicken AM279340, domestic goose AY387655 and duck AY854375.

The PCR profile consisted of 4 min at 94ºC following 35 cycles of 40s at 94ºC, 40s at 56ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 25 µl reaction contained 0.2 units of Taq polymerase (Bioline), 1x kit-supplied PCR buffer, 1.5 mM $MgCl_2$, 0.02% gelatine, 5% DMSO, 0.12 mM of each dNTP, 10 pmol of each primer and, approximately, 25 ng of genomic DNA. Sequencing reactions were carried out using the Big Dye 1.1 Terminator technology and labelled fragments

were resolved in a 3100 automated sequencer (Applied Biosystems). DNA sequences were aligned and edited using the software BioEdit (Hall 1999). Primer design was tested using Oligo 6.0 (Molecular Biology Insights).

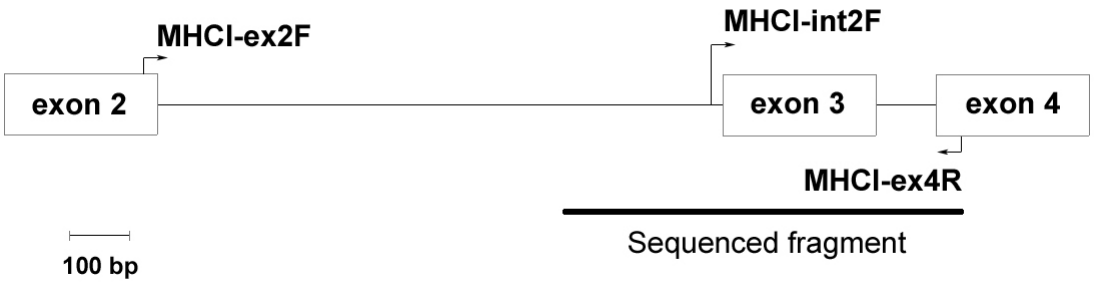


Fig 1. Schematic illustration of part of an MHC class I gene of hawks and allies. The position of the primers used in this study is indicated by arrows.

Molecular cloning and sequencing analysis

Investigation of variation at MHC loci requires separating the different PCR amplification products, either because of the possibility of amplifying more than one locus, or because individuals are likely to be heterozygous for these loci. Cloning and sequencing protocols followed those described for raptor MHC class II genes (see Alcaide et al. 2007 and Alcaide et al. 2008 for details). Polymorphism statistics within species were generated using the software DNAsp (Rozas et al. 2003). Putative amino acid sequences were obtained after alignment to the chicken BF1 gene (Shaw et al. 2007). The phylogenetic relationships of class I sequences were visualized through Neighbour Net networks built in the software Splitstree 4 (Huson & Bryant 2006).

Inference of positive selection in the presence of recombination

Genetic hallmarks of positive selection at functionally important amino acid sites are identified from an excess of non-synonymous substitutions (d_N) over synonymous substitutions (d_S), where $\omega = d_N/d_S > 1$. The use of phylogenetic

methods, such as those implemented in the PALM package (Yang 200), are believed to cause high numbers of false positives when high levels of recombination are operating (Anisimova et al. 2003). The recently developed software OmegaMap (Wilson & McVean 2006) permits to infer positive selection in the presence of recombination. Previous analyses of positive selection at MHC class II sequences in lesser kestrels have already demonstrated that Omegap is less prone to overestimate the number of amino acid sites experiencing positive selection. Thus, we followed the same analytical protocol used for the kestrel class II data set to investigate diversifying selection at MHC class I sequences (see Alcaide et al. 2008 for details)

Table 1. Birds of prey in which exon 3 sequences from MHC class I loci have been isolated. The taxonomy follows Brooke and Birkhead (1991).The number of different exon 3 sequences isolated and the number of individuals analysed per species are indicated. The codes here proposed will be employed for the naming of MHC sequences following the nomenclature recommended by Klein et al. (1990).

Species Family	No. of clones analysed per individual	Exon 3 Seqs (No of individuals)	GenBank Acc. No	Species code	Country of Origin
Eurasian Kestrel *Falco tinnunculus* *Falconidae*	6	23 (25)	EU120698-722	Fati	Spain
Lesser Kestrel *Falco naumanni* *Falconidae*	6	18 (25)	EU120664-79	Fana	Spain
Black-shouldered Kite *Elanus caeruleus* *Accipitridae*	8	4 (1)	EU120680-83	Elca	Spain
Spanish Imperial Eagle *Aquila adalberti* *Accipitridae*	8	9 (8)	EU120684-87	Aqad	Spain
Eurasian Black Vulture *Aegypius monachus* *Accipitridae*	8	4 (1)	EU120688-91	Aemo	Spain

Andean Condor *Vultur gryphus Cathartidae*	8	6 (8)	EU120692-94	Vugr	Argentina
Eagle Owl *Bubo bubo Strigidae*	1	1 (1)	EU120697	Bubub	Spain

RESULTS

Amplification of MHC class I fragments and complete exon 3 sequences

We successfully sequenced part of a genomic MHC class I fragment spanning exons 2 to 4 (~1.4 kb as estimated in 1.5 % agarose gels) in one Spanish Imperial Eagle *Aquila adalberti* (EU120724), one Bearded Vulture *Gypaetus barbatus* (EU120725) and one White-backed Vulture *Gyps africanus* (EU120723). The length of intron 2 and intron 3 was estimated at about 1 kb and 75 bp, respectively (see Fig. 1). The length of intron 2 appears much longer in birds of prey than in the chicken BF1 and BF2 genes (228 bp, Shaw et al. 2007). However, the alignment with genomic class I sequences of the chicken, duck and goose (see above) revealed that part of the intron 2 sequence flanking exon 3 is quite well conserved across different avian orders. Nevertheless, intron 3 sequences were quite divergent and exhibited an extremely high GC content (> 70%), precluding optimal primer design and walking. We therefore designed a new degenerate primer (MHCI-int2F: CATTTCCCTYGTGTTTCAGG) sitting in the conserved flanking region of intron 2, which we used in conjunction with the reverse primer MHCI-ex4R (see above). We predicted this approach would be successful given the small size of intron 3 (Fig. 1). Subsequently, we amplified a fragment of about 450 bp in all the species summarized in Table 1 using primers MHCI-int2F and MHCI-ex4R, following the same PCR protocol described above

MHC class I polymorphism and gene duplications

The number of MHC sequences isolated per individual ranged from 1 to 4 as measured by cloning and sequencing of PCR amplicons. Consequently, the

number of putative MHC class I loci amplified per species was estimated to range from 1 to 2. Genetic variation at several species of raptors is summarized in Table 1. The amino acid sequences of 23 class I alleles isolated from the Eurasian kestrel *Falco tinnunculus* are shown in Figure 2.

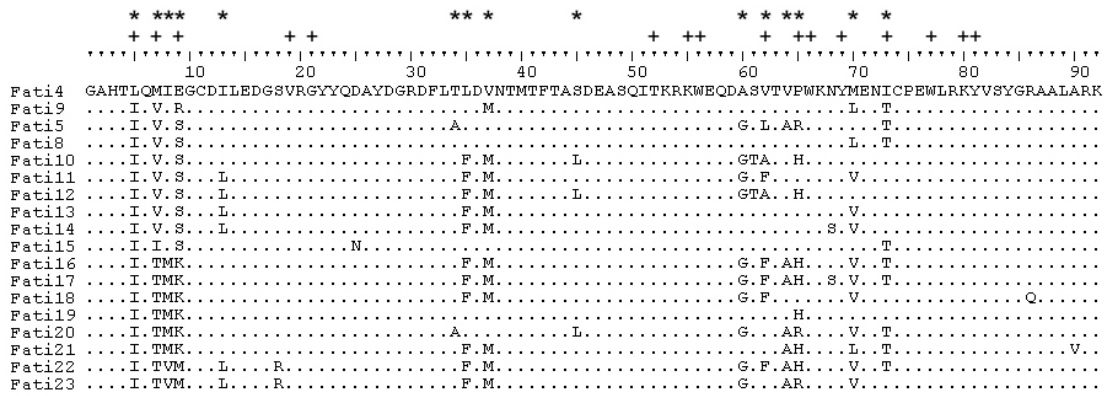


Fig 2. Alignment of putative amino acid sequences of 18 class I exon 3 alleles of the Eurasian Kestrel. *Dots* indicate identity with the top sequence. *Asterisks* on top indicate PBRs under strong positive selection in Eurasian Kestrels revealed by PAML (>0.95 posterior probabilities). Crosses indicate amino acid residues that are known to interact with antigens in the PBR of the human HLA-A2 molecule (Bjorkman et al. 1987).

None of the DNA sequences reported here showed any signs of nonfunctionality, such as stop codons or frameshift mutations. On the other hand, the phylogenetic network constructed from the genomic class I sequences of birds of prey failed to identify any kind of orthologous relationships among putative class I loci, regardless whether we analyzed exon 3, the flanking sequence composed of intron 3 and part of exon 4, or the entire sequence spanning exons 3 to 4 (see Fig. 3).

Test of selection

The mean value per codon of the selection parameter across the entire exon 3 was set at $\omega = 3.82$. The mean amount of population recombination per codon ($\rho = 0.44$) showed to greatly exceed the mean amount of population mutation ($\theta = 0.010$). Thus, this evidence for a predominant role of intragenic recombination and/or gene conversion during the evolutionary history of exon 3 suggests that

inferring selection at this locus without also assuming recombination may be inappropriate.

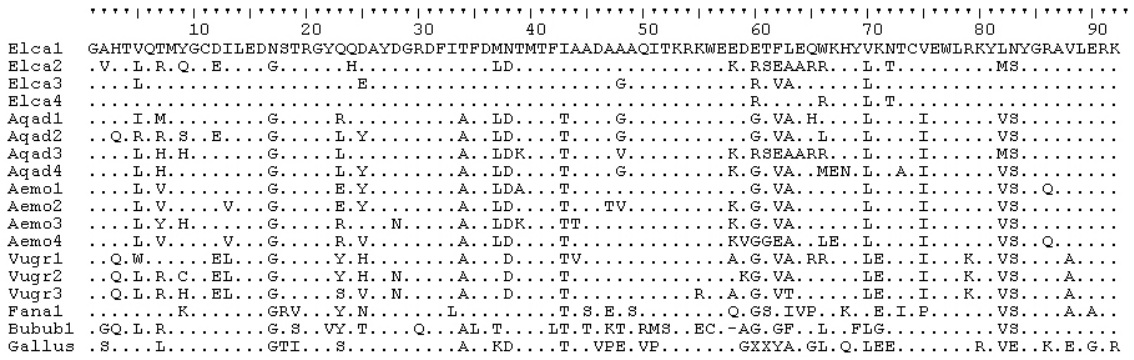


Fig 3. Complete amino acid sequences of class I exon 3 alleles in 6 raptor species and the chicken Gallus gallus. Dots indicate identity with the top sequence. See table 1 for species code

DISCUSSION

To our knowledge, this is the first study isolating and reporting polymorphism patterns in classical MHC class I genes of birds of prey and one of the very few studies of class I gene structure in non-model avian species. Based on our PCR approach, the genomic architecture and complexity of raptor MHC class I genes support previous data collected for class II genes (see Alcaide et al. 2007). Hence, the MHC of birds of prey seems closer to the relatively simple and compact MHC of the chicken (Kauffman et al. 1999) than to that of passerines. In this respect, we have found evidence for a low number of class I loci (1-2) in comparison to passerine species where genetic evidence for up to seven class I loci has been reported within the same individual (e.g. Witzell et al. 1999b, Westerdahl et al. 2004a). However, our estimate is likely biased downward since PCR approaches might selectively amplify particular genes in multigene families (Wagner et al. 1994), and Southern blots would help resolve this issue further (Westerdahl et al. 1999, Wittzell et al. 1999, Edwards et al. 2000). On the other hand, the lack of stop codons or frameshift mutation in any coding region suggests a low incidence of pseudogenes. Pseudogenes, as indicated by stop codons or disrupted open reading frames, have been commonly documented in

passerines for both class I and II (Westerdahl et al. 1999, Hess et al. 2000, Edwards et al. 2000) but appear almost absent in other avian groups (e.g. Kauffman et al.1999, Ekblom et al. 2003, Alcaide et al. 2007).

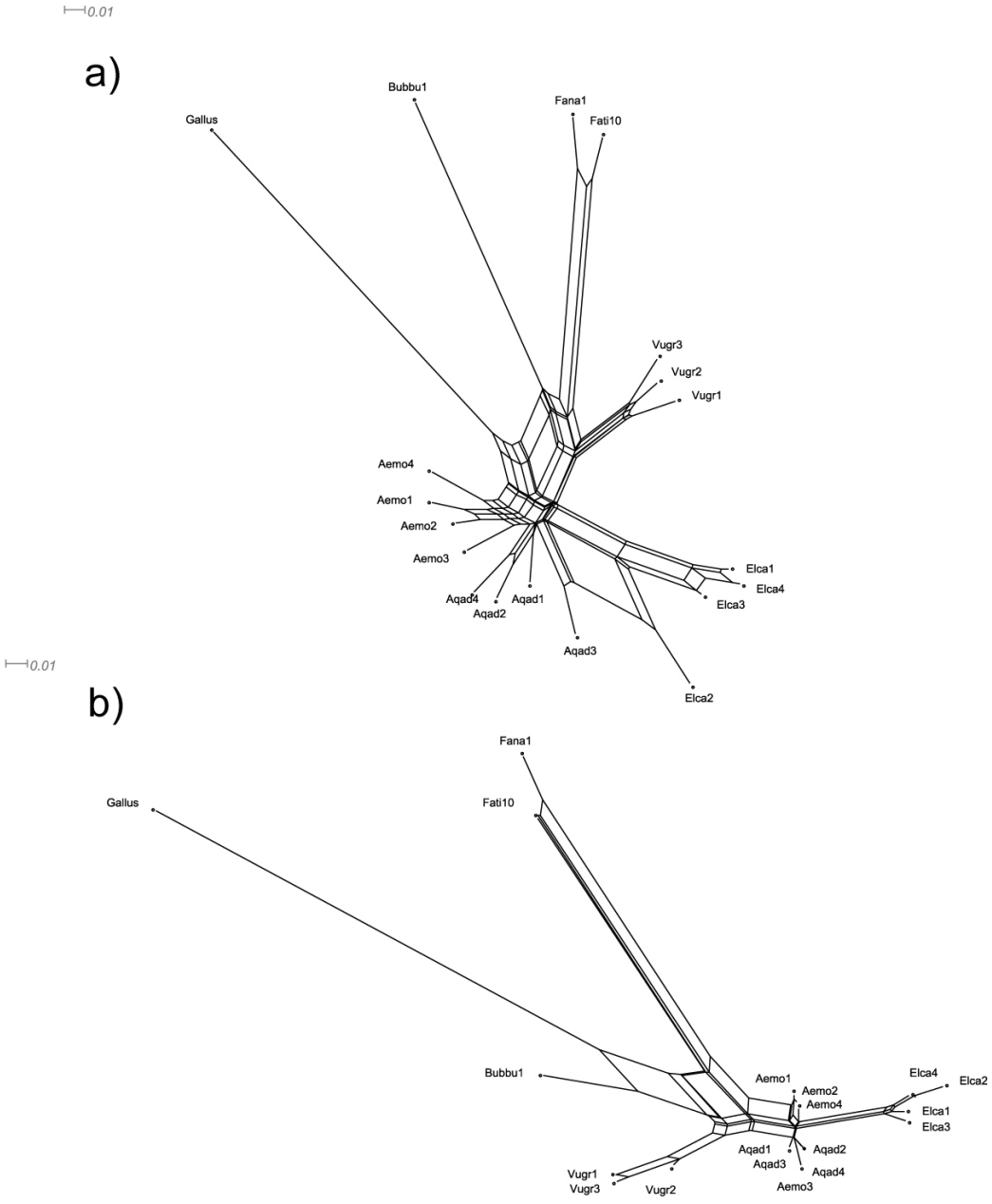


Fig 4. A Neighbor-net constructed from genomic class I sequences of the entire exon 3 (a) and intron 3 plus part of exon 4 (b) that were isolated in different species of birds of prey. The same sequence from the chicken *Gallus gallus* is used as outgroup.

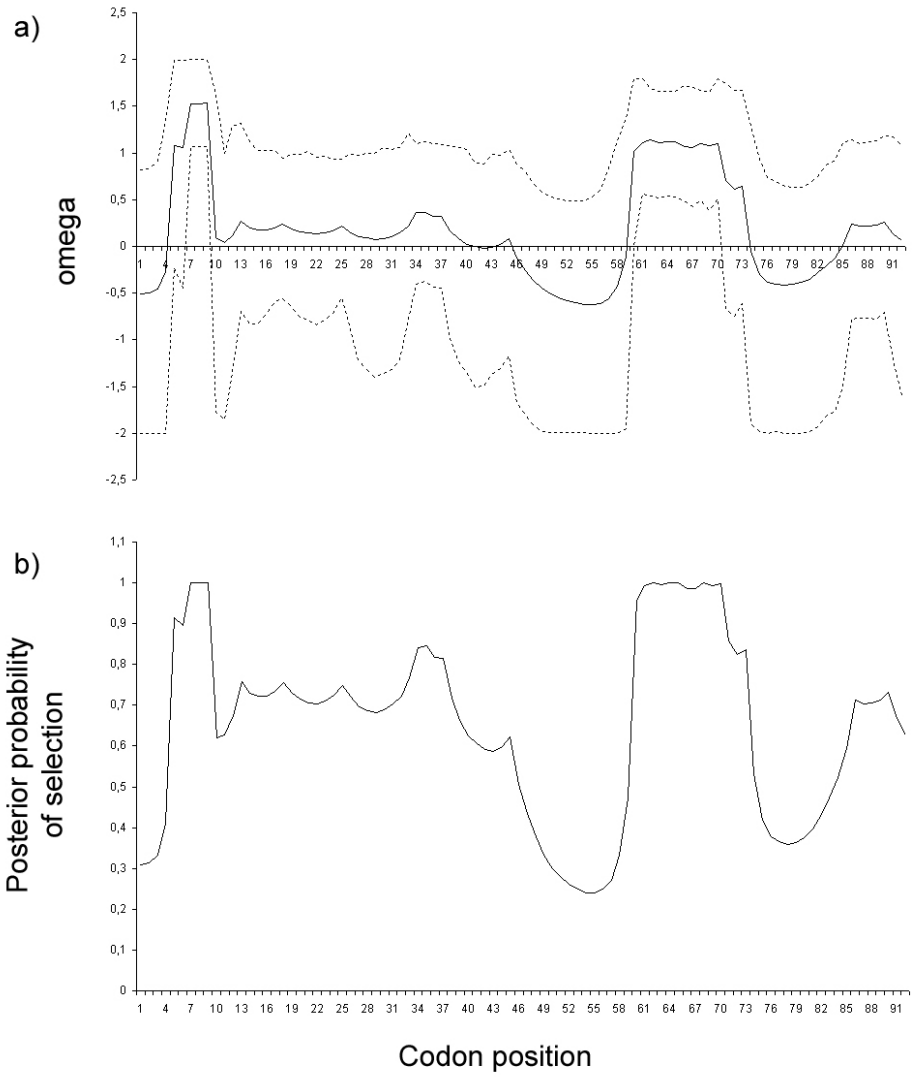


Fig 5. a) Spatial variation in the logarithm of the selection parameter ω across the third exon of a classical MHC class I gene of the Eurasian Kestrel. Parameter estimates were carried out in the software package OmegaMap using an objective set of prior distributions (Wilson and McVean 2006). The sitewise mean (solid line) and 95% HPD intervals (dotted lines) are shown. **b)** Spatial variation in the posterior probability of positive selection.

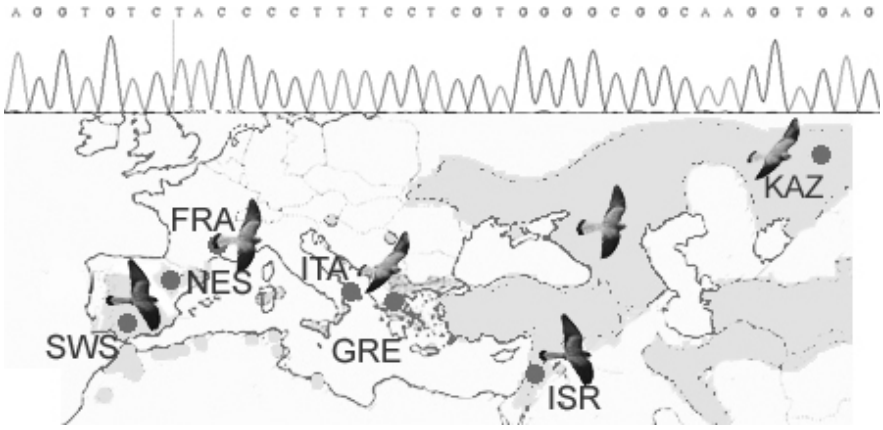
Our survey of MHC polymorphism has revealed high genetic diversity in several raptor species. Ongoing research in other birds of prey such as the Egyptian vulture *Neophron percnopterus* is currently reporting more than 10 class I alleles (Agudo and Alcaide, unpublished data). Positive selection at the same amino acid sites that are known to comprise the PBR of the human HLA-A2 molecule (Bjorkman et al. 1987) indicates that balancing selection is operating. Although we have not performed gene expression analyses in this study, other

studies have generally observed a correlation between signatures for balancing selection and level of expression of MHC genes (Zoorob 1990, Jacob et al. 2000). Finally, the lack of orthologous relationships among putative different class I loci support the concerted evolution hypothesis proposed for the avian MHC (see also Edwards et al. 1995a, Witzell et al. 1999a, Alcaide et al. 2007), or possibly post-speciation gene duplication (Edwards et al. 1999). This latter possibility should be considered, since our taxon sampling was low, with large periods of time between speciation events in the tree.

In conclusion, the molecular methods and sequence data collected in this paper should contribute to a better understanding of the evolutionary significance and conservation implications of the MHC in birds of prey. Moreover, since the primers designed for this study are targeting highly conserved regions across class I genes, similar fragments in other avian groups are likely to be cross-amplified successfully. Given that MHC genes may decisively determine pathogen and parasite resistance (Edwards and Potts 1996; Hedrick 2001), this study may also aid in the preservation of genetic diversity in raptors.

Capítulo 7

Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the Lesser Kestrel *(Falco naumanni)*



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ABSTRACT

Understanding the selective forces that shape genetic variation in natural populations remains a high priority in evolutionary biology. Genes at the major histocompatibility complex (MHC) have become excellent models for the investigation of adaptive variation and natural selection because of their crucial role in fighting off pathogens. Here we present one of the first data sets examining patterns of MHC variation in wild populations of a bird of prey, the lesser kestrel, *Falco naumanni*. We report extensive polymorphism at the second exon of a putatively functional MHC class II gene, *Fana*-DAB*1. Overall, 103 alleles were isolated from 121 individuals sampled from Spain to Kazakhstan. Bayesian inference of diversifying selection suggests that several amino acid sites may have experienced strong positive selection (ω= 4.02 per codon). The analysis also suggests a prominent role of recombination in generating and maintaining MHC diversity (ρ=4Nc = 0.389 per codon, θ= 0.017 per codon). Both the Fana-DAB*1 locus and a set of eight polymorphic microsatellite markers revealed an isolation-by-distance pattern across the Western Palaearctic (r = 0.67; P = 0.01 and r = 0.50; P = 0.04, respectively). Nonetheless, geographical variation at the MHC contrasts with relatively uniform distributions in the frequencies of microsatellite alleles. In addition, we found lower fixation rates in the MHC than those predicted by genetic drift after controlling for neutral mitochondrial sequences. Our results therefore underscore the role of balancing selection as well as spatial variations in parasite-mediated selection regimes in shaping MHC diversity when gene flow is limited.

INTRODUCTION

Genetic diversity is widely considered essential for the evolutionary and adaptive potential of populations and species. Many studies have therefore aimed at providing insights into genome-wide diversity using a relatively short array of neutral loci (reviewed by Coltman and Slate 2003, DeWoody and DeWoody 2005). Although variation at supposedly neutral DNA markers such as microsatellites or mtDNA has great potential for inferring population connectivity and relatedness (e.g. Paetkau et al. 1995, Martínez-Cruz et al. 2004, Godoy et al. 2004, Fredsted et al. 2005, Alcaide et al. 2005), their suitability for detecting adaptive variation and as surrogates for genetic variation in fitness-related loci is limited (e.g. Crandall et al. 2000, Aguilar et al. 2004, Jarvi et al. 2004). Furthermore, local adaptation often requires restricted gene flow, and thus, investigating variation at genes under selection may be useful for unravelling population subdivision as well (e.g. Miller et al. 1997, Miller & Whitler 1997, Miller et al. 2001). The major histocompatibility complex (MHC) has become an excellent model for the investigation of adaptive variation in vertebrates (see recent reviews by Sommer 2005, Piertney and Oliver 2006). The MHC is a multigene family involved in the development of adaptive immune responses against pathogens (Klein 1986). MHC genes encode cell-surface glycoproteins that bind and present short peptides (i.e. antigens) to specialized cells of the immune system in order to trigger appropriate immune reactions including antibody production or destruction of antigen-presenting cells. Genetic variation at MHC genes largely determines what foreign peptides an individual is capable of responding to, and thus, is thought to influence individual fitness and long-term survival of populations (Hughes 1991, Hughes & Nei 1992).

Several evolutionary mechanisms have been suggested to generate and maintain extraordinary levels of polymorphism at the MHC (e.g. Robinson et al. 2000). Thus, some studies have documented a major role of intragenic

recombination and gene conversion against de novo point mutations (e.g. Richman et al. 2003). On the other hand, two main types of balancing selection, 'heterozygote advantage' and 'frequency-dependent selection', are also thought to be important in maintaining the high levels of MHC variability needed to counteract selection pressures imposed by pathogens (Hedrick 1999; Bernatchez & Landry 2003). Additionally, other nonmutually exclusive modes of selection have dealt with spatial and/or temporal variations in parasite selection regimes (Hill 1991), MHC-dependent mate choice (Penn & Potts 1999) and maternal–foetal interactions (Clarke & Kirby 1966; Edwards & Hedrick 1998).

The usually highly polymorphic second exon of MHC class II B genes has been widely studied in vertebrates because this locus encodes the functionally important peptide-binding region (PBR) involved in the immune response against bacteria and parasites (e.g. Musolf et al. 2004; Bos & DeWoody 2005; Miller et al. 2005; Wegner et al. 2006). In birds, most studies of the MHC have focused mainly on galliform species or passerines (Edwards et al. 1998; Witzell et al. 1999; Ye et al. 1999; Bonneaud et al. 2004a; Jarvi et al. 2004), with few examples of other avian groups (Bollmer et al. 2007; Ekblom et al. 2007). In this respect, the isolation of avian MHC genes has been traditionally assumed to be laborious and time-consuming because of the substantial variation in gene organization even between closely related species. Furthermore, many species possess multiple MHC loci (e.g. Westerdahl et al. 2004a), and concerted evolution among paralogous genes (e.g. Edwards et al. 1995a; Witzell et al. 1999) has challenged the construction of locus-specific primers. However, a recent and extensive characterization of MHC class II B genes in birds of prey showed that the structure of genes from species belonging to the same raptor family is quite well conserved (Alcaide et al. 2007). Although concerted evolution precluded the design of locus-specific primers in several species (see Alcaide et al. 2007), the number of gene copies was generally low (1–3). At first sight, the complexity and

diversity of MHC genes of birds of prey would resemble that of the widely studied chicken MHC, which appears to be small and compact, containing only enough expressed genes to ensure resistance against common pathogens (i.e. 'minimal essential MHC hypothesis', Kaufman & Salomonsen 1997). However, the use of polymerase chain reaction (PCR) approaches in this previous study cannot dismiss an underestimation of the number of gene copies in a multigene family (e.g. Wagner et al. 1994). Among the raptor species investigated, those belonging to the genus *Falco* provided the best chance for investigating patterns of MHC variation at single polymorphic and positively selected MHC loci.

The lesser kestrel *Falco naumanni* is one of the most widely studied bird species. During the last century, habitat transformations led to the extinction of the species from several locations of its breeding range in Eurasia, practically disappearing in others (Biber 1990). Changes in land use and agricultural practices have been implicated as the main causes of population decline (Tella et al. 1998) of this habitat-specialist falcon inhabiting steppe and pseudosteppe ecosystems (Cramp & Simmons 1980; Ferguson-Lees & Christie 2001). As a result, the breeding range of the philopatric lesser kestrel became more patchy. Genetic divergence among fragments may be thus expected to follow an isolation-by-distance pattern that would be in agreement with strong philopatry and restricted dispersal over short distances (see Negro et al. 1997; Serrano et al. 2001; Serrano & Tella 2003). Such restrictions in gene flow might predict an increase in the chance for local adaptations that could be reflected in functionally important genes such as those belonging to the MHC. On the other hand, the smaller population sizes brought on by population decline could thwart the effects of selection, leaving a more random pattern.

Our aim in this study, one of the first examining MHC diversity in wild populations of a bird of prey, was of comparing patterns of variation at MHC loci

and supposedly neutral markers (microsatellites and mtDNA) in order to investigate the extent of local adaptations at evolutionary relevant genes when gene flow is limited. Our study is geographically broad, including several populations across Eurasia, from Spain to Kazakhstan. As far as we know, geographical variation in MHC genes has only been studied previously in a few bird species: the great snipe (*Gallinago media*, Ekblom et al. 2007), the red grouse (*Lagopus lagopus scoticus*, Piertney 2003), South Island robin (*Petroica australis australis*, Miller & Lambert 2004a) and the little greenbul (*Andropadus virens*, Aguilar et al. 2006). Besides providing valuable data concerning diversity at functionally important genes and conclusion on the relevance of the MHC, this study also assesses the suitability of MHC genes as potential genetic markers to establish the origin of vagrant or captive individuals, undoubtedly, one of the most exciting scopes in molecular ecology.

MATERIALS AND METHODS

Study Species and Populations

The lesser kestrel (Aves: Falconidae) is a small migratory falcon whose breeding range covers mid-latitude and low altitudes of Eurasia. Here, this facultatively colonial falcon can be found in human structures holding up to 100 breeding pairs when they are surrounded by agricultural land. Lesser kestrels are known to winter in the savannah and grass plains of Africa, with the most numerous aggregations recorded in the southern region of the continent (Cramp & Simmons 1980; Ferguson-Lees & Christie 2001).

We investigated geographically distinct breeding populations of lesser kestrels across Eurasia: southwestern Spain (SWS), central-western Spain (CWS), northeastern Spain (NES), France (FRA), Italy (ITA), Greece (GRE), Israel (ISR) and Kazakhstan (KAZ) (see Table 1, Fig. 1). Only one individual per nest

was analysed, and therefore, individuals were presumably unrelated. Number of individuals analysed per population is shown in Table 1. Birds were caught during the 2002 and 2003 breeding season, and thus, our estimates are unlikely to be influenced by temporal variation in selection patterns. Blood samples from Iberian birds were preserved in absolute ethanol, and feathers pulled from the birds' back were stored in paper envelopes or plastic bags and kept at 4 °C when sampling individuals from the remainder breeding distribution. The DNA purification protocol we used follows that described by Gemmell & Akiyama (1996). Blood and feather tips were digested by incubation with proteinase K for at least 3 h. DNA purification was carried out by using 5M LiCl, organic extraction with chloroform-isoamylic alcohol (24:1) and DNA precipitation with absolute ethanol. Pellets obtained were dried and washed twice with 70% ethanol, and later stored at –20 °C in 0.1 mL of TE buffer.

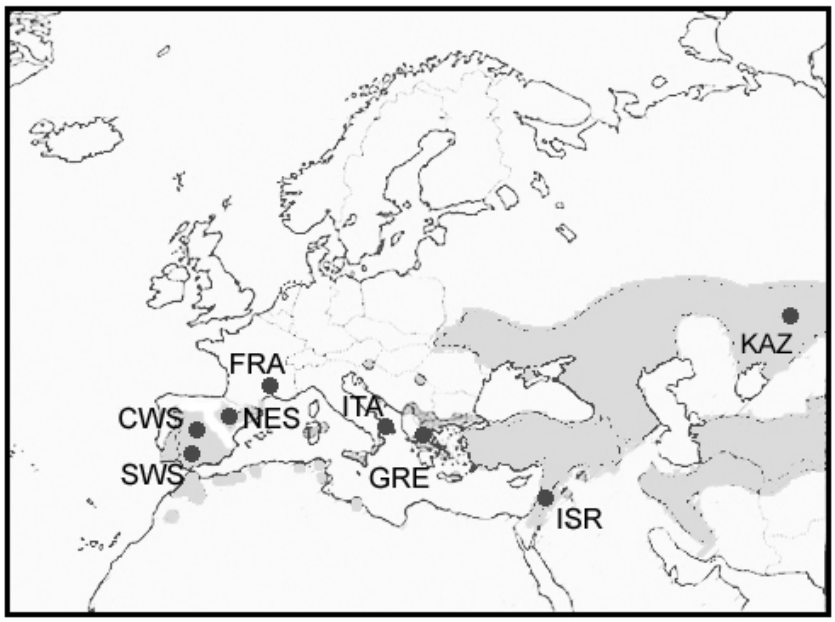


Fig.1. Breeding distribution of the Lesser Kestrel in the Western Palearctic (grey areas). Populations investigated in this study are indicated by black dots.

Microsatellite genotyping

We amplified nine microsatellites that were isolated originally in the peregrine

falcon *Falco peregrinus* (Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89, Fp107 CL58 and Cl347; Nesje *et al*. 2000; see Appendix). For each locus, PCR was carried out in a PTC-100 Programmable Thermal Controller (MJ Research) using the following PCR profile: 35 cycles of 40 s at 94 °C, 40 s at 55 °C, 40 s at 72 °C and finally, 4 min at 72 °C. Each 11 μL reaction contained 0.2 U of *Taq* polymerase (Bioline), 1× PCR manufacturer-supplied buffer, 1.5 mm $MgCl_2$, 0.02% gelatine, 0.12 mm of each dNTP, 5 pm of each primer and, approximately, 10 ng of genomic DNA. Forward primers were 5'-end labelled with HEX, TET or 6-FAM. Amplified fragments were resolved on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Conformity to Hardy–Weinberg expectations and linkage disequilibrium was analysed using genepop (Raymond & Rousset 1995).

Table 1. Sampled populations and number of birds typed at microsatellites (μsats), mtDNA control region sequences (CR) and *Fana*-DAB1* (MHC). The most abundant MHC alleles at each location are indicated. MHC alleles were named following the nomenclature recommended by Klein et al. (1990).

Population (Code)	Number of birds typed μsats/CR/MHC	Year of sampling	Number of MHC alleles	Most frequent MHC alleles
SW-Spain (SWS)	69/8/25	2002	33	Fana2 (20%) Fana19 (12%)
CW-Spain (CWS)	76/0/0	2002	-	-
NE-Spain (NES)	68/8/25	2002	28	Fana2 (16%) Fana19 (16%)
France (FRA)	26/0/16	2002	18	Fana2 (15%) Fana1 (12.5%)
Italy (ITA)	26/8/16	2003	18	Fana2 (29%) Fana1 (12.5%) Fana3 (12.5%)
Greece (GRE)	21/8/16	2003	22	Fana2 (12.5%) Fana1 (9.3%)

				Fana29 (9.3%)
Israel (ISR)	34/16/17	2003	20	Fana36 (15%)
				Fana42 (11%)
Kazakhstan (KAZ)	7/0/7	2003	12	Fana77 (14%)

Mitochondrial DNA sequencing

We amplified a 262-bp fragment (excluding primers) of the mitochondrial control region (CR) that has previously been shown to contain a high number of polymorphic sites in the *Falco* species (e.g. Nittinger et al. 2007). The selected fragment extends from the central part of the CR to the repetitive section adjacent to the trRNA (positions 15 814–16 014 in F. peregrinus, Accession no. AF090338). Overall, mtDNA samples from 16 birds hatched in Spain, 16 birds from Italy and Greece as well as 16 birds from Israel were sequenced (Table 1). Previous analyses utilizing cytochrome b sequences documented a strong pattern of genetic differentiation between Asian and Mediterranean populations of lesser kestrels (Wink et al. 2004). Thus, we believe it unnecessary to amplify control regions sequences from the birds sampled in Kazakhstan. The PCR in these experiments was carried out using primers CRFalF1: 5′-GCTTCACAGGTGACCCTTC-3′ and CRFalR1: 5′-GAT-GTGAATTTTGGCGGG-3′. The PCR profile consisted of 35 cycles of 40 s at 94 °C, 40 s at 52 °C, 40 s at 72 °C and finally, 4min at 72 °C. Each 20 μL reaction contained 0.2 U of Taq polymerase (Bioline), 1× PCR manufacturer-supplied buffer, 1.5 mm MgCl2, 0.02% gelatine, 0.12 mm of each dNTP and 5pm of each primer. Since the yield of PCR products was generally low when amplifying mitochondrial sequences from feather tips, we performed post re-amplifications to increase the concentration of the PCR template to be sequenced as well as negative controls in the PCR experiments in order to detect contaminations. Sequencing reactions were carried out using the BigDye 1.1 Terminator technology, and labelled fragments were subsequently resolved in a 3100 automated sequencer (Applied Bio-systems).

The co-amplification of nuclear copies of mitochondrial sequences (numts) was detected through the analysis of sequencing chromatograms. Some individuals showing one or two double peaks were discarded and substituted by new individuals until we reached the sample sizes given above. Since avian erythrocytes are enriched for nuclear DNA and depleted for mitochondrial DNA, the co-amplification of putative numts was more frequently found in DNA extracted from blood samples (four cases out of 20 individuals analysed) than in DNA extracted from feather tips (two cases out of 34 individuals analysed). Unambiguous sequences were aligned using the software BioEdit (Hall 1999) and basic statistics of mtDNA diversity, including nucleotide and haplotype diversity, were calculated in Dnasp (Rozas et al. 2003).

MHC class II genotyping

The entire second exon of an MHC class II B locus, which we here designate as *Fana*-DAB*1, was amplified using primers Fal2FC (5'-CCTCCCTGTACAAACAGAG-3') and Fal2RC (5'-GTGGCACTGGGAAACSTG-3'), which sit in the flanking introns 1 and 2, respectively (see Alcaide *et al*. 2007 for more details). Overall, 121 kestrels were genotyped from Spain to Kazakhstan (Table 1). The PCR was carried out in a PTC-100 Programmable Thermal Controller (MJ Research) using the following PCR profile: 1 cycle of 4 min at 94 °C, 35 cycles of 40 s at 94 °C, 40 s at 56 °C, 40 s at 72 °C and finally, 4 min at 72 °C. Each 25 μL reaction contained 0.4 U of *Taq* polymerase (Bioline), 1× PCR manufacturer-supplied buffer, 1.5 mm $MgCl_2$, 0.02% gelatine, 0.12 mm of each dNTP, 10 pm of each primer, 5% DMSO and, approximately, 25 ng of genomic DNA. Investigation of variation at MHC loci requires separating the different PCR amplification products, either because of the possibility of amplifying more than one locus, or because individuals are likely to be heterozygous at many sites in these loci. After PCR clean-up in Microcon centrifuge tubes (Millipore), PCR products were cloned into bacterial plasmids using the PGEM-T easy vector system II (Promega). Clones

were screened for the expected insert size in 1.5% agarose gels by running a second PCR with M13 primers. Six to eight positive clones per individual were selected at random for sequencing analysis. Sequencing reactions were carried out using the BigDye 1.1 Terminator technology and labelled fragments were subsequently resolved in a 3100 automated sequencer (Applied Biosystems).

MHC class II sequences were aligned and edited using BioEdit 7.0.5.2 (Hall 1999). Following Edwards *et al*. (1995), rare sequences found only once and differing by less than 3 bp from a redundant sequence of the same PCR product were considered artefacts of PCR errors and were assumed to have already been sampled. Since recombination of cloned PCR products is an additional source of artefacts (Bradley & Hillis 1996), direct sequencing of uncloned PCR products was used to check for agreement of polymorphic sites with cloned sequences. All alleles found only in one individual were verified by performing a second typing of that individual. Polymorphism statistics were generated using the software Dnasp (Rozas *et al*. 2003). Putative amino acid sequences were obtained after alignment to the chicken B-LBII (Zoorob *et al*. 1990).

Estimating diversifying selection in the presence of recombination

There are many tests for selection on MHC genes, each appropriate for different time scales over which selection acts (reviewed by Garrigan & Hedrick 2003). The selection parameter ω measures the ratio between non-synonymous substitutions (d_N) and synonymous substitutions (d_S) along coding sequences. An excess of non-synonymous substitutions over synonymous substitutions is related to positive selection, where $\omega>1$. By contrast, functional constraints in protein sequences are indicated by values of $\omega<1$. Maximum likelihood methods have been widely used to test for the presence of codons affected by positive selection and to identify those sites (e.g. Yang et al. 2000). Nevertheless, the use of

phylogenetic methods to identify sites experiencing diversifying selection in the presence of high levels of recombination is believed to cause high numbers of false positives (Anisimova et al. 2003). In this respect, high recombination rates at the MHC have been commonly documented (e.g. Richman et al. 2003, Edwards & Dillon 2004, Miller & Lambert 2004b) and consequently, ω values might be overestimated. We therefore used the recently developed programme OmegaMap (Wilson and McVean 2006), which permits inference of positive selection in the presence of recombination. OmegaMap employs a Bayesian population genetics approximation to the coalescent theory that co-estimates the selection parameter ω and the recombination rate ($\rho = 4N_e c$) along the sequence in order to incorporate evolutionary uncertainty. Positional variation in ω across exon 2 was investigated using a sliding window of 10 codons (approximately 10% of the total, see Wilson & McVean 2006). Analyses were conducted using an objective set of priors (i.e. those that do not represent any previous information about the values of different parameters considered in the model). Following the author's recommendation (see more details in Wilson & McVean 2006), the probable values of the mutation rate (μ) and the transition/transversion rate ratio (κ) were adjusted to follow improper_inverse distributions (starting values for μ and κ were set at 0.1 and 3.0, respectively), and the selection parameter (ω) and the recombination rate (ρ) were adjusted to follow inverse distributions in the range between 0.01 and 100. Means for ω, ρ, and the population mutation rate ($\theta = 4N\mu$) per codon were calculated using the posterior distributions generated with the objective prior set. Two MCMC chains were run for 500,000 iterations, with a 50,000 iteration burn-in. After paired chains were checked for convergence (i.e. two independent runs should match within an acceptable degree of error when comparing in a plot the mean and higher and lower 95% highest posterior densities for ω against codon position), they were merged to infer posterior distributions over ω.

Estimates of population differentiation

The extent of population differentiation at supposedly neutral microsatellite markers was calculated according to the traditional F_{ST} estimate using the software GENETIX 4.04 (Belkhir et al. 1996-2004). On the other hand, the substantial variability commonly found at MHC genes has highlighted the statistical inadequacy of analyzing individual DNA or amino acid sequences for some problems. This fact has been addressed, for instance in humans, by grouping alleles into supertypes attending to shared binding motifs (e.g. Lund et al. 2004). Even though only a few amino acid differences are known to confer different degrees of protection against pathogens (e.g. Hill 1998, Froeschke & Sommers 2005, Bonneaud et al. 2006a), closely related alleles are thought to have similar peptide binding properties (e.g. Trachtenberg et al. 2003). Thus, the relative frequencies of certain allelic lineages, rather than individual alleles, may reflect adaptation to local pathogen communities. We therefore calculated the nucleotide-sequence based estimate of genetic differentiation K_{ST} (Hudson et al. 1992) for MHC and mitochondrial sequences using the software DNAsp (Rozas et al. 2003). In addition, clustering of class II alleles was visualized through Neighbour Net networks that were built in the software Splitstree 4 (Huson & Bryant 2006) using maximum likelihood distances. In this respect, phylogenetic networks are believed to provide a useful representation of the genetic relationships among sequences when recombination is operating as compared to traditional phylogenetic trees. Finally, isolation by distance was investigated through Mantel tests that were carried out in the programme GENETIX 4.04. After introducing a matrix containing both genetic and demographic data, P-values were calculated using 10,000 permutations. Geographic distance was calculated according to a straight line connecting each pair of sampled populations.

RESULTS

*Genetic diversity at nine microsatellite loci, mtDNA-CR sequences and Fana-DAB1**

We found 103 alleles across 9 microsatellite markers and 327 genotyped kestrels. Average observed heterozygosity was 0.66. No significant evidence of linkage disequilibrium was reported between any pair of loci analysed. Only locus Fp107 departed significantly from Hardy-Weinberg expectations and was subsequently removed from further analysis. This locus consistently showed heterozygosity deficits that are likely related to the presence of null alleles (see also Nesje et al. 2000). Six different mtDNA-CR haplotypes were found in 48 lesser kestrels sampled across the Mediterranean (GenBank Accession No: EU525933-EU525938). The polymorphism survey at mtDNA sequences revealed 13 segregating sites corresponding to 13 point mutation events, 5.4 nucleotide differences on average (0.41 per site) between unique alleles (*k*) and a nucleotide diversity (π per site) of 0.021. At the MHC class II locus *Fana* DAB*1, we isolated 103 alleles from 121 kestrels (GenBank Accession No: EF370767-370788 and EU107667-EU107746, see Fig.2). Average heterozygosity was 0.98. The polymorphism statistics derived from the analysis of our MHC Class II DR β exon 2 data set revealed 70 variable sites (S), 23.32 nucleotide differences on average (0.33 per site) between unique alleles (*k*), 85 nucleotide differences (1.214 per site) between all alleles and a nucleotide diversity among all alleles (π per site) of 0.086. All unique sequences differed by at least one non-synonymous substitution in the PBR, which suggests that they might also differ in their antigen binding properties. None of the MHC sequences here reported showed any signs of nonfunctionality, such as stop codons or frameshift mutations. We consistently found one or two different MHC alleles per individual.

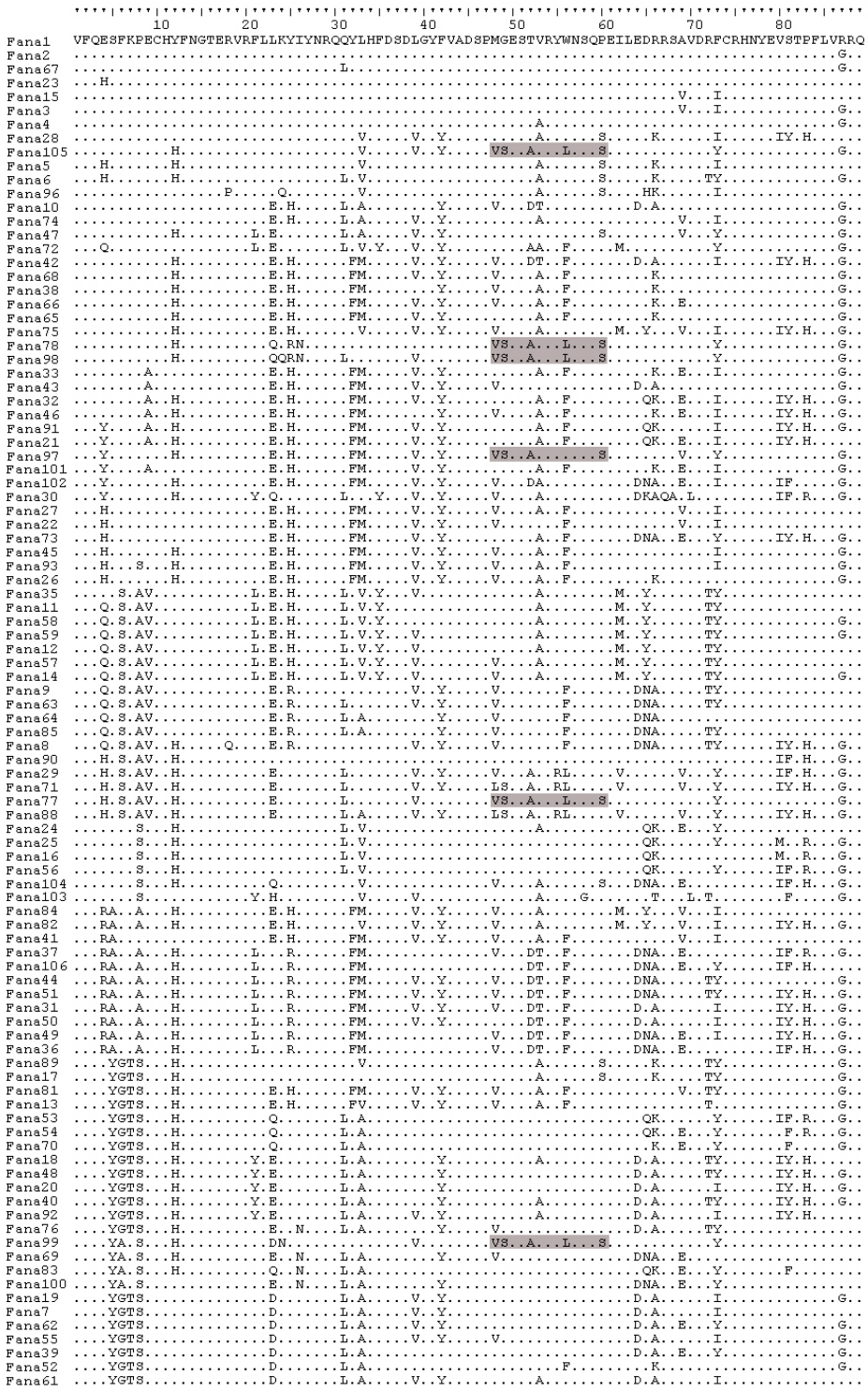


Fig. 2. Inferred amino acid sequences of the *Fana*-DAB1* locus. Dots indicate identity with the top sequence. An amino acid motif exclusively found in Kazakhstan is outlined in grey.

*Positive selection and recombination rates at Fana-DAB*1*

Genetic analysis performed in DNAsp revealed significant deviations from neutral expectations in the frequency spectrum of segregating sites within the kestrel MHC, with an excess of high frequency sites (Tajima's D=2.37, P<0.05). Analyses performed in OmegaMap revealed a mean value per codon of ω=4.02. Spatial variation in the selection parameter ω across exon 2 is represented in Figure 3, where several amino acid sites display a hallmark of positive selection. The mean amount of population recombination per codon (ρ=0.389) greatly exceeds the mean amount of population mutation (θ=0.017). These results suggest that the accumulation of new recombinants exceeds that of new mutations by at least one order of magnitude. The Neighbour-Net network presented in Fig. 5 also reveals a complex pattern suggesting multiple recombination events during the evolutionary history of the locus.

Patterns of population differentiation at neutral and adaptive loci

Pairwise estimates of population differentiation at eight microsatellite markers revealed significant evidence of isolation by distance across the Mediterranean breeding distribution of lesser kestrels (r = 0.50, P = 0.04; Fig. 5). We excluded the population from Kazakhstan (N = 7) from this analysis because of the low reliability of microsatellite data when sample sizes are small. Significant evidence for isolation-by-distance patterns was also found after analysing the *Fana*-DAB*1 locus (r = 0.67, P = 0.01; see Fig. 5). Nonetheless, pronounced differences regarding the frequencies of the most abundant MHC alleles, as well as the relative frequencies of each allelic lineage, contrast with the uniform distribution of allelic frequencies that we found at microsatellites (see Table 1, Figs 4 and 6).

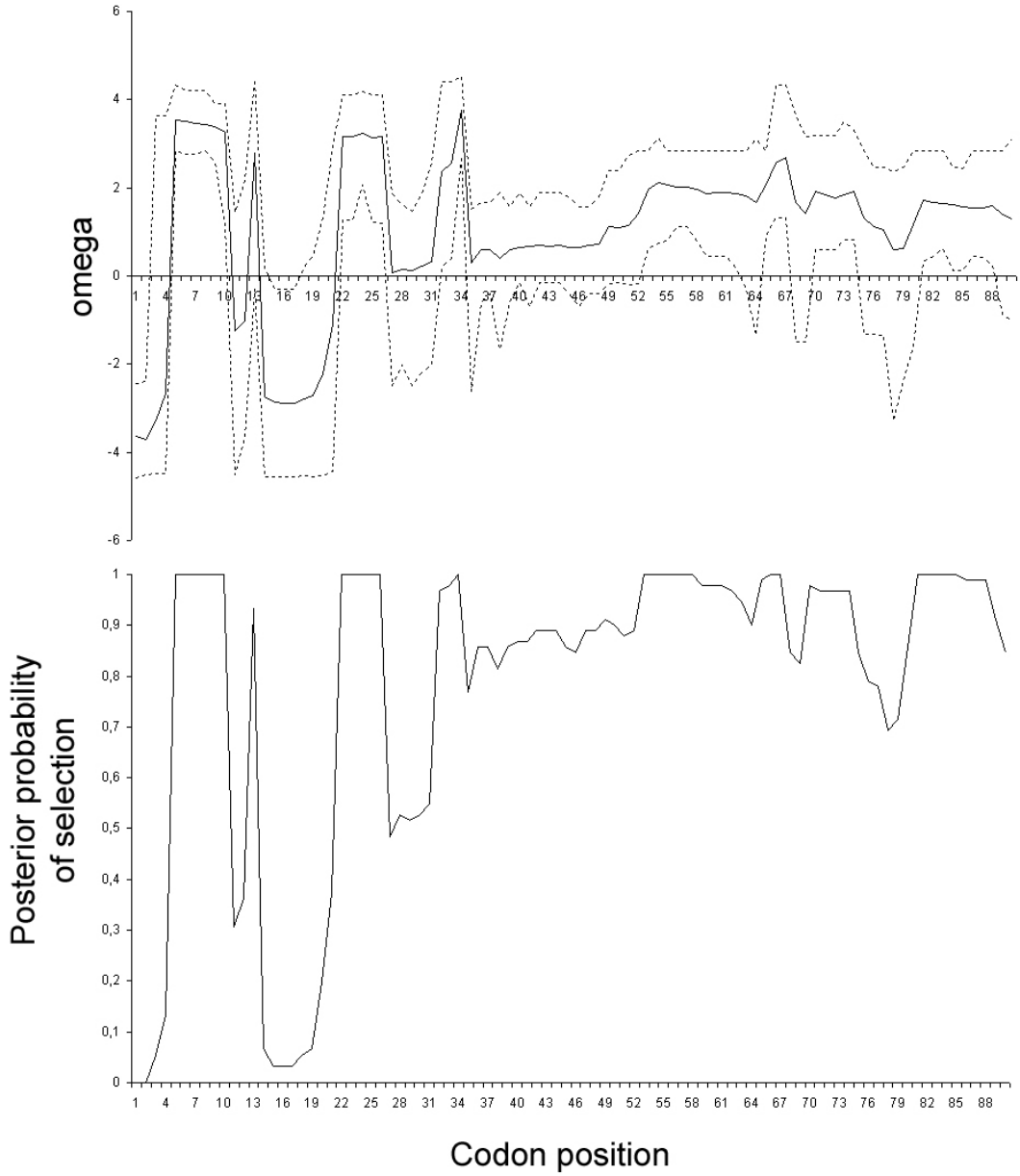


Fig. 3 (a) Spatial variation in the logarithm of the selection parameter ω across the second exon of a classical MHC class II gene of the lesser kestrel. Parameter estimates were carried out in the software package omegamap using an objective set of prior distributions (Wilson & McVean 2006). The sitewise mean (solid line) and 95% HPD intervals (dotted lines) are shown. (b) Spatial variation in the posterior probability of positive selection.

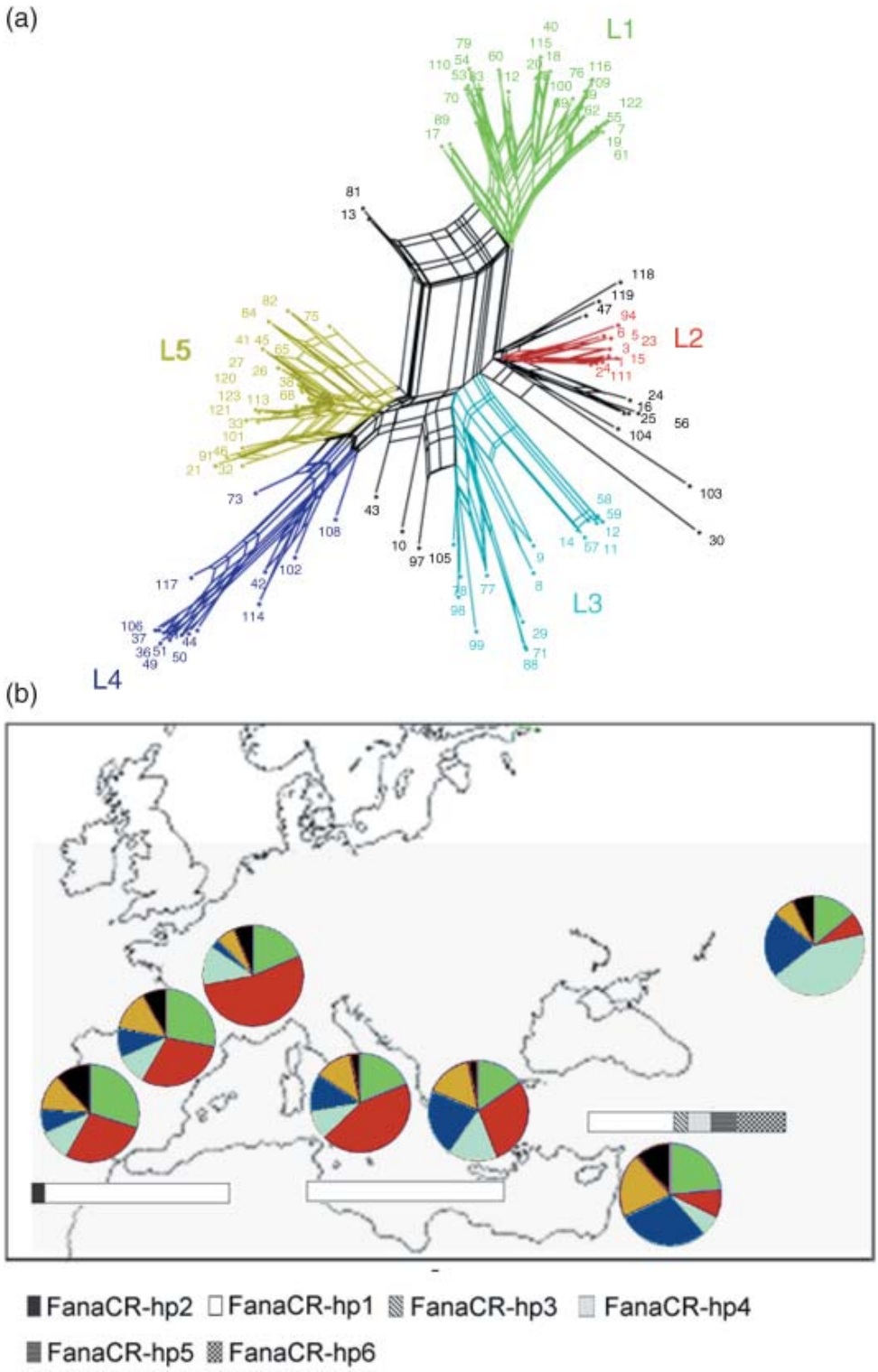


Fig. 4 (a) Neighbour-Net constructed from exon 2 sequences isolated in the lesser kestrel. Seven allelic lineages are proposed taking into account the clustering of class II sequences and considering the presence of abundant MHC alleles within clusters. (b) Spatial variation in the lesser kestrel MHC (coloured circles) as a means of partial contributions of different allelic lineages. Geographical variation at mtDNA-CR sequences (bars) is also shown.

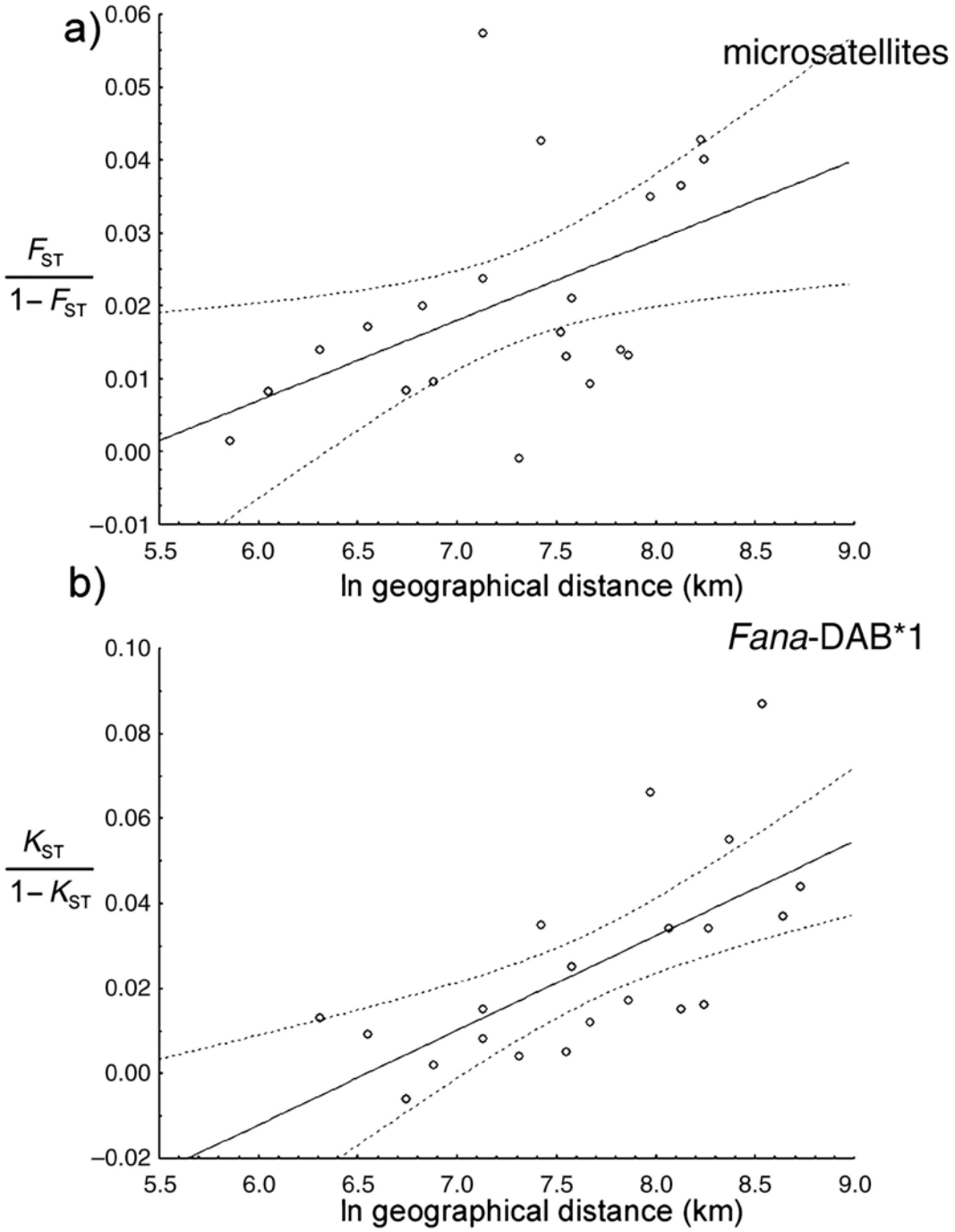


Fig. 5 (a) Correlation between the extent of genetic differentiation at eight microsatellite markers and geographical distance in Mediterranean populations of lesser kestrel (r = 0.50, P = 0.04). (b) Correlation between the extent of genetic differentiation at *Fana*-DAB*1 and geographical distance when analysing sampled populations from Spain to Kazakhstan (r = 0.67, P = 0.01). 95% confidence intervals are indicated by dotted lines.

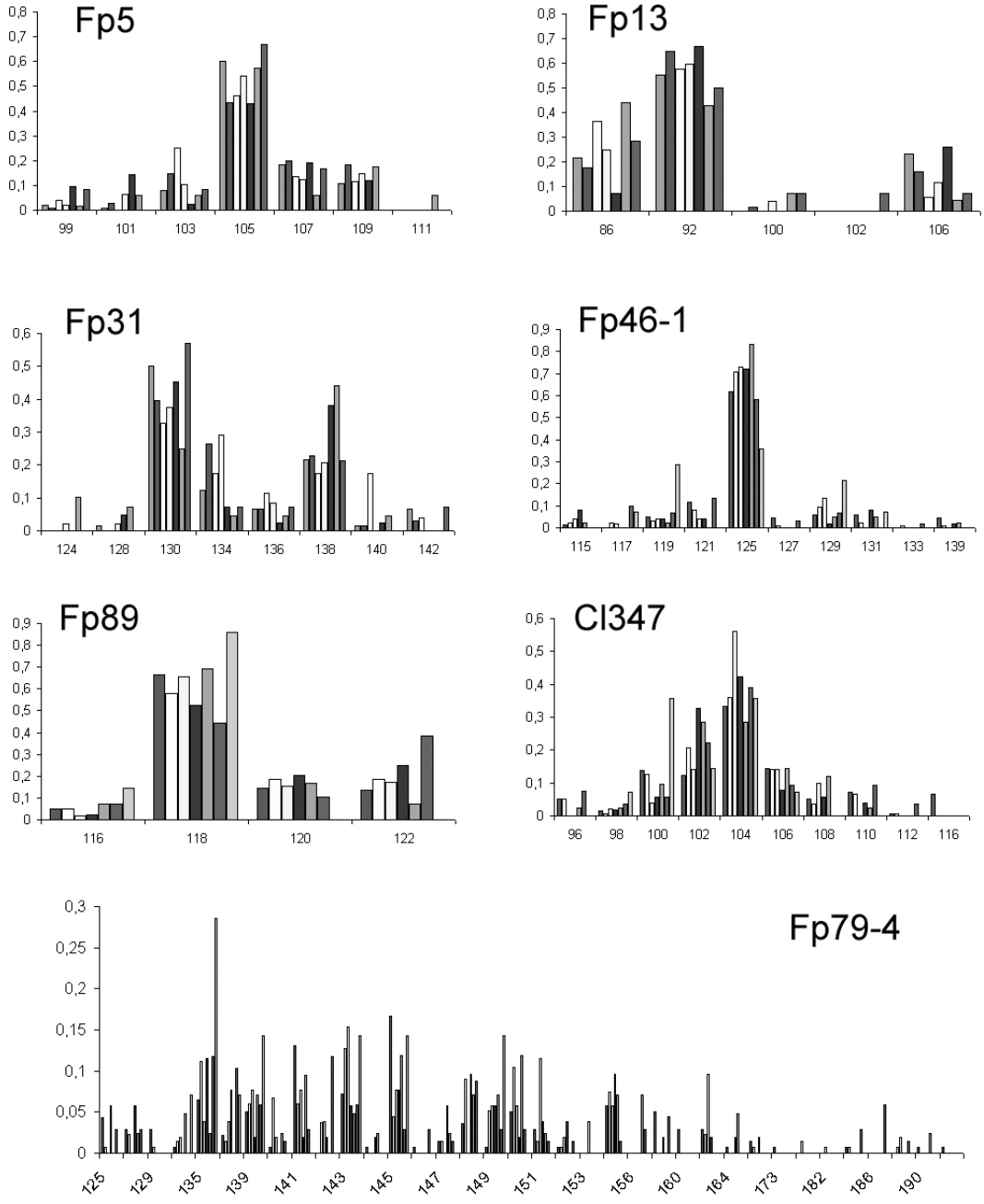


Fig.6. Distribution of allelic frequencies at seven polymorphic microsatellite markers in the lesser kestrel. Sampled populations follow a western-eastern direction (Left-SWS-NES-FRA-ITA-GRE-ISR-KAZ-Right, Fig. 1). Allele size in bp is given in the X-axis.

For instance, 13 class II sequences isolated from seven kestrels sampled in Kazakhstan were not previously registered in any Mediterranean kestrel. Moreover, we found an exclusively abundant amino acid motif in Kazakhstan (amino acid positions 48–60; see Fig. 2). By contrast, the chance of finding private alleles at high frequencies when analysing microsatellite markers was low

(see Fig. 6). Genetic divergence at the MHC fits better into geographical variation at mitochondrial control region sequences (see Fig. 4). Thus, we found one haplotype uniformly distributed across the Western and Central Mediterranean that was also abundant in the most distant Mediterranean population of Israel. Nevertheless, we found in Israel new haplotypes not previously reported in European populations that might represent Asian haplotypes. The K_{ST} estimate for CR sequences between the most distant Mediterranean populations of Spain and Israel was 0.17 ($P < 0.05$).

DISCUSSION

Ours is one of the first studies examining MHC diversity in wild populations of a bird of prey. We have reported exceptionally high levels of polymorphism at a putatively functional and expressed MHC class II locus in the lesser kestrel, *Fana*-DAB1*. Analysis of the entire second exon revealed no stop codons or frameshifts mutations as well as genetic evidence for balancing selection and recombination. Whereas Bayesian analysis of diversifying selection in the presence of recombination seems to be more conservative than maximum likelihood methods (see Alcaide et al. 2007 for a comparison), several amino acid sites of exon 2 showed to have experienced strong positive selection (Fig. 3). Although we have not performed gene expression analysis, research in this topic has observed a strong correlation between signatures for balancing selection and expression (e.g. Zoorob 1990, Jacob et al. 2000). In addition, whereas many studies in birds have been unable to examine allelic diversity at a single MHC locus (Edwards et al. 1998; Hess et al. 2000; Bonneaud et al. 2004a), or have encountered low polymorphism at MHC loci (Hess et al. 2000; Gasper et al. 2001; Aguilar et al. 2006), we have been able to focus exclusively on variation at a single highly polymorphic MHC locus. In fact, the level of expression and functional relevance of different loci, as well as locus identity of alleles is unknown in many bird studies (i.e. Edwards et al. 1998, Hess et al. 2000, Bonneaud et al. 2004a),

making appropriate population genetic analyses difficult. Hence, the specific amplification of a single locus exhibiting strong positive selection may turn the lesser kestrel into an excellent model species for the investigation of the evolutionary significance of MHC genes and its relationship to adaptive variation. The amplification of one single locus in this species is also supported by an ongoing study in which the segregation of MHC alleles from parents to the offspring is fitted to a single model of biparental inheritance. Hence, we found some homozygous genotypes in the offspring when the parents shared at least one allele (Alcaide et al. unpublished data). At the very least, MHC allelic composition and heterozygosity at the genomic level can therefore be readily tested in detail in kestrels and related species, for instance, in relation to resistance/susceptibility to parasite infections and other fitness-related traits such as reproductive performance. The role of heterozygosity at the MHC has been rarely documented in natural populations in detail (e.g. Hedrick et al. 2001, Arkush et al. 2002, Froeschke & Sommer 2005), although experiments in mice under laboratory conditions are exemplary in this regard (Penn et al. 2002).

Both microsatellite markers and the MHC locus *Fana*-DAB1* revealed an isolation-by-distance pattern in our study area that would be in agreement with population fragmentation and with apparently limited dispersal abilities in the philopatric lesser kestrel (Negro et al. 1997, Serrano et al. 2001, Serrano & Tella 2003). Nonetheless, the lack of private alleles at high frequencies across microsatellites contrasts with the pronounced differences in the frequencies of MHC alleles or allelic lineages between European and Asian populations (see Fig. 5 and 6). Such genetic divergence may be consequence of historically limited gene flow among both areas, a fact that is supported by preliminary mitochondrial data in the form of cytochrome *b* sequences (Wink et al. 2004) as well as by our control region sequences (Fig. 5). On the other hand, it is perhaps not surprising that the microsatellite loci showed uniformly lower levels of

differentiation than *Fana*-DAB1*. These two sets of loci differ not only in selective regimes but also in mechanism of mutation, with microsatellites showing levels of variation that many have argued can compromise studies of even closely related populations. Back-mutation and homoplasy can be common in microsatellite alleles, and the high variation within population can result in artificially low levels of population divergence (e.g. Charleswoth 1998). Thus, direct comparison of population patterns of microsatellites and MHC is ultimately complicated by their very different mechanisms of evolution. A more appropriate comparison would be, for example, *Fana*-DAB1* with anonymous loci or introns, which, like the MHC, are genotyped by sequencing and evolve primarily by point mutation. Such a study would better identify the true causes of differences between MHC and the neutral portion of the lesser kestrel genome. The contrast in K_{ST} values between CR sequences and the *Fana*-DAB1* locus after comparing the two distributional borders in the Mediterranean (0.17 vs. 0.015) is instructive in this regard. Accordingly to the equation K_{ST} = 1 / 1+4Nm, we expect K_{ST} for mitochondrial sequences to be four times higher than for a nuclear gene since the population size of the former is ¼ of the later. Nonetheless, our results suggest that fixation rates in CR sequences is at least one order of magnitude higher than those reported for MHC coding sequences. These results should be explained in part because balancing selection at the MHC may have mitigated the effects of genetic drift (but see for instance Miller & Lambert 2004a when dealing with small populations)

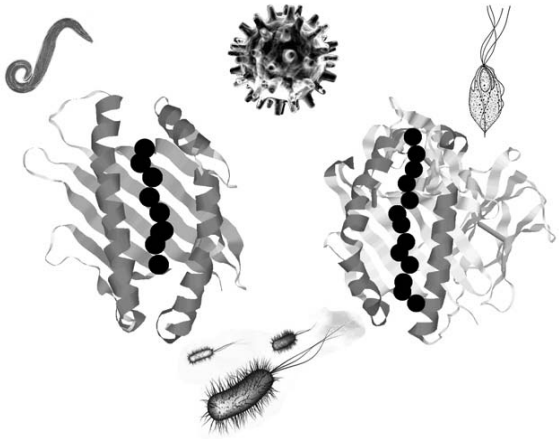
Restricted gene flow is a crucial condition to favour local adaptation, and therefore, spatial variation in parasite selection regimes may cause MHC polymorphism in accordance with Hill's hypothesis (1991) (see also Hedrick 2002). Recently, Ekblom and co-workers (2007) explained spatial patterns of MHC class II variation in the great snipe *Gallinago media* as a result of local adaptation to different ecologically distinct distributional regions (i.e. mountain

populations vs. lowland populations). By contrast, lesser kestrels are known to inhabit similar steppes and pseudo-steppes ecosystems across Eurasia (Cramp & Simmons 1980, Ferguson-Lees & Christie 2001) and our results would be better explained attending to limited gene flow translated into different adaptations to local pathogen communities as an example of geographically varying co-evolution (see Thompson 2005). In this sense, several studies have shown that the degree of population structure of parasites is related to that of the host species exploited (e.g., Blouin et al. 1995, Criscione & Blouin 2007), while others have documented situations in which the host displayed low genetic differentiation but parasite populations were strongly structured (e.g. McCoy et al. 2005).

Finally, strong geographical variation at the MHC suggests the potential of locally selected MHC alleles or allelic lineages to resolve the origin of captive or vagrant individuals. The lesser kestrel has a direct implication in this regard since knowledge about the composition of the different wintering grounds in Africa is of high relevance in conservation. Hence, MHC alleles could be used in combination with mtDNA to unravel migration routes. At the very least, cytochrome *b* sequences implicated South Africa as an important wintering ground of Asian populations, with no trace for European birds (Wink et al. 2004). Nevertheless, Wink and co-workers found some degree of haplotype mixing in both breeding and wintering populations, and at this point, MHC alleles may improve assignment of individuals. In the same line, our CR sequences give no resolution in European populations and shared haplotypes have been found in Israel. Since the intronic sequences flanking the second exon of the class II B genes are highly conserved at the family level (Alcaide et al. 2007), this highly polymorphic region can be successfully cross-amplified in a large variety of species as a means of obtaining fast and valuable genetic information. Moreover, the size of the amplified fragment (about 300 bp) would allow the investigation of non-invasive samples involving degraded DNA.

Capítulo 8

MHC diversity in kestrels is related to host traits driving differential exposure to pathogens



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Manuscrito para enviar a *Plos Pathogens*

Background. Extensive genetic variation at the Major Histocompatibility Complex (MHC) is thought to have evolved as a means of fighting off a broad spectrum of infectious diseases. However, it is still poorly known how pathogen burdens drive MHC diversity. Here, we surveyed a whole community of pathogens and parasites (35 taxa including viruses, bacteria, fungi, protozoan, metazoan and nematodes) in two closely related, sympatric birds of prey (Eurasian and lesser kestrel) differing in ecology and life history, and therefore, with variable expected levels of host exposure.

Principal findings. Although specific host traits such as geographic range, habitat, diet as well as migratory and breeding social behaviour seem to explain the differential infection by particular pathogens, overall pathogen diversity, richness and prevalence was higher in Eurasian than in lesser kestrels. Differences were higher with respect to viruses and bacteria, which are among the most virulent pathogens. Accordingly, we found higher number (64 vs. 49 alleles) as well as more divergent MHC class I and class II haplotypes in Eurasian than in lesser kestrels. Differences were only statistically significant in those codons comprising the antigen binding regions, and the lack of significant differences at 8 microsatellites discards an effect of effective population size. The lowest pathogen burdens as well as the smallest but still quite divergent set of MHC sequences were found in Eurasian kestrels from the Canary Islands (16 alleles), where the rates of allele fixation at MHC loci seem to have occurred faster than at microsatellites.

Conclusions / Significance. Different ecological requirements and life history traits of hosts are shaping in a complex way their wide community of pathogens, which in turn, may be driving MHC variability. Therefore, several hypotheses must be tested together to ascertain variability in pathogen infections and host resilience. In this respect, MHC diversity may constitute a good predictor of host

susceptibility to infections, especially in a globalizing world where species are increasingly exposed to new pathogens and emerging diseases.

INTRODUCTION

Genetic diversity at functionally important genes such as those belonging to the major histocompatibility complex (MHC) is widely believed to influence the evolutionary and adaptive potential of populations and species (Sommer 2005, Piertney & Oliver 2006). This multigene family plays a central role in the immune system of vertebrates (Klein 1986). In particular, MHC genes code for cell-surface glycoproteins that bind foreign peptides for their presentation to specialised cells of the immune system, which subsequently trigger adequate immune responses. The recognition of foreign antigens bound to MHC class II proteins initiate antibody synthesis against bacterial or parasite proteins. On the other hand, MHC class I molecules bind peptides derived from the processing of intracellular pathogens, such as viruses and some protozoa, and promote the destruction of the antigen-presenting cell. Genetic variation at MHC genes largely determines the number of foreign antigens an individual is capable of responding to, and thus, MHC diversity is thought to decisively influence individual fitness and long-term persistence of populations (Hughes & Nei 1992). The selective pressures imposed by infectious agents have turn MHC genes into the most polymorphic coding loci described so far (Robinson et al. 2001), and consequently, this huge variability has focussed the attention in evolutionary biology and conservation genetics given that MHC variability may reflect important adaptive processes within and between populations and species. The intensity of selection is especially significant in those amino acid positions belonging to the peptide-binding region (PBR), a highly variable extracellular groove that determines the specificity of MHC molecules. Balancing and diversifying selection, in conjunction with MHC-dependent mate choice, are the most widely accepted evolutionary mechanisms aimed at maintaining the high levels of MHC polymorphism needed to counteract pathogen-mediated selective pressures (reviewed by Sommer 2005, Piertney & Oliver 2006).

Whether extraordinary levels of MHC polymorphism are intended to cope with a broad spectrum of potential infections, the strength of diversifying selection at MHC loci is expected to be driven by the richness and virulence of pathogen species to which hosts are exposed, which in turn, must be related to both host's life histories and ecological conditions. In this respect, it has been documented the influence of temperature clines in the world-wide distribution and virulence of pathogens (Clarke and Gaston 2006). Patterns of habitat use and range distribution are thought to determine the extent and cohabitation period of host-parasite interactions in the same line (Tella et al. 1999). Species that only thrive within a range of environmental conditions are also believed to hold lower but more specialized pathogen burdens than generalist species with a broad tolerance to environmental conditions (Dobson & McCallum 1997). In addition, migratory species are commonly exposed to at least two different pathogen faunas during their annual cycle (Hubalek 2004), whilst resident species only have to face one. High prevalence of pathogens in socially-breeding species has been attributed to high transmission rates in the colonies (Tella 2002) as well. An axis of body size, developmental period and life span of hosts has been positively related, on the other hand, to their immunocompetence (Tella et al. 2002) and, therefore, with lower parasite infections. As another example, risks of infections are expected to differ among species with different feeding ecologies given that prey items constitute a potential source of microbial agents (Friend and Franson 1999, Lumeij et al. 2000).

Elucidating sources of variability in pathogen pressure that can be related to MHC evolution are of great interest. However, studies associating environmental and ecological factors with MHC diversity in natural populations are notably few and have been mainly restricted to humans (Prugnolle et al. 2005) and fishes (Wegner et al. 2003, Simková et al. 2006, Dionne et al. 2007, Blair et al. 2007). In birds, even though it is widely recognised that pathogen pressures

greatly varies among species with different ecology and life histories, the majority of research efforts have been focused on the inter-specific comparative study of immunocompetence surrogates, such as immune organs (Møller & Erritzoe 1996) and particular immune responses (Møller et al. 2001, Tella et al. 2002), without relating them to actual pathogen burdens and MHC-related hallmarks of selection. The very few examples linking avian pathogens to the MHC have dealt with a single host-pathogen association, usually involving malaria infections (Bonneaud et al 2006b, Loiseau et al. 2008).

As far as we know, we present here the first study that simultaneously investigates detailed sequence data for both MHC class I and class II genes in relation to a whole community of parasites and pathogens, including viruses, bacteria, fungi, protozoan, metazoan and nematodes (thereafter termed pathogens for simplicity), in wild populations of two closely related bird species with contrasting life histories and ecological requirements. We investigated three subspecies of the Eurasian kestrel *Falco tinnunculus* and the phylogenetically related lesser kestrel *Falco naumanni* (Groombridge et al. 2002). While the lesser kestrel is an habitat-specialist, estenophagous, colonial and migratory falcon, the sympatric European subspecies of the Eurasian kestrel *Falco t. tinnunculus* is mostly considered a cosmopolitan territorial breeder, euriphagous, sedentary and habitat generalist species (Cramp & Simmons 1980). We expected that differences in ecology and life histories between these two species (see details in Table S1) would determine a differential exposure to pathogens, according to the hypotheses outlined above, and that these differences would translate into different selection patterns acting on MHC polymorphism. Since our compiled hypotheses predict alternative pathogen pressure outcomes (in terms of diversity, richness and/or prevalence, see Table 1), and different kinds of pathogens may greatly vary in their virulence and selective forces on hosts, we had not a single clear prediction on which species would show greater MHC variability. In addition,

we also sampled two island subspecies of the Eurasian Kestrel, *Falco t. dacotiae and Falco t. canariensis*, for which were expected to hold lower MHC diversity and prevalence of infections (Table 1) because of the demographic and genetic constraints typically associated with insularity, which affects both communities of infectious agents and their hosts (Dobson & McCallum 1997, Clifford et al. 2006). Finally, MHC variability was examined in conjunction with patterns of neutral variation (microsatellites) to discard the confounding effects of effective population size (Alcaide et al. 2008).

Table S1. Ecological profiles and life history traits of the Eurasian kestrel (*Falco tinnunculus*) and the lesser kestrel (*Falco naumanni*) (see Cramp & Simmons 1980 for more details)

	Falco tinnunculus	***Falco naumanni***
Body mass	140 – 300 g	100 – 200 g
Clutch size	3 – 6 eggs	3 – 5 eggs
Incubation period	27 – 29 days	28 – 29 days
Nestling period	27 – 32 days	28 days
Life span	16 yr	13 yr
Niche amplitude		
Altitudes	0-5000 m	0-2750 m
Habitats	Wide tolerance - Generalist (steppes and pseudosteppes, semi-deserts, low dense forests, urban environments)	Specialist Steppes and pseudosteppes, urban environments
Nests	Mostly cavity-nester, using cliffs, human structures and trees. Also in corvid nests and exceptionally on the ground	Cavity-nester, using mostly human structures, exceptionally in cliffs, on the ground or in tree holes

Distributional range in the Western Paleartic Resident (black areas) Migrant breeding (grey areas)		
	Breeding latitudes: up to 70ºN	Breeding latitudes : 30-50º N
Breeding system	Usually solitary breeder	Mostly colonial
Migratory status	Sedentary (i.e. Canary Islands) , partially sedentary (i.e. Iberian Peninsula) or short-medium distance migrator (i.e. North Europe)	Trans-Saharian, long-distance migrator
Diet	Euriphagous (small mammals, birds, reptiles and insects)	Estenophagous (Insect specialist)

RESULTS

Surveys of pathogen diversity, richness, and abundance

Results from the pathogen screening are detailed in Table S2, while estimates of pathogen diversity, richness, prevalence and individual richness are compiled in Table 2. The highest values of these pathogen burden indicators were found in Eurasian kestrel adults sampled in the continent (*F. t. tinnunculus*), being much higher than those reported for sympatric lesser kestrel adults (*F. naumanni*). Fledglings showed lower values than adults but slight differences between the two species. Adult Eurasian kestrels sampled from the island subspecies (*F.t. dacotiae*

and F.t. canariensis) had the lowest values, comparable to continental nestlings and much lower than continental Eurasian kestrel adults.

Table S2. Prevalence (percentage of individuals infected) of 35 avian pathogens in lesser (*Falco naumanni*) and Eurasian kestrels (*Falco tinnunculus*). Results are separated for each age class and subspecies. Sample sizes are in bold.

	PREVALENCE					
	Adult Birds				**Nestlings**	
	Falco t. tinnunculus	***Falco t. dacotiae***	***Falco t. canariensis***	***Falco naumanni***	***Falco t. tinnunculus***	***Falco naumanni***
Fungi	**N=40**	**N=20**	**N=17**	**N=45**	**N=244**	**N=175**
Candida albicans	25	15	23.6	33.3	25.4	4
Bacteria	**N=40**	**N=20**	**N=17**	**N=45**	**N=244**	**N=175**
Campylobacter spp	10	0	0	22.2	7	1.1
E. coli enterotoxigenic strain	30	5	17.7	6.7	11.9	21.1
Mycobacterium avium	2.50	0	0	0	0	0
Pasterella multocida	17.5	0	0	0	1.2	1.1
Pseudomonas aeruginosa	5	0	0	0	2	0
Salmonella spp	22.5	30	17.7	20	5.3	14.2
Chlamydophila psittaci	52.5	15	11.8	37.8	34	26.3
Mycoplasma spp	40	20	23.6	44.4	32.4	46.8
Viruses	**N=40**	**N=20**	**N=17**	**N=45**	**N=244**	**N=175**
Adenovirus	35	0	0	26.7	7	1.7
Circovirus	0	0	0	0	0	0

Herpesvirus	7.5	0	0	0	0	0
Influenzavirus (IH5)	0	0	0	0	0	0
Influenzavirus (IH7)	12.5	0	0	8.9	1.2	9.1
Influenzavirus (IH9)	0	0	0	0	0	0
Paramixovirus	40	0	0	8.9	7.8	11.4
Polyomavirus	0	0	0	0	0	0
Poxvirus	12.5	30	29.5	24.4	7	10.8
Reovirus	5	0	0	20	8.2	1.1
West Nile virus	25	50	47.1	55.5	16.4	12.6
Hemoparasites	**N= 40**	**N=20**	**N=17**	**N=45**	**N= 244**	**N=175**
Haemoproteus spp	0	5	0	0	0	0
Leucocytozoon spp	22.5	15	35.4	20	19.2	35.4
Plasmodium spp	0	10	17.6	0	0	0
Trypanosoma spp	0	5	5.9	0	0	0
Trichomonas	**N= 40**	**N=20**	**N=17**	**N=45**	**N= 244**	**N=175**
Trichomonas gallinae	40	25	17.7	42.2	10.2	4.6
Intestinal parasites	**N=40**	**N=5**	**N=4**	**N=25**	**N=100**	**N=101**
Coccidia						
Caryospora spp	42.5	40	0	44	12	9.1
Eimeria spp	22.5	0	0	28	4	4
Isospora spp	17.5	0	0	0	0	0

Cestodes						
Cladotaenia spp	10	0	0	20	5.0	4.0
Unciunia spp	0	40	75	0	0	0
Nematodes						
Serratospiculum spp	0	0	0	0	0	0
Porrocaecum spp	15.0	0	0	56.0	8.0	9.1
Ascaridia spp	50.0	40.0	0	0	4.0	0
Capillaria spp	27.5	0	25.0	80.0	2.0	19.9
Cyrnea spp	30	20.0	25.0	0	5.0	6.0

Table 2. Diversity of pathogens (Shannon-Wiener index), richness (number of pathogen species), prevalence (percentage of individuals infected by at least one pathogen), and individual richness (mean number of pathogens per individual host) of pathogens infecting the different species, subspecies and age classes of kestrels sampled.

	Adult Birds				**Nestlings**	
	Falco t. tinnunculus	***Falco t. dacotiae***	***Falco t. canariensis***	***Falco naumanni***	***Falco t. tinnunculus***	***Falco naumanni***
Diversity	3.37	1.97	2.28	2.58	1.98	1.88
Richness	26	16	14	19	23	20
Prevalence	3.37	1.97	2.28	2.58	1.98	1.88
Individual richness	4.05	1.97	2.28	3.67	2.10	1.93

A categorical Principal component Analysis (CatPCA) allowed us to assess the associations of pathogens differentially infecting species/subspecies and age classes. The CatPCA yielded four dimensions with an eigenvalue greater than 1 that accounted for 64% of the variance (Table 3).

Table 3. Results from the CatPCA indicating the correlation (component loading coefficient, values >0.5 in bold) between groups of pathogens and the resulting four dimensions (D). The variance explained by each pathogen group is shown in brackets.

Pathogens	D1	D2	D3	D4	% variance
Viruses	**0.664 (0.441)**	-0.044 (0.002)	-0.262 (0.068)	-0.204 (0.042)	0.553
Bacteria	**0.665 (0.442)**	-0.149 (0.022)	-0.205 (0.042)	0.266 (0.071)	0.577
Haematozoa	0.253 (0.064)	**0.644 (0.415)**	-0.003 (0.000)	-0.532 (0.283)	0.762
Mycoplasma	0.073 (0.005)	0.396 (0.157)	0.512 (0.262)	**0.601 (0.362)**	0.786
C. psitacci	0.496 (0.246)	-0.362 (0.131)	0.176 (0.031)	0.182 (0.033)	0.441
T. gallinae	0.120 (0.014)	**0.591 (0.349)**	-0.446 (0.199)	0.393 (0.154)	0.717
C. albicans	0.359 (0.129)	0.126 (0.016)	**0.668 (0.446)**	-0.247 (0.061)	0.652
Eigenvalue	1.342	1.092	1.049	1.005	4.488
% variance	19.168	15.600	14.985	14.363	64.116

The first dimension (D1) revealed high loading for viruses and bacteria and hence defines a gradient of infection combining the prevalence and number of species of these pathogens. The second dimension (D2) showed high values for haematozoa and *T. gallinae* and, therefore, it defines a gradient of protozoan infection. The third (D3) and fourth (D4) dimensions included high values for *C. albicans* (the only fungi identified) and *Mycoplasma* sp., respectively (Table 2). Factor scores of the individual birds in each dimension showed several statistical differences between groups (species/subspecies) and ages (Table 4).

Table 4. Results from MANOVAs testing differences between species/subspecies and age classes in the four dimensions derived from a categorical PCA that condense variation in the pathogen community. Significant results are in bold.

	Species/subspecies		Age		Species x Age	
Dimension	*F*	*P*	*F*	*P*	*F*	*P*
Species						
D1	9.272	**0.002**	123.544	**<0.0001**	0.945	0.331
D2	4.060	**0.044**	3.450	0.064	0.026	0.873
D3	.206	0.650	3.606	0.058	7.013	**0.008**
D4	2.543	0.111	1.093	0.296	8.853	**0.003**
Subspecies						
D1	22.429	**<0.0001**				
D2	1.466	0.230				
D3	0.412	0.523				
D4	12.476	**0.001**				

Continental Eurasian kestrels were more infected by viruses and bacteria (D 1) than lesser kestrels, both in fledglings and adults (Fig. 2). Contrarily, the infection by protozoans (D 2) was higher in lesser than in Eurasian kestrels (Fig. 2), although the significance of the difference was much lower than in the case of viruses and bacteria. Infection mostly headed by *C. albicans* (D 3) and *Mycoplasma* sp. (D 4) showed differences between species only for nestlings, with higher incidence in Eurasian and lesser kestrel respectively (Fig. 2). Regarding island effects, adult Eurasian kestrels from the continent showed higher infection by viruses and bacteria (D 1) and *Mycoplasma* sp. (D 4) than those from the Canary Islands (Fig 2).

When looking at infections by particular pathogens, post-hoc univariate comparisons of prevalences showed many significant differences between adults

and nestlings of both Eurasian and lesser kestrels in the continent, adults always showing higher prevalences than fledglings (Table S2).

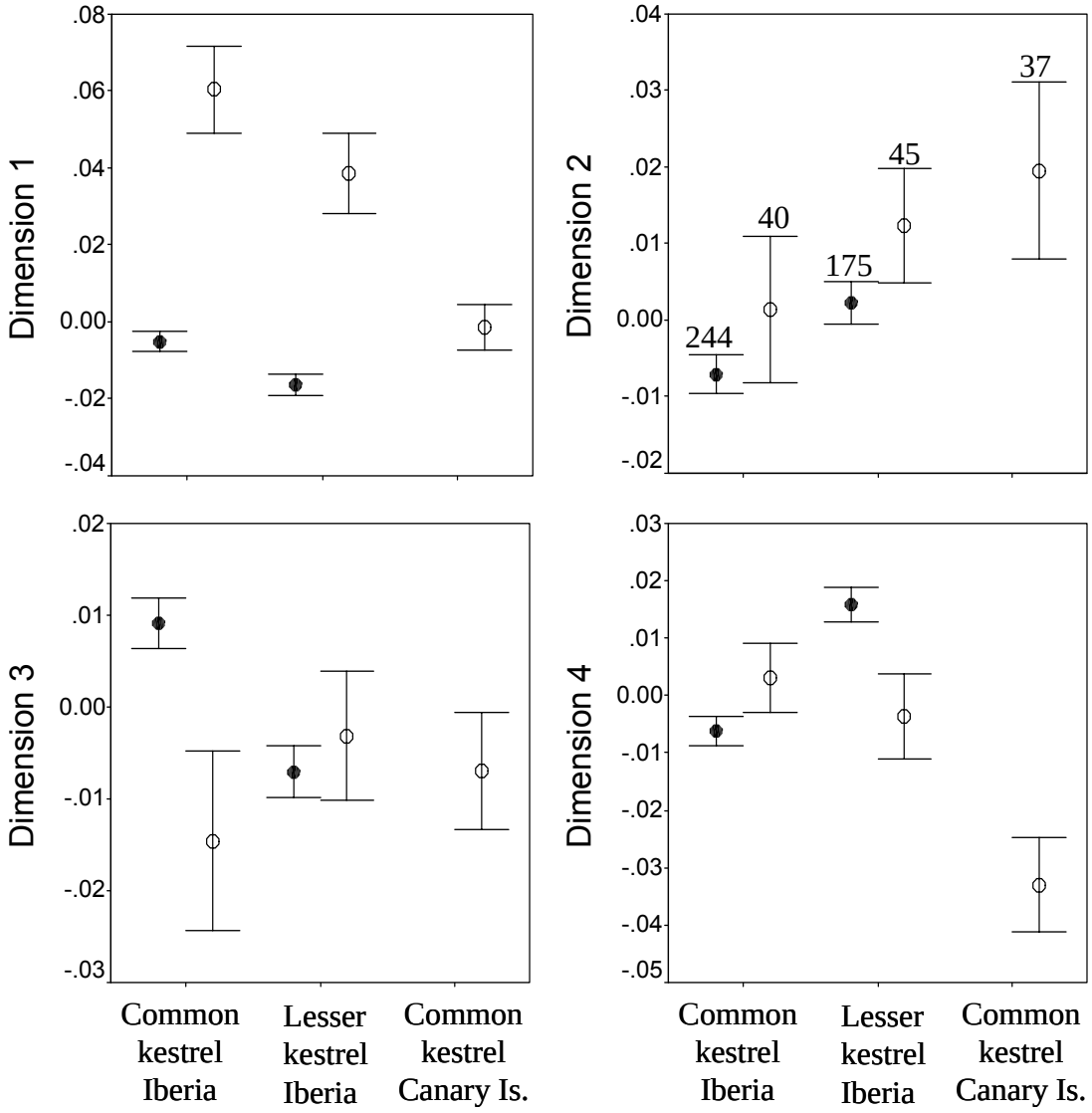

Figure 2. Differences between species/subspecies and age classes in the four dimensions derived from a categorical PCA that condense variation in the pathogen community. Black dots represent adults and white dots fledglings. Sample sizes are shown in the plot for D2.

Fledgling Eurasian kestrels showed significant higher prevalences of *Leucocytozoon*, *C. albicans*, *Campylobacter* sp., *P. aeruginosa*, *C. psittaci*, adenovirus and reovirus than lesser kestrels, while the contrary was found for *T. gallinae*, *E.coli* enterotoxigenic, *Mycoplasma* sp., Influenzavirus IH7 and poxvirus

(Table S2). Adult Eurasian kestrels showed a higher prevalence of *P. multocida*, *E.coli* enterotoxigenic and paramixovirus than lesser kestrels, while the latter was more frequently infected by reovirus and West-Nile virus. A higher prevalence of multiple pathogens was found in Eurasian kestrels adults from the continent than from Canary Islands (Table S2). Only *Plasmodium* and West-Nile virus infected more frequently island subspecies.

Genetic diversity at microsatellites and MHC loci

Detailed polymorphism statistics at microsatellites and MHC loci are summarized in Table 5. Average microsatellite diversity per individual was not significantly different after comparing the two species of kestrels living in the continent. Average homozygosity by loci was 0.1725 for lesser kestrels and 0.1625 for Eurasian kestrels (t=-0.3797, df=48, P=0.71). In contrast, island kestrels showed significant lower genetic diversity than mainland Eurasian kestrels (Homozygosity by loci estimates: 0.26 vs 0.1626, respectively; t=3.44, df=28, P=0.001). Island subspecies were clumped together given that we did not find significant differences at neutral (F_{ST}<0) or adaptive loci (K_{ST}<0). Kestrel MHC sequences are deposited in GenBank (Acc No. EU120698-EU120722, EF370767-370788 and EU107667-EU107746).

MHC amino acid diversity per site ranged from 0.05 (conserved site) to 0.22 (the most polymorphic site) (see Fig. 2). After comparing paired values of the amino acid diversity parameter *d* at each PBR codon position for both class I and class II loci, we found statistically significant evidence of higher amino acid diversity within the mainland population of Eurasian kestrels than in the lesser kestrel (Wilcoxon matched-pairs test: W+=478.5, W-=187.5, N=36, P=0.023). In contrast, amino acid diversity differences at non-PBR codons was not

significant (Wilcoxon matched-pairs test: W+=199.5, W-=265.5, N=30, P=0.50). We believed that a similar analysis comparing continental and insular populations is not adequate because of the lack of evolution in sympatry and the influence of founder events during island colonization.

DISCUSSION

One of most cited implication underlying MHC theory outlines the role of infectious agents in driving diversifying selection at functionally important loci (reviewed by Sommer 2005, Piertney & Olivier 2006, Acevedo-Whitehouse & Cunningham 2006). However, few studies have demonstrated clear positive correlations between MHC diversity and pathogen species richness in wild populations so far, being clear evidence in this respect restricted to humans and fishes. Thus, Prugnolle and co-workers (2005) showed that genetic diversity at the HLA-B gene was notably influenced by local diversity of intracellular pathogens in human populations. In a similar way, a recent study in the Atlantic salmon (Dionne et al. 2007) reports a positive correlation between the temperature of rivers, which affects the richness and virulence of pathogen communities, and MHC class II diversity across a latitudinal gradient in Eastern Canada. As far as we know, only these two studies have taken into account the roles of neutral evolutionary forces linked to demographic processes and population structure (see Piertney & Oliver 2006). Other studies have shown positive correlations between MHC diversity and parasite diversity in different populations of three-spinned sticklebacks (Wegner et al. 2003) and several species of cyprinid fishes (Simková et al. 2006), but no additional analyses of genetic variation at neutral loci were performed. In birds, MHC variation has been mainly put into the context of different demographic histories (e.g. Richardson & Westerdahl 2003, Hansson & Richardson 2005), local adaptations (e.g. Ekblom et al. 2007, Alcaide et al. 2008), disassortative mating (e.g. Freeman-Gallant et al. 2002, Ekblom et al. 2004, Richardson et al. 2005, Bonneaud et al. 2006b), and

resistance/susceptibility to infectious diseases (e.g. Bonneaud et al. 2006a, Loiseau et al. 2008). Up to the date, ours is the first study that associates detailed sequence polymorphism at both MHC class I and class II genes to extensive surveys of pathogen and parasite diversity in wild avian populations. Moreover, whereas the majority of studies extrapolate the amino acid positions comprising the PBR in humans (e.g. Ekblom et al. 2003, Dionne et al. 2007), we used detailed analyses identifying positively selected amino acid sites within the kestrel MHC (Alcaide et al. 2007, Alcaide et al. 2008, Alcaide et al. CG).

This study shows higher number as well as more divergent alleles on average in the MHC of the continental subspecies of the Eurasian kestrel than in the lesser kestrel (Table 2). Even though the population of the Eurasian kestrel in Spain practically doubles that of the lesser kestrel (Martí & del Moral 2003), we did not find significant differences regarding genetic variation at 8 polymorphic and supposedly neutral microsatellite markers. In addition, further analyses showed that amino acid diversity was significantly different at the codons comprising the PBR, but not at non-PBR sites (Fig. 3). Therefore, our estimates of MHC diversity are not likely influenced by effective population sizes. On the contrary, these results suggest a higher incidence of diversifying selection acting on MHC genes of the Eurasian kestrel due to a higher exposition to pathogens. While fledglings of both Eurasian and lesser kestrels show similar and low pathogen burdens, probably due to the small opportunities to pathogen transmission since hatching until fledging time, both the diversity, richness, and prevalence of pathogens are markedly larger in adult Eurasian than in their sympatric adult lesser kestrels. The larger exposure of Eurasian kestrels to viruses and bacteria (D1 in Fig. 2), which comprise the most virulent pathogens among the wide array we surveyed, might have decisively contributed to its larger MHC diversity.

Several ecological factors and life history traits may explain different risks of infection among host species (Table 1). Most of the work done to date centred on a single or reduced group of pathogen species to test the different hypotheses which could explain such variability. Our approach at the pathogen community lever, however, rather than disentangling hypotheses, supports several of them acting together in a complex way that may explain overall differences in pathogen burdens. For instance, the truly cosmopolitan character of the Eurasian kestrel may have increased the diversity of infectious agents individuals have been exposed to during its evolutionary history (Tella et al. 1999), whilst the lesser kestrel became a steppe-specialist species with more restricted geographic range and habitat uses. In fact, the bacteria *Pseudomonas aeruginosa* and *Mycobacterium avium*, Herpesviruses, as well as intestinal parasites of the genus *Ascaridia*, *Isospora*, and *Cirnea* were exclusively found to infect Eurasian kestrels (Table S2). In contrast, there was no infection found in lesser kestrels that was not detected in Eurasian kestrels as well. Moreover, the broader feeding spectrum displayed by Eurasian kestrels may decisively increase risk of infections when compared to the insect-specialist lesser kestrel, thus explaining the higher prevalences in the former of enterotoxigenic *E.coli*, *Pasterella*, and *Salmonella* which are transmitted from birds, mammal, and reptile prey respectively (Friend and Franson 1999). On the other hand, lesser kestrels were more infected by pathogens such as *Trichomonas*, *E coli*, *Mycoplasma* spp, IH7, and poxvirus, which horizontal transmission among nestlings is enhanced by the close proximity of nests and even the frequent nest-switching of fledglings in the colonies (Tella et al. 1997). The common presence of other species breeding in lesser kestrel colonies, such as domestic pigeons (*Columba livia*, Forero et al. 1996), may increase transmission rates of *Trichomonas and Mycoplasma* to kestrel nests. Finally, the higher prevalence of West Nile virus in adult lesser kestrels may be reasonably related to its migratory behaviour (Hubálek 2004).

Besides pathogen-driven selection regimes, the degree of genetic exchange is expected to determine the extent of local adaptations in open populations. A recent study on lesser kestrels has shown isolation by distance patterns across the Western Palearctic when analysing MHC class II B sequences (Alcaide et al. 2008). Thus, restricted gene flow may favour directional selection of some allelic lineages over others. Conversely, the population of Eurasian kestrels in the Western Palearctic shows high levels of genetic uniformity (Alcaide et al. in second revision, *Heredity*). This finding suggests higher levels of gene flow between populations of Eurasian Kestrels, a fact that would limit the fixation of MHC alleles because of local selection. Nonetheless, although restricted gene flow would be in agreement with a comparably lower number of MHC alleles in the lesser kestrel, the stronger genetic hallmark of diversifying selection at the PBR of the Eurasian kestrel could not be explained by neutral evolutionary forces but because of pathogen and parasite-mediated selective pressures.

Finally, the comparison between mainland and insular subspecies of the Eurasian kestrel constitutes the strongest support for the role of the diversity and amount of infectious agents in driving diversifying selection at MHC sequences. Our surveys of pathogens in islands failed to detect up to 14 types of bacterial, viral, and parasitic infections commonly hosted by continental kestrels. Moreover, among pathogens shared by continental and island kestrels, significant differences in prevalences were higher in continental ones in eight out of ten comparisons. In addition, while neutral selective forces such as population bottlenecks and founder events have provoked the loss of about 25% of microsatellite diversity in the Canary Islands, the fixation rates at MHC sequences seems to have occurred three times faster (Table 4). These results would highlight the inadequacy of using neutral markers as surrogates for genetic variation in fitness-related loci in some situations (see also Aguilar et al. 2004,

Jarvi et al. 2004). Since diversifying selection might be constrained by locally impoverished pathogen communities, we believe that natural selection has promoted the fixation of the most efficient MHC alleles. However, and in agreement with studies on great reed warblers (Richardson & Westerdahl 2003), selection has preserved high genetic divergence. The average number of nucleotide differences between unique alleles in island subspecies has increased in the case of class II alleles but not in the case of class I alleles. These genetic data are congruent with a comparably higher incidence of bacterial species in the Canary Islands (4 out of 7 species isolated in mainland kestrels) in relation to viral infections (only 2 out of 7 types of viral infections isolated in mainland kestrels).

In conclusion, this is one of the very first studies performed over wild avian populations which have related detailed MHC sequence polymorphism to ecological determinants presumably linked to different degrees of pathogen and parasite exposure. Our results support higher levels of MHC variation in generalist rather than in specialist species, as well as low MHC diversity in islands. More research should therefore be encouraged to determine whether reduced MHC diversity may emerge as an additional cost of specialization. In a context of emerging diseases because of global change and human activities (e.g. LaDeau et al. 2007), this study alerts about different degrees of susceptibility to infectious diseased linked to MHC variation. In agreement with the already documented massive extinctions in islands (e.g. Warner 1968, Van Riper et al. 1986), the most dramatic consequences may be derived from the introduction of exotic pathogens and parasites throughout immunologically naïve species (Wikelski et al. 2004, Carrete et al. 2008).

MATERIALS AND METHODS

Study Species and Populations

We sampled continental Eurasian and lesser kestrels from different locations in Spain and the two island subspecies of Eurasian Kestrels inhabiting the Canarian Archipelago (Fig. 1). The Spanish continental population of Lesser Kestrels is estimated at 12,000-20,000 breeding pairs, whilst that of the Eurasian Kestrel is believed to be constituted by 25,000-30,000 breeding pairs (Martí & del Moral 2003). Insular subspecies are represented by 4,000-5,000 for *Falco t. canariensis* and about 400 breeding pairs in *Falco t. dacotiae*.

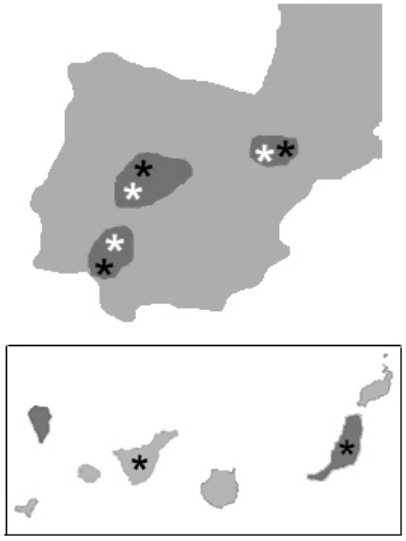

Figure 1. Sampled locations of the Eurasian kestrel (black asterisks) and the lesser kestrel (white asterisks) for genetic analyses. The origin of the individuals sampled for pathogen surveys is indicated by dark grey areas.

Biological samples for genetic analyses and pathogen surveys

For MHC and microsatellite determination, we genotyped 25 Lesser Kestrels hatched in large colonies (>10 breeding pairs), 25 Eurasian Kestrels raised by solitary breeding pairs, and 25 island kestrels including both adults and nestlings. All individuals come from different nests and thus were presumably unrelated given the low rates of extra-pair paternity even in the colonial lesser kestrel

(Alcaide et al. 2005). About 8-9 continental kestrels were sampled for each of the geographic locations indicated by asterisks in Figure 1. Twelve island kestrels were sampled in Fuerteventura and 13 birds were sampled in Tenerife. About 100 µl of blood preserved in 96% ethanol were digested by incubation with proteinase K for at least 3 hours. DNA purification was carried out by using 5M LiCl organic extraction method with chloroform-isoamylic alcohol (24:1) and further DNA precipitation using absolute ethanol. Pellets obtained were dried and washed twice with 70% ethanol, and later stored at –20º C in 0.1-0.2 ml of TE buffer.

For pathogen surveys, adult kestrels were captured from different locations (see Fig. 1) at the nests or using bal-chatri traps, while nestlings were sampled at the nest when they were close to fledge (i.e., ca. 25-days old). As for genetic analyses, we sampled only a fledgling per nest in colonies of lesser kestrels and unrelated breeding pairs in the case of Eurasian kestrels. Only adult birds from the Canary Islands were obtained (see Table S2 for the number of individuals sampled for each species and subspecies). We collected oral and cloacae swabs as well as faecal samples. About 0.1 ml of blood taken from the brachial vein was stored in absolute ethanol for screening pathogens through PCR. Two blood smears were immediately taken and later fixed in the laboratory using absolute ethanol. Individuals for the genetic and species richness surveys were sampled in the same populations during a short period of time (2002-2006). Hence, we did not expect artefacts derived from the analysis of birds which had not been included in the genetic survey. In fact, recent analyses of population structure at MHC class II loci in Lesser Kestrels has shown high levels of genetic uniformity throughout the Iberian Peninsula (Alcaide et al. 2008), which suggests similar pathogen-mediated selective pressures.

Microsatellite and MHC genotyping

Eight microsatellite markers (Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89, Fp107; Nesje et al. 2000, Cl347 see appendix) were amplified using the following PCR profile: 35 cycles of 40s at 94ºC, 40s at 55ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 11 µl reaction contained 0.2 units of Taq polymerase (Bioline), 1x PCR manufacturer supplied buffer, 1.5 mM MgCl2 , 0.02% gelatine, 0.12 mM of each dNTP, 5 pmol of each primer and, approximately, 10 ng of genomic DNA. F-Primers were 5'-end labelled with HEX, NED or 6-FAM. Amplified fragments were resolved on an ABI Prism 3100 Genetic Analyser and further scored using the programmes Genotyper and GeneMapper (Applied Biosystems).

We amplified complete exon 2 sequences of an MHC class II B gene and complete exon 3 sequences of a classical MHC class I gene (see Alcaide et al. 2007, Alcaide et al. CG) using the following PCR profile: 1 cycle of 4 min at 94ºC, 35 cycles of 40s at 94ºC, 40s at 54ºC (for class I loci) or 56ºC (for class II loci), 40s at 72º C and finally, 4 min at 72ºC. Each 25 µl reaction contained 0.4 units of Taq polymerase (Bioline), 1x PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP, 10 pmol of each primer, 5% DMSO and, approximately, 25 ng of genomic DNA. Investigation of variation at MHC loci requires separating the different PCR amplification products because of individuals are likely to be heterozygous. After PCR clean-up in Microcon centrifuge tubes (Millipore), PCR products were cloned into bacterial plasmid using the PGEM-T easy vector system II (Promega). Clones were screened for the expected insert size in 1.5 % agarose gels by running a second PCR with M13 primers. Six to eight positive clones per individual were selected at random for sequencing analysis. Sequencing reactions were carried out using the Big Dye 1.1 Terminator technology and labelled fragments were subsequently resolved in a 3100 automated sequencer (Applied Biosystems).

Estimates of genetic diversity at neutral and adaptive loci

Individual genetic diversity at microsatellites was measured as a means of homozygosity by loci estimates (Aparicio et al. 2006) and compared using un-paired t-tests. MHC sequences were aligned and edited using BioEdit 7.0.5.2 (Hall 1999). Those cloned sequences differing in no more than 2 base pairs with respect to a redundant sequence were considered PCR artefacts. Since recombination of cloned PCR products is an additional source of artefacts (Bradley and Hillis 1996), direct sequencing of uncloned PCR products was used to check for agreement of polymorphic sites with cloned sequences. All alleles found only in one individual were verified by performing a second typing of that individual. Polymorphism statistics were generated using the software DNAsp ver 4.20 (Rozas et al. 2003).

MHC amino acid diversity for both species of continental kestrels evolving in sympatry was estimated for PBR and non-PBR codons separately by means of the diversity index *d* calculated using the programme DIVAA (Rodi et al. 2004). Conserved regions are characterised by low values of *d*, whilst highly polymorphic positions display high values of *d*. A discrepancy between PBR and non-PBR diversity would provide evidence concerning the intensity of selection acting specifically on antigen binding sites of MHC molecules. Putative amino acid sites conforming the PBR of MHC class I and class II molecules in kestrels, i.e. those displaying strong positive selection via an excess of non-synonymous over synonymous nucleotide substitutions, were previously identified using maximum likelihood and Bayesian methods (see Alcaide et al. 2007, Alcaide et al. 2008, Alcaide et al. CG).

Pathogen detection

We studied the presence/absence in each kestrel of 35 organisms recognised as potential avian pathogens, including protozoa, bacterial, viral, fungal, haematozoan, protozoan and metazoan species (Table S2). We therefore covered almost the whole pathogenic community, with the only exception of ectoparasites. The two most common ectoparasites of kestrels are the haematophagous louse flies (Hippoboscidae: *Ornithophila gestroi*) and Carnus flies (*Carnus hemapterus*), which we were unable to correctly sample because they infect nestlings at a very narrow period of growth (Tella 1996). Although unusual, large infestations of louse flies may affect host fitness (Bize et al. 2004). Nonetheless, the low prevalence and abundance of both louse and Carnus flies in kestrels suggests weak effects on their hosts (Tella 1996, Tella et al. 1997, see also Dawson and Bortolotti 1996). On the other hand, the feather-eating Mallophaga mites (Insecta) are very rare in kestrels (authors, unpublish. data), and feather mites (Acari) are considered mutualistic rather than pathogenic (Blanco et al. 2001). Therefore, the no inclusion of these scarce ectoparasites should not affect our study on relationships between pathogen burdens and MHC diversity.

Pathogenic oral fungi *(Candida albicans)* were grown on standard fungical media composed of Agar Sabouraud by incubating at 37ºC for 48 hours. Pathogenic oral *(Pasterella multocida)* and cloacal bacteria *(Salmonella sp., Campylobacter jejuni,* enterotoxigenic *Escherichia coli and Pseudomonas aeruginosa)* were cultured on 5% sheep blood agar, chocolate agar and McConkey agar to avoid *Proteus sp*. overgrowth. Plates were incubated at 37ºC at both normal atmospheric and microaerophilic (10% CO_2) conditions for 24 hours. Suspected colonies were subsequently subcultured on appropriate medium and identified using multi-substrate identification strips (API 20 E; BioMerieux) (see

Blanco et al. 2007 for details). *Campylobacter* colonies were identified through PCR-RFLP of the flagellin gene A (Petersen & Newell 2001. *Mycobacterium avium* was determined by PCR and culture (Aranaz et al, 1997). The presence of other pathogenic microorganisms in blood was determined using PCR-based methods. Thus, the detection of *Chlamydia psittaci* follows the procedure described by Schettler et al. (2003) and *Mycoplasma spp*. were identified as suggested by Mekkes & Feberwee (2005) and Turcsányi et al. (2005). The presence of poxvirus, the paramyxovirus causing the Newcastle disease, the serotypes H5, H7 and H9 of the avian influenza, adenovirus, circovirus, herpesvirus, polyomavirus reovirus and West Nile virus was determined following the PCR-based methods available in the literature (Tadese & Reed 2003, Cardoso et al. 2005, Kiss et al. 2006, Zhang et al. 2006, Schrenzel et al. 2007; Hsu et al. 2006, Farkas et al. 2007, Hofle et al 2008, Potti et al, 2008).

Blood parasites (Haematozoa) were checked through both traditional microscopic screening of blood smears (Tella et al. 1999) and PCR-based methods (Hellgren et al. 2004, Stone et al. 2005) to increase the accuracy of our approach (see Cosgrove et al. 2006, Valkiunas et al. 2006). We looked for the protozoa *Trichomonas gallinae* in the crop mucosa collected with swabs and stored in warm sterile physiologic solution as well as through PCR detection (Grabensteiner & Hess 2006). Fresh faecal samples were examined for coccidian species (Protozooa) by oocyst sporulation with 2.5% potassium dichromate during fourteen days (Forbes et al. 2005), followed by zinc sulfate flotation. For the detection of metazoan helminths (trematodes, acantocephalans, cestodes and nematodes) we used the flotation method with zinc sulphate solution as well as the slide direct examination procedure (Greiner et al. 1994, Clyde & Patton 2001).

Host sample sizes for age classes and species/subspecies were usually large enough (> 15 individuals, range 17-244 except in two cases, see Table S2) to allow obtaining reliable estimates of pathogen prevalences, and thus they were suitable for statistical comparisons (Jovani & Tella 2006).

Analyses of variability in pathogen infection

As a first exploratory overview, we calculated the richness (number of pathogen species), diversity (using the Shannon-Wiener index), prevalence (percentage of birds infected by at least one pathogen) and individual pathogen richness (number of pathogen species per individual) for each species, subspecies and age-class of kestrels sampled. Going deeper, we then tested for differences in the prevalence of each pathogen between continental lesser and common kestrels (considering nestlings and adults separately), as well as between continental and insular adult common kestrels (pooling both island subspecies: *F. t. dacotiae* and *F .t. canariensis*) by means of contigency tables and exact probabilities. Additionally, in an attempt to objectively condense the original variable set of pathogens, we pooled them in groups with biological sense according to their phylogeny and location of infections. We considered only pathogens determined from blood by PCR in order to reach an enough sample size with complete data for all individuals. Thus, we used the number of bacterial (Phylum Actinobacteria, Proteobacteria), virus, and haematozoa (protozoan from blood, Phylum Apicomplexa, Euglenozoa) genera, as well as the presence or not of *Chlamydophila psittaci* (Phylum Chlamydiae), *Mycoplasma* sp. (Division Firmicutes), the fungi *Candida albicans* (Phylum Ascomycota) and *Trichomonas gallinae* (protozoan from the oral cavity, Phylum Metamonada), infecting each individual kestrel. On this data set, we conducted Categorical Principal Components Analysis (CatPCA) to obtain mutually uncorrelated composite factors or dimensions (Meulman et al. 2004). Factor scores of the objects on the

resulting dimensions were extracted and used as dependent variables of MANOVA to test for differences between species/subspecies and age (nestlings and adults).

Capítulo 9

Maintaining MHC alleles at high frequencies: the role of breeding performance and genetic inheritance in lesser kestrels

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ABSTRACT

The major histocompatibility complex (MHC) has become a valuable source of genetic variation for testing selection hypothesis related to fitness associated traits. The frequency-dependent selection theory has become one of the most cited evolutionary mechanisms to explain the extensive polymorphism patterns commonly found at MHC loci. This phenomenon leads to cyclic fluctuations of allele frequencies translated into low rates of allelic fixation, but only a handful of empirical studies conducted for wild species have documented selection in favour of rare alleles. In the present study, the integration of genetic and productivity data in captive and free-ranging populations of lesser kestrels has supported contemporary positive selection of common MHC haplotypes. We found a higher incidence of common class II alleles within the most successful breeding individuals kept in captivity (N=41 individuals) than in 50 nestlings hatched in wild colonies (0.66 vs 0.43, P<0.01) or other breeding birds (N=52 individuals) from the captive stocks (0.66 vs .0.44, P=0.012). After controlling for genetic variation at neutral loci, our results were not attributable to founder events or differences in individual genetic diversity. In addition, significant deviations in Mendelian proportions were reported for common MHC (N=204 nestlings, P<0.001) but not for abundant microsatellites alleles. Positive selection at frequent or still increasing class II sequences may have evolved as a means of maintaining largely enough reservoirs of selectively advantageous alleles when loci are extremely polymorphic. Finally, we discuss the possible implications derived from this study in relation to the genetic management of captive and reintroduction programs.

INTRODUCTION

During the last two decades, the major histocompatibility complex (MHC) has become the paradigm of how natural selection shapes and maintains extraordinary levels of genetic variation at functionally important genes (reviewed by Bernatchez & Landry 2003, Piertney & Oliver 2006). MHC genes code for cell-surface glycoproteins directly involved in the development of immune responses in vertebrates (Klein 1986). Thus, pathogen-mediated selective pressures have been suggested to drive the evolution of MHC molecules during the arms race between pathogens and their hosts. Resilience against a broad spectrum of infectious diseases is thought to be promoted by a high number of MHC variants, a fact that is supported by the documenting of the most polymorphic coding regions described so far within the MHC (e.g. Robinson et al. 2005). In particular, the majority of studies have focused attention on genetic diversity at the exon 2 of MHC class II B genes and the exons 2 and 3 of classical MHC class I genes (e.g. Westerdahl et al. 2004a, Prugnolle et al. 2005, Mainguy et al. 2006, Dionne et al. 2007, Alcaide et al. 2008, but see Acevedo-Whitehouse & Cunningham 2006). These loci encode the amino acid chains that shape the highly variable peptide binding region (PBR) involved in the presentation of microbial antigens to specialized cells of the immune system. The recognition of foreign peptides bound to MHC molecules subsequently triggers the development of adaptive immune responses aimed at controlling pathogen and parasite infections (reviewed by Sommer 2005).

Several evolutionary mechanisms have been proposed to maintain the high levels of MHC diversity needed to counteract the selective pressures imposed by pathogens. Two main types of balancing selection, 'heterozygote advantage' and 'frequency-dependent selection' have been the most widely investigated so far (reviewed by Bernatchez & Landry 2003, Sommer 2005) . The 'heterozygote

advantage' hypothesis predicts that individuals carrying different MHC alleles may be able to cope with a more diverse pathogen fauna than homozygous because of a broader array of antigen-binding properties. On the other hand, the 'frequency-dependent selection' hypothesis predicts that common MHC alleles might be selected against as soon as novel pathogenic strains avoid the most extended host immune defences. However, only a handful of empirical studies conducted for wild populations have provided clear empirical evidence supporting any of these evolutionary frameworks (reviewed by Sommer 2005, Piertney & Oliver 2006).

Selectively advantageous MHC alleles are expected to increase their frequencies in open populations because they should differentially enhance survival and reproductive parameters in some individuals. Besides being associated with resistance/susceptibility to lethal infectious diseases (e.g. Nagaoka et al. 1999, Westerdahl et al. 2005, Bonneaud et al. 2006a), the MHC has been widely related to fundamental stages of reproduction including mate choice (reviewed by Tregenza & Wedell 2000, Piertney & Oliver 2006), embryo development (Wedekind et al. 1996, Edwards & Hedrick 1998, Skartein et al. 2005) and expression of secondary sexual traits (von Schantz et al. 1995, Ditchkoff et al. 2001). In the same line, female preferences for genetically diverse males or certain MHC haplotypes are thought to increase offspring fitness (e.g. Reusch et al. 2001, Ekblom et al. 2004).

In the present study, we looked for MHC class II sequences (exon 2) at high frequencies in lesser kestrels *Falco naumanni*. This species is highly suitable for this purpose because one single polymorphic and positively selected MHC class II locus can be specifically amplified via PCR (see Alcaide et al. 2008). This fact solves many of the statistical barriers usually associated to avian MHC, which includes the amplification of gene duplicates, pseudogenes or low polymorphic

loci (Edwards et al. 1998, Hess et al. 2000, Gasper et al. 2001, Bonneaud et al. 2004a, Aguilar et al. 2006). In accordance with the frequency-dependent selection theory, the most common MHC alleles may be decreasing or still increasing their frequencies in the population depending on the selective pressures exerted by pathogens at the present evolutionary time frame. To test whether common MHC alleles are currently selectively favoured or not, we investigated the genetic profiles of the most prolific breeding individuals kept in captivity. In addition, natural selection is expected to cause deviations from typical Mendelian proportions, and thus, we investigated the segregation of MHC haplotypes from parents to fledglings. In this respect, the relative homogeneity of environmental and management conditions within captive stocks may minimize biases linked to stochastic processes on fitness parameters (e.g. Serrano et al. 2005). We additionally used genetic variation at 8 polymorphic microsatellite markers and the inheritance of abundant microsatellite alleles to control for the effect of neutral evolutionary forces. Besides providing valuable empirical data concerning the selective mechanisms shaping patterns of adaptive genetic variation, this study also shed light on the implications of MHC composition on fitness-related traits.

MATERIALS AND METHODS

Study species and populations

The lesser kestrel *Falco naumanni* is a small migratory falcon usually associated to urban and rural environments, where colonies of up to 100 breeding pairs can be usually found in old human structures such as churches, castles or abandoned farmhouses (Cramp & Simmons 1980, Negro 1997). Lesser kestrels are patchily distributed across low and mid elevations of Eurasia, extending their breeding range from Portugal to China. We investigated two geographically distinct wild populations of lesser kestrels in Spain and three captive populations that were

maintained to provide individuals for reintroduction programs (DEMA in Extremadura, GREFA in Madrid and TORREFERRUSA in Catalonia) (see Fig. 1).

Fig. 1. Breeding distribution of the lesser kestrel in Spain (shaded areas). The location of captive breeding populations is indicated by asterisks.

DNA purification

Blood and feathers were taken from adult birds and nestlings. Blood samples were preserved in absolute ethanol and feathers pulled from the bird's back were stored in paper envelopes or plastic bags and kept at 4º C. The DNA purification protocol we used follows that described by Gemmell and Akiyama (1996). Blood and feathers tips were digested by incubation with proteinase K for at least 3 hours. DNA purification was carried out by using 5M LiCl, organic extraction with chloroform-isoamylic alcohol (24:1) and DNA precipitation with absolute ethanol. Pellets obtained were dried and washed twice with 70% ethanol, and later stored at –20º C in 0.1ml of TE buffer.

Microsatellite genotyping

We amplified eight microsatellite markers originally isolated in the peregrine falcon *Falco peregrinus.* Loci Fp5, Fp13, Fp31, Fp46-1, Fp79-4 and Fp89 were

available from the study carried out by Nesje and co-workers (2000). Two set of primers flanking two microsatellite sequences available in GenBank (AF448412 and AF448411, respectively) were additionally designed. Locus Cl347 was amplified using primers Cl347Fw: tgtgtgtgtaaggttgccaaa and Cl347Rv: cgttctcaacatgccagttt). Locus Cl58 was amplified using primers Cl58Fw: tgtgtctcagtggggaaaaa and Cl58Rv: tgctttggtgctgaagaaac). For each locus, the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following PCR profile: 35 cycles of 40s at 94ºC, 40s at 55ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 11 µl reaction contained 0.2 units of Taq polymerase (Bioline), 1x PCR manufactured-supplied buffer, 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP, 5 pmol of each primer and, approximately, 10 ng of genomic DNA. F-Primers were 5'-end labelled with HEX, TET or 6-FAM. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyser (Applied Biosystems). Conformity to Hardy-Weinberg expectations and linkage disequilibrium was analysed using GENEPOP (Raymond & Rousset 1995).

MHC genotyping

The second exon of a positively selected and putatively functional MHC class II β locus was amplified using the following PCR profile (see Alcaide et al. 2007 and Alcaide et al. 2008 for details): 1 cycle of 4 min at 94ºC, 35 cycles of 40s at 94ºC, 40s at 56ºC, 40s at 72º C and finally, 4 min at 72ºC. Each 25 µl reaction contained 0.4 units of Taq polymerase (Bioline), 1x PCR manufacturer-supplied bufffer (Bioline), 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP, 10 pmol of each primer, 5% DMSO and, approximately, 20 ng of genomic DNA. PCR products were clean-up in Microcon centrifuge tubes (Millipore) and further cloned into bacterial plasmid using the PGEM-T easy vector system II (Promega). This step was necessary taking into account that individuals are likely to be heterozygous and thus, alleles are needed to be separate before sequencing.

Clones were screened for the expected insert size in 1.5 % agarose gels by running a second PCR with M13 primers. Six to eight positive clones per individual were selected at random for sequencing analysis. Recombination during cloning procedures can be translated into PCR artefacts (Bradley & Hillis 1996), and thus, direct sequencing of uncloned PCR products was used to check for agreement of polymorphic sites. Sequencing reactions were carried out using the Big Dye 1.1 Terminator technology and labelled fragments were subsequently resolved in a 3100 automated sequencer (Applied Biosystems).

Survey of MHC and microsatellite diversity in free-ranging populations of lesser kestrels

We amplified complete exon 2 sequences from 50 lesser kestrels hatched in wild colonies located in south-western Spain (N = 25 individuals) and north-eastern Spain (N = 25 individuals). All individuals belonged to different nests, and therefore, they were presumably unrelated. Cloned MHC sequences were aligned and edited using the programme BioEdit 7.0.5.2 (Hall 1999). Following Edwards and co-workers (1995), those sequences differing less than 3 bp from a redundant sequence were discarded since they must be the results of PCR errors. Class II alleles found only in one individual were verified by performing a second typing of that individual. The phylogenetic relationships between MHC sequences were visualized using the software SplitsTree 4 (Huson & Bryant 2006). Our aim at this point was obtaining the distribution of allele frequencies at the MHC class II in the Spanish population of lesser kestrels. The finding of MHC sequences at high frequencies will allow us to establish hypothesis and predictions concerning selection in favour or against particular MHC alleles or allelic lineages. The number and frequency distribution of microsatellite alleles were calculated using the software GENETIX 4.04 (Belkhir et al. 1996-2004. In addition, individual

microsatellite diversity was measured as a means of homozygosity by loci estimates (Aparicio et al. 2006).

Selection of breeding kestrels kept in captivity

We investigated the genetic profiles of 41 lesser kestrels which repeatedly registered high breeding success in captivity. We focused on the best three consecutive years of reproduction of each bird to avoid variance associated to lack of experience or senescence. Given that breeders perform partial or total removal of the eggs to induce a second an even a third clutch (Pomarol et al. 2004), we considered the ratio between the total number of fledglings and the total number of eggs laid per pair as an indicator of reproductive fitness. Fifty-two breeding birds were additionally sampled at random from the three captive populations. The average egg-to-fledgling success was 0.84 for highly successful breeding birds and 0.44 for randomly chosen breeding individuals (Table 1). The influence of common MHC alleles over the egg-to-fledgling ratio was also investigated in 27 breeding pairs which were mated during at least three years in a row. We used a binomial Generalized Linear Mixed Model in which the number of eggs was introduced as a binomial denominator in the response variable and the identity of the captive population was treated as a random term to account for non-independence of birds sampled within the same breeding stock. The number, properties and origin of the lesser kestrels investigated in this study are shown in Table 1.

Inheritance of MHC alleles from parents to fledglings

We investigated the segregation of MHC alleles from parents to the offspring in 25 kestrel families raised in captivity as well as 15 kestrel families sampled from two wild colonies located in south-western Spain. We used a minimum sample size of 4 nestlings per family to allow that all possible combinations of alleles can occur.

Sample size per family ranged from 4 to 10 nestlings. Overall, 204 nestlings were analysed during the 2004-2007 breeding seasons.

Table 1. Breeding parameters and frequencies of common MHC class II sequences in lesser kestrels sampled from wild colonies and captive breeding populations.

Origin of individuals analysed	N	Frequencies of common clusters of MHC sequences	Egg-to-fledgling Success
Nestlings hatched in wild colonies	**50**	**0.43**	**-**
Successful breeding individuals from DEMA	21	0.67	0.87
Successful breeding individuals from GREFA	10	0.65	0.77
Successful breeding individuals from TORREFERRUSA	10	0.65	0.86
All successful breeding individuals from captive stocks	**41**	**0.66**	**0.84**
Randomly sampled breeding individuals from DEMA	22	0.50	0.51
Randomly sampled breeding individuals from GREFA	18	0.52	0.34
Randomly sampled breeding individuals from TORREFERRUSA	12	0.21	0.42
All randomly sampled breeding individuals from captive stocks	**52**	**0.44**	**0.44**

Since parental MHC alleles were known from cloning techniques, the MHC class II locus was directly sequenced in nestlings. This approach is adequate taking into account that the average genetic divergence between unique alleles is above 24 nucleotide differences (see Alcaide et al. 2008). Observed versus expected frequencies of common MHC alleles in the offspring were subsequently compared using non-parametric Wilcoxon matched-paired signed-rank tests. We

controlled for genetic inheritance of common microsatellites alleles by analysing paternity data of 43 families of lesser kestrels from two previous studies (Alcaide et al. 2005, Alcaide et al. CG).

RESULTS

Genetic variation at microsatellites and MHC class II sequences in free-ranging populations

Polymorphism statistics at 8 microsatellite markers are summarized in Table 2. No marker departed significantly from Hardy-Weinberg expectations. Up to 44 different MHC class II sequences (GenBank Accession No: EF370767-370788 and EU107667-EU107746) were isolated from 50 lesser kestrels and only one bird was homozygous. The most abundant MHC alleles were Fana2 (17%), Fana19 (12%) and Fana1 (6%). The alleles Fana1 and Fana2 only differed in one single and non-synonymous nucleotide substitution (see also Alcaide et al. 2008). Although it is currently thought that only a few amino acid differences may confer different degrees of protection (e.g. Hill 1998, Froeschke & Sommers 2005, Bonneaud et al. 2006a), closely related alleles are expected to have similar antigen binding properties (e.g. Trachtenberg et al. 2003, Meyer-Lucht & Sommer 2005). Thus, we investigated the relative frequencies of the two most abundant clusters of MHC sequences (see Fig. 2, in red), rather than individual alleles. The average number of nucleotide differences within each cluster was 2.53 for similar alleles to Fana1 and Fana2, and 4.00 for similar alleles to Fana19. The combined frequency of the two most abundant clusters of MHC alleles was 0.43. Previous analyses of population structure within the Iberian Peninsula revealed genetic uniformity regarding the distribution of allele frequencies at the MHC class II locus (see Alcaide et al. 2008 for details).

Table 2. Polymorphism statistics at 8 microsatellite markers in the Spanish population of lesser kestrels. The most frequent alleles at some markers are indicated

Locus	No. of alleles	Size Range (bp)	He	Ho	Common alleles
Fp5	6	99-109	0.67	0.65	105
Fp13	4	86-106	0.58	0.56	92
Fp31	7	128-142	0.70	0.71	130
Fp46-1	10	115-139	0.57	0.56	125
Fp79-4	41	125-192	0.94	0.92	
Fp89	4	116-122	0.52	0.49	118
Cl58	6	118-123	0.46	0.46	
Cl347	11	96-116	0.79	0.79	104

Allele frequencies of the MHC class II locus and individual microsatellite diversity within the most successful breeding individuals kept in captivity

The relative frequencies of common MHC sequences were significantly higher within the most successful breeding birds kept in captivity than in nestlings sampled at random in wild colonies (0.66 vs 0.43; *Mann-Whitney U-test*, U=692.5, z=2.65, two-tailed P=0.008) and other reproductive birds from the captive stocks (0.66 vs 0.44; *Mann-Whitney U-test*, U=763; z=2.34; two tailed P=0.02). Females showed a slightly higher incidence of common MHC sequences than males (0.69 for females, N=22 vs 0.63 for males, N=19), but such differences were not statistically significant (*Mann-Whitney U-test*, U=223.5; z=-0.37; two tailed P=0.71). No significant differences were found between wild birds and non-highly successful captive breeding kestrels (0.44 vs 0.43; *Mann-Whitney U-test*, U=1310.5; z=-0.07; two tailed P=0.94). In addition, results from the binomial GLMM analysis showed a strong positive relationship between the eggs-to-fledgling ratio and the number of common MHC alleles (estimate ± SE = 0.4698 ± 0,0857; $F_{1,22}$ = 30.07, P < 0.0001). Concerning genetic variation at

supposedly neutral microsatellites, average homozygosity by loci within the most successful captive birds was not significantly different when compared to the set of 50 wild nestlings that were MHC-typed (H_L:0.31 vs 0.33; *t-test*, t=0.589; df=87; P=0.56).

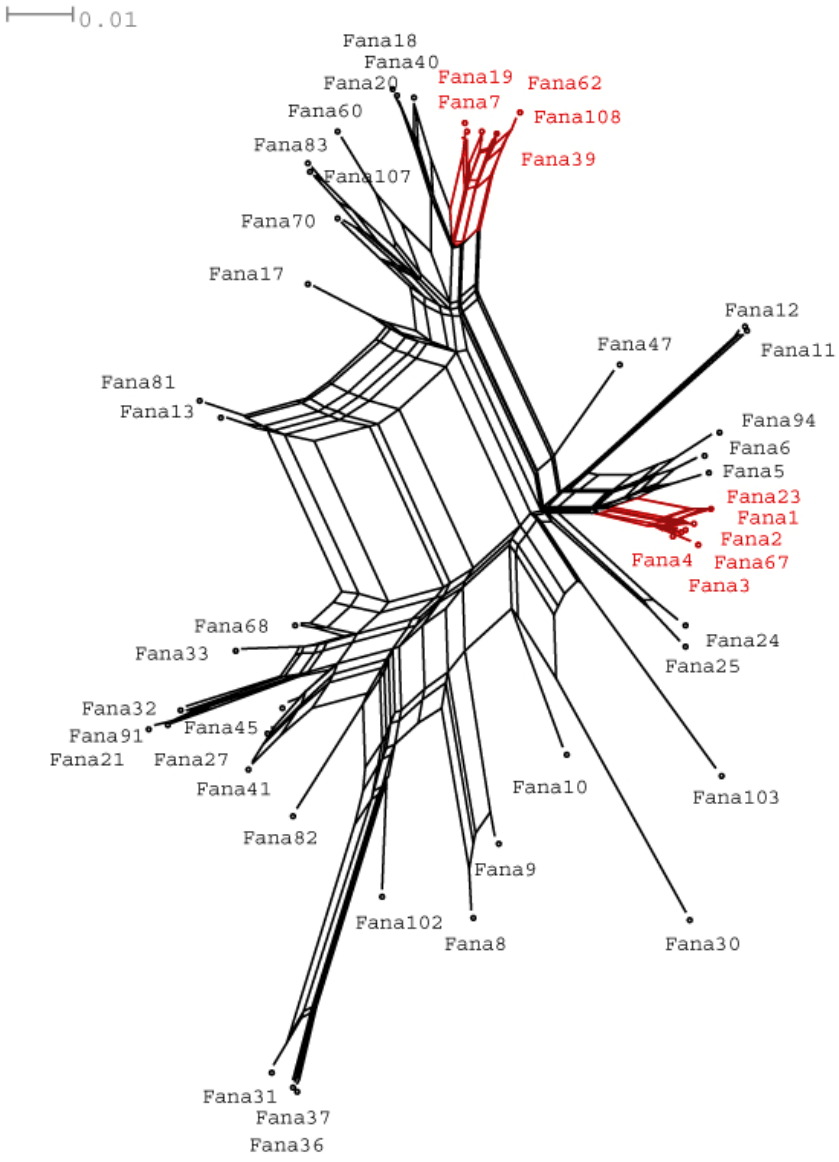

Fig. 2. Neighbor-net network built from MHC class sequences of the Lesser Kestrel using maximum likelihood distances. The two most abundant clusters of closely related alleles found in Spain are indicated in red.

Genetic inheritance of common MHC and microsatellite alleles from parents to the offspring

We found that the frequencies of common MHC alleles in fledglings were significantly higher than expected according to typical Mendelian proportions (Non-parametric Wilcoxon matched-pairs signed-rank test: W-=45, W+=483, N=33, P<0.001). Results remained significant when we restricted the analysis to captive or wild individuals alone (P<0.001). In contrast, we did not detect significant differences between expected and observed frequencies when the most abundant alleles at six polymorphic microsatellites were analyzed (Non-parametric Wilcoxon matched-pairs signed-rank test: W-=170, W+=265, N=29, P=0.31).

DISCUSSION

This study is one of the firsts that provides empirical evidence for positive selection of common MHC alleles. To date, the very few published studies that have been conducted for free-ranging vertebrate populations have shown negative frequency-dependent selection (e.g. Paterson et al. 1998, Froeschke & Sommer 2005, Harf & Sommer 2005, Meyer-Luncht & Sommer 2005, Schad et al. 2005). Our results rely on a crucial component of individual fitness such as breeding output. In this regard, we found a higher incidence of common MHC haplotypes within the best captive breeding kestrels, as well as significant deviations from typical Mendelian proportions in their genetic inheritance from parents to fledglings. Conversely to the majority of studies, and particularly those conducted on birds, statistical support for these two findings was facilitated here by two conditions that have been rarely met in open populations of non-laboratory species. Firstly, the specific amplification of one single polymorphic and positively selected MHC locus (Alcaide et al. 2007, Alcaide et al. 2008), and secondly, the acquisition of detailed sequence data allowing the identification of clusters of closely related alleles. In this respect, it is broadly assumed the

difficulty to harness adequate statistical support in many MHC studies due to extensive or low polymorphism (e.g. Hess et al. 2000, Gasper et al. 2001, Lund et al. 2004) and the lack of an adequate allelic assignment to locus (e.g. Ekblom et al. 2004, Bowen et al. 2006).

Understanding the genetic cues linked to variance in breeding performance is one of the most fascinating topics in evolutionary biology. In birds, individual genetic diversity has been correlated to reproductive fitness components such as clutch size (Ortego et al. 2007b) or hatching failure (Bensch et al. 1994, Kempenaers et al. 1999, Mackintosh and Briskie 2003). The majority of studies have however investigated genetic variation at a short array of supposedly neutral genetic markers. These kinds of approaches are currently considered as poor surrogates of individual fitness, specially in outbred populations or when clear association between some neutral markers and fitness-related loci can not be clearly demonstrated (e.g. Balloux et al. 2004, Aguilar et al. 2004, Hansson et al. 2004a, DeWoody and DeWoody 2005). This fact may explain the extremely weak or even the lack of statistical relationships found in some studies, and the probably strong bias through no published studies documenting negative results (reviewed by Coltman & Slate 2003). In this context, and contrary to a recent study achieved in wild colonies of lesser kestrels (Ortego et al. 2007b), our estimates of breeding output can not be explained by variances in individual genetic diversity. Nevertheless, considering the low strength of the correlation showed by Ortego and co-workers, statistical support would probably require a much larger sample size.

To date, the role of functionally important MHC loci on breeding performance in birds has been mainly restricted to patterns of mate choice, extra-pair paternity rates or genetic compatibility (e.g. Ekblom et al. 2004, Richardson et al. 2005, Bonneaud et al. 2006b). The increase of common MHC alleles within

the most successful breeding kestrels kept in captivity would suggest that these alleles are the most selectively advantageous at the present evolutionary time frame. The possibility that our results were affected by founder events is rejected by the lack of any F_{ST} value significantly different from zero when comparing patterns of microsatellite variation between captive and source natural populations (Alcaide et al. in thrid review, *Conservation Genetics*). Further, differences in MHC frequencies between captive and wild populations are exclusively significant when analysing highly successful captive birds.

Infectious diseases have already been suspected to dramatically decrease productivity rates in captive populations of poultry and waterfowl (e.g. Yoder & Hofstad 1964, Hwang 1965, Pomeroy & Nagaraja 1991), and in lesser kestrels as well (Colás et al. 2002). In this respect, it is also currently assumed that there is a trade-off between the costs of triggering an immune response and those allocated to energetically expensive physiological functions such as reproduction (e.g. Ardia et al. 2003, Sanz et al. 2004, Uller et al. 2006, Weil et al. 2006, Marzal et al. 2007, but see Williams et al. 1998). Thus, selectively advantageous MHC alleles are expected to favour energetic investments in reproductive effort. In lesser kestrels, individual fitness of males and females may be important considering that, in this species, both sexes contribute to eggs incubation (e.g. Donázar et al. 1992). Poor conditions of parents have been suggested as one of the principal causes of hatching failure in some bird species, including lesser kestrels (Serrano et al. 2005). On the other hand, it is known that maternal immunity is transferred to the embryo via the amniotic fluid and the egg yolk. The role of maternal antibodies should be crucial before the development of the nestling own immune system (reviewed by Grindstaff et al. 2003). We have not found, however, significant higher frequencies of common MHC alleles in females than in males. In any case, the fact that females may be selecting males with

selectively advantageous alleles can not be dismissed (see for instance data on the Great Snipe *Gallinago media*, Ekblom et al. 2004).

From an immunological perspective, the first weeks of nestling's life are considered the most critical, coinciding with the education of immune cells in a specialized avian immune organ called bursa of Fabricius (Wakenell 1999). The inheritance of selectively advantageous MHC alleles may be translated into a more efficient immune system during a period where pathogen infections can seriously compromise nestling's fitness and survival (e.g. Wiese et al. 1977, Mills et al. 1999, Potti et al. 2002, Bonneaud et al. 2004b).This fact may explain the increased productivity rates, measured as the egg-to-fledgling ratio, at both individual (Table 1) and breeding pair level. Biased survival in favour of new-borns birds linked to common MHC alleles is supported by statistically significant deviations from Mendelian proportions in fledglings. Nonetheless, more research is still needed to determine whether selection is acting mainly through selective fertilizations (e.g. Skarstein et al. 2005), embryo development or after hatching.

Finally, the relevant incidence of common MHC alleles within the most successful breeding individuals as well as their preferential inheritance in fledglings have direct implications for the genetic management of captive and reintroduction programs. A previous study relying on patterns of variation at supposedly neutral microsatellites has documented significant decreased average heterozygosities and increased inbreeding coefficients in reintroduced populations in relation to the captive demes from which released bird come from. This fact was attributed to large variances in reproductive success among breeding individuals (Alcaide et al. in third revision, *Conservation Genetics*). The investigation of MHC data alerts about a more pronounced founder effect acting on functionally important genes demanding high levels of genetic diversity. This study therefore provides valuable data in relation to the debate about whether

preservation of genetic diversity at MHC loci during captive breeding and reintroduction programs should be mandatory or not (see Hughes 1991, Miller & Hedrick 1991). Our findings in this respect strongly encourage the spatial and temporal diversification of fledglings from different broods and different captive flocks, as well as the promotion of immigration to minimize genetic impoverishment at evolutionary relevant MHC loci in reintroduced populations.

In conclusion, this study shows increased egg-to-fledgling success in association with common or increasing clusters of closely related MHC class II alleles. We suggest that these mechanisms should counteract the fluctuations linked to stochastic processes that could mitigate the frequencies of selectively advantageous alleles when loci are extremely polymorphic. That is the case of the MHC class II B gene of the lesser kestrel, where more than 100 different alleles have been isolated (Alcaide et al. 2008). In any case, we expect pathogen-mediated selection regimes to considerably vary in time in order to balance rates of allele fixation (see for instance Westerdahl et al. 2004b). Athought hatching failure and nestling survival are undoubtedly two clear fitness-related parameters, this study demands future research aimed at demonstrating lower pathogen and parasite incidence in individuals showing the abundant MHC motifs proposed here.

CONCLUSIONES

1. La segregación de alelos de microsatélite de acuerdo a las leyes de Mendel ha confirmado una baja incidencia de paternidades extra-pareja en el cernícalo primilla así como el primer caso documentado de fertilización mixta.

2. Las altas tasas de filopatría así como los sesgos en los patrones de dispersión hacia distancias geográficas cortas no se traducen en el desarrollo de patrones de diferenciación genética a pequeña escala en el cernícalo primilla. Este hallazgo pone de manifiesto la idoneidad de combinar tradicionales metodologías de seguimiento basadas en la captura-marcaje-recaptura de individuos con innovadoras técnicas genéticas que permiten inferir indirectamente el grado de conectividad en poblacionales fragmentadas.

3. Aunque la fragmentación de las poblaciones a pequeña escala no ha sido suficientemente importante con respecto a las capacidades dispersivas de la especie, la pérdida de hábitat promueve la diferenciación genética a escala continental en una especie típicamente esteparia como el cernícalo primilla. Esta hipótesis es soportada tras el contraste proporcionado por el cernícalo vulgar, una especie filogenéticamente próxima, simpátrica y generalista, que no ha sufrido el declive poblacional de origen antropogénico que han experimentado muchas especies en Eurasia.

4. Si bien el cernícalo vulgar emerge como una especie panmíctica a escala continental, el cernícalo primilla ha evidenciado de forma significativa patrones de aislamiento por distancia. No obstante, el intercambio de

individuos entre poblaciones parece haberse mantenido a unas tasas lo suficientemente elevadas como para evitar el empobrecimiento genético de la especie. Esto sugiere que la diversidad genética de las poblaciones de cernícalo primilla sólo ha de verse seriamente mermada tras cuellos de botella poblacionales acompañados de un aislamiento geográfico pronunciado.

5. La evaluación de los reproductores utilizados para la cría en cautividad del cernícalo primilla no ha detectado un empobrecimiento genético que pudiera explicar la alta incidencia de fallos en la eclosión de huevos que frecuentemente se producen en las poblaciones cautivas. Sin embargo, si se han detectado pérdidas significativas en la transmisión de la diversidad genética desde los stocks cautivos a los individuos liberados en los programas de reintroducción. Este fenómeno es atribuido a la elevada heterogeneidad en la contribución de cada una de las parejas reproductoras, lo que se traduce a su vez en un descenso del tamaño efectivo de la población.

6. Con el fin de garantizar niveles adecuados de diversidad genética que aumenten la viabilidad de las poblaciones reintroducidas a largo plazo, aconsejamos la liberación de juveniles criados en cautividad no relacionados genéticamente en la misma localización así como el fomento del reclutamiento de individuos salvajes.

7. La aplicación de técnicas moleculares basadas en la PCR han permitido el aislamiento y caracterización de genes polimórficos de MHC de clase II y clase I en las grandes familias de aves de presa. Estos marcadores genéticos, sometidos a fuertes presiones selectivas inducidas por

patógenos, suponen una valiosa aportación en los campos de la ecología evolutiva y genética de la conservación para este grupo taxonómico globalmente amenazado.

8. Análisis detallados del polimorfismo en las secuencias de MHC reportaron fuertes presiones selectivas sobre determinados aminoácidos, así como un papel predominante de procesos recombinatorios en la generación de dicho polimorfismo. En lo relativo a organización y estructura genética, las aves de presa se asemejan más a los galliformes que a los paseriformes, lo cual incluye un bajo número de duplicaciones génicas y una ausencia detectable de pseudogenes. Desde un punto de vista evolutivo, se hallaron evidencias a favor de los mecanismos de evolución concertada y evolución transespecífica del polimorfismo.

9. El cernícalo primilla ha mostrado más de 100 variantes alélicas en un sólo gen de MHC de clase II. La divergencia entre secuencias nucleotídicas se ajusta a un modelo de aislamiento por distancia congruente con la existencia de adaptaciones locales y flujo génico restringido. El grado de estructuración genética en el MHC es mucho más pronunciado que el exhibido por marcadores neutrales de microsatélite, exhibiendo estos últimos altos niveles de homoplasia. Este hallazgo apoyaría por tanto las hipótesis que sugieren variaciones espaciales en las presiones selectivas ejercidas por patógenos como una de las causas del elevado polimorfismo en los genes del MHC.

10. La diversidad genética en genes del MHC parece estar en consonancia con determinantes ecológicos y de historia vital que delimitan las interacciones entre las comunidades de patógenos y sus hospedadores.

Extensos análisis en comunidades de patógenos en el cernícalo primilla y 3 subspecies del cernícalo vulgar han demostrado una posible relación directa entre la diversidad de patógenos a los que se enfrenta el hospedador y los niveles de polimorfismo genético en genes del MHC. Las estimaciones en variación genética adaptativa permitirían en cierta manera predecir distintos grados de vulnerabilidad en un contexto actual de enfermedades infecciosas emergentes.

11. Por último, esta tesis aporta evidencias significativas que sugieren selección contemporánea de alelos comunes de MHC de clase II. Nuestros análisis muestran un incremento significativo de alelos frecuentes en individuos reproductores excelentes, así como desviaciones significativas en las proporciones Mendelianas durante la segregación de dichos alelos de padres a hijos. Estos mecanismos deben mantener reservas suficientes de alelos selectivamente ventajosos como mecanismo compensatorio ante las posibles variaciones generadas por procesos estocásticos cuando los genes son extremadamente polimórficos.

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