JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2012.02529.x

SHORT COMMUNICATION

Colonizing the world in spite of reduced MHC variation

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Keywords:

adaptive potential;
Falco genus;
immunity;
MHC diversity;
pathogen-mediated selection.

Abstract

The major histocompatibility complex (MHC), which harbours the most polymorphic vertebrate genes, plays a critical role in the host–pathogen coevolutionary arms race. However, the extent to which MHC diversity determines disease susceptibility and long-term persistence of populations is currently under debate, as recent studies have demonstrated that low MHC variability does not necessarily hamper population viability. However, these studies typically assayed small and decimated populations in species with restricted distribution, thereby making inferences about the evolutionary potential of these populations difficult. Here, we show that MHC impoverishment has not constrained the ecological radiation and flourishing of falcons (Aves: Falconidae) worldwide. We found two remarkably different patterns of MHC variation within the genus *Falco*. Whereas MHC variation in kestrels (the basal group within the genus) is very high, falcons exhibit ancestrally low intra- and interspecific MHC variability. This pattern is not due to the inadvertent survey of paralogous genes or pseudogenes. Further, patterns of variation in mitochondrial or other nuclear genes do not indicate a generalized low level of genome-wide variability among falcons. Although a relative contribution of genetic drift cannot be completely ruled out, we propose the falcons went through an evolutionary transition, driven and maintained by natural selection, from primarily highly variable towards low polymorphic and slow-evolving MHC genes with a very specific immune function. This study highlights that the importance of MHC diversity cannot be generalized among vertebrates, and hints at the evolution of compensatory immune mechanisms in falcons to cope with emerging and continuously evolving pathogens.

Introduction

It is widely assumed that decreased variation at adaptive loci may have negative effects on individual fitness and long-term population survival, as these populations would have reduced potential for future adaptation to environmental change (Allendorf & Luikart, 2007). Genes of the major histocompatibility complex (MHC) are thought to play an essential role in the adaptive immune response of jawed vertebrates by coding for molecules that recognize and present antigenic peptides to T lymphocytes, thereby initiating an adaptive immune response (Klein, 1986). The extensive polymorphism and unusual persistence of MHC alleles are clearly of adaptive significance, and their maintenance is mainly promoted by balancing selection resulting from host–pathogen coevolution (Sommer, 2005; Spurging & Richardson,

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2010). Theory predicts that populations demonstrating higher MHC diversity can respond to a broader spectrum of pathogens (Sommer, 2005; Meyer-Lucht & Sommer, 2009). However, evidence that loss of MHC variation negatively affects population survival is so far equivocal (Acevedo-Whitehouse & Cunningham, 2006).

In an increasing number of cases, the observed patterns apparently do not follow the predictions of MHC diversity; that is, populations do not always show increased disease susceptibility or viability despite eroded MHC polymorphism (Mikko & Andersson, 1995; Babik *et al.*, 2005, 2009; Radwan *et al.*, 2010; Castro-Prieto *et al.*, 2011). However, many of these studies focused on populations that are small and confined to their original geographic distributions; thus, population response to novel and potentially harmful pathogens is difficult to determine for species with potential to encounter a greater range of immune challenges. Nevertheless, dramatic negative impacts, including population declines and even extinctions, have certainly been documented, for example, when endemic avian species occupying isolated archipelagos were exposed to novel pathogens, such as malaria or poxvirus, causing diseases transmitted by introduced mosquito vectors (e.g. Wikelski *et al.*, 2004).

Here, we investigated the evolution of MHC genes in the genus *Falco* by using a comprehensive phylogenetic approach that includes species with very restricted distributions, such as the Mauritius kestrel (*F punctatus*), as well as some with near-global distribution (Table S1), such as the peregrine falcon (*F. peregrinus*). The genus *Falco* (Falconiformes: Falconidae) is represented by 37 species of small- to medium-sized predatory birds that occur on all continents except Antarctica (Cramp & Simmons, 1980; del Hoyo *et al.*, 1994). They are present in most habitats, from tundra to desert to tropical forest. We compiled data from twelve *Falco* species located at both basal (hereafter kestrels) and more diverged steps (hereafter falcons) in the mitochondrial DNA-based phylogeny proposed for this avian clade (Roulin & Wink, 2004) (see Figs S1 and S2). We found two remarkably different patterns of MHC class I and MHC class II variation within the genus. Whereas kestrels fit the theoretical framework and usually display extremely high MHC variability (e.g. Alcaide *et al.*, 2010), falcons, in turn, exhibited an ancestrally low intra- and interspecific MHC diversity. This surprising pattern, which has not precluded the ecological radiation of falcons and their successful colonization of most habitats worldwide, begs an explanation. We thus investigated whether the generalized low MHC variation across the most diverged species of the genus could have resulted from (i) the inadvertent survey of paralogous genes or the degeneration of functional genes into pseudogenes, or (ii) the outcome of demographic events. The rejection of those hypotheses would point to a predominant role of natural selection in the observed pattern.

Materials and methods

DNA/RNA isolation

DNA extracts were obtained using the HotSHOT (Truett *et al.*, 2000) or salt extraction protocols (Talbot *et al.*, 2011). In most cases, we used blood samples, except for a few instances: in the case of peregrine falcons, four of the DNA samples were extracted from muscle tissue, four from museum skins (skin and feather; Aleutian Islands), one from eggshell membrane (Aleutian Islands) and one from an egg (Fiji). Two of the Merlin (*F. columbarius*) samples were from feathers and one hobby (*F. subbuteo*) from museum skin (toe pad). Total RNA from the spleen of a freshly dead Eleonora's falcon *F. eleonorae* was isolated following the procedure described by Chomczynski & Sacchi (1987). Tissue was homogenized into a solution containing 4 M guanidine-isothiocyanate, 25 mM sodium citrate, 0.5% sodium dodecyl sulphate and 100 mM β-mercaptoethanol. After an organic extraction based on the addition of phenol and chloroform–isoamyl alcohol (24 : 1), the pellet was washed twice with 70% ethanol and resuspended in RNAase-free Milli-Q water.

MHC typing and sequence analyses

We amplified the third exon of a single MHC class I gene using the primers MHCI-int2F and MHCIEx4Rv (Alcaide *et al.*, 2009) or MHCI-INT18 (5′-CAGGGGCT CACACAATACAG-3′) and MHC-ex395R (5′-GGCAG TACAAGGTCAGCGTCCC-3′). The second exon of a single MHC class II B gene was amplified according to Alcaide *et al.* (2007) using the primers Fal2FC and Fal2RC. Genomic fragments spanning exon 2 to exon 3 were amplified using the primers Fal2FC and RapEx3CR (Alcaide *et al.*, 2007). PCR products were sequenced according to the Big Dye technology (Applied Biosystems) and resolved into an ABI3130xl (Applied Biosystems, Foster City, CA, USA), or universal tailed simultaneous bidirectional cycle sequencing (SBS, LI-COR, Inc., 1999; see Steffens *et al.*, 1993; Oetting *et al.*, 1995), using procedures similar to those reported in the study by Talbot *et al.* (2011) and resolved on a LI-COR 4200 or 4300 automated sequencer (LI-COR, Inc., 1999).

Resolving the gametic phase of the MHC class II locus was straightforward, as the majority of the falcons analysed were homozygous and alleles in heterozygotes differed in no more than two point mutations (see Table 1). However, the MHC class I locus was slightly more polymorphic in some species, such as the gyrfalcon and peregrine falcon. Here, heterozygotes were inferred from the sequence data but confirmed through single-strand conformational polymorphism (SSCP; Sunnucks *et al.*, 2000) using automated procedures modified from

Table 1 Polymorphism statistics at MHC class I and MHC class II genes across different species of (a) kestrels and (b) falcons. See Fig. S1 for the phylogenetic relationships among species. Na, number of different alleles at a given locus (the number of different amino acid sequences is indicated in parentheses); *k*, average number of nucleotide differences between alleles; and N, number of individuals genotyped.

	MHC class I (exon 3)					
Species	Na	K	N	Populations sampled	References	GenBank Acc. Nos.
(a) Kestrels						
Falco naumanni	> 80	9.15	> 80	Portugal, Spain, France, Italy, Greece, Israel	Alcaide *et al*., 2010; A. Rodríguez & M. Alcaide, unpublished data	JF831086-JF831120
Falco tinnunculus (continental)	23 (23)	10.99	25	Spain	Alcaide *et al*., 2010	EU120696- EU120722
Falco tinnunculus (insular)	6 (6)	8.45	25	Canary Islands	Alcaide *et al*., 2010	EU120696- EU120722
Falco punctatus	1 (1)	0	4	Mauritius Islands	This study	JN613279
(b) Falcons						
Falco peregrinus	5 (2)	1.00	30	Fiji, Tasmania, Australia, Alaska, Greenland, Russia, Argentina, Chile, Falkland Islands, Spain, Northern Africa	This study	JN613264, JN613269-72
Falco eleonorae	3 (2)	4.66	32	Canary Islands, Greece	This study	JN613263, JN613265-66
Falco rusticolus	4 (4)	3.83	8	Alaska, Canada	This study	JN613273-76
Falco cherrug	2 (2)	2.00	3	United Arab Emirates	This study	JN613277
Falco fasciinucha	1 (1)	0	2	Zimbabwe	This study	JN613278
Falco subbuteo	2 (2)	2.00	1	Spain	This study	JN613267-68
Falco biarmicus	NA	NA	NA	NA	NA	NA
Falco columbarius	NA	NA	NA	NA	NA	NA
Falco concolor	NA	NA	NA	NA	NA	NA
Falco femoralis	NA	NA	NA	NA	NA	NA
	MHC class II B (exon 2)					
(a) Kestrels						
Falco naumanni	>100	22.68	>100	Spain, France, Italy, Greece, Israel, Kazakhstan	Alcaide *et al*., 2008, 2010	EF370839–370864; EU10767-EU107746; HQ418344-HQ402921
Falco tinnunculus (continental)	41 (41)	24.31	25	Spain	Alcaide *et al*., 2010	EU118314-EU118359
Falco tinnunculus (insular)	10 (10)	25.78	25	Canary Islands	Alcaide *et al*., 2010	EU118314-EU118359
Falco punctatus	1 (1)	0	5	Mauritius Islands	This study	
(b) Falcons						
Falco peregrinus	3 (3)	1.50	63	Fiji, Tasmania, Australia, Alaska, Greenland, Russia, Canada, Northern Africa, South American migrants	Alcaide *et al*., 2007; this study	EF370947
			1	Spain	Alcaide *et al*., 2007; this study	EF370948
			22	Northern Africa, South American residents (Argentina, Chile, Falkland Islands)	This study	JN613255
Falco eleonorae	2 (1)	1.00	32	Canary Islands, Greece	This study	JN613254, JN613256
Falco rusticolus	1 (1)	0	12	Alaska, Canada	This study	JN613259
Falco cherrug	1 (1)	0	3	United Arab Emirates	This study	JN613262
Falco fasciinucha	1 (1)	0	2	Zimbabwe	This study	JN613261
Falco subbuteo	1 (1)	0	1	Spain	This study	JN613258
Falco biarmicus	2 (2)	2.00	1	Unknown	Alcaide *et al*., 2007	EF370949–370950
Falco columbarius	1 (1)	0	3	Alaska	This study	JN613260
Falco concolor	1 (1)	0	1	Bahrain	This study	JN613257
Falco femoralis	1 (1)	0	1	Unknown	Alcaide *et al*., 2007	EF370988

Dahse *et al.* (1998). We employed the same forward and reverse universal tailed primers used in the sequencing reactions. PCR amplifications of the SSCP product were carried out in a final volume of 10 μL reaction mixture containing 2–100 ng genomic DNA, 0.2 mM dNTPs, 5 *p*mole unlabelled primer, 1.5 *p*mole IRD-labelled universal primer, 0.1 μg BSA, 1× PCR buffer (Perkin Elmer Cetus I) and 0.3 units *Taq* polymerase (Promega, Madison, Wisconsin, USA). PCRs were began at 94 °C for 2 min and continued with 40 cycles each of 94 °C for 30 s; 50 °C for 30 s; 72 °C for 60 s and concluded with a 30-min extension at 72 °C. PCR-amplified SSCP products were diluted approximately five-fold (2 μL of PCR product to 9 μL standard formamide-loading dye) and denatured for 4 min at 94 °C. The fluorescently labelled PCR products were electrophoresed on a 48-well 0.5× mutation detection enhancement (MDE) gel (Lonza) containing 0.5× MDE gel solution, 0.6× TBE, 0.005% to 10% APS and 0.0005% TEMED. Gel electrophoresis was carried out with 0.6× TBE at room temperature (22 °C; motor speed 1; power settings: voltage 2000 V, current 30 mA, power 6 W) for 12 h on a LI-COR 4200 automated sequencer. Two individuals homozygous for different MHC class I alleles, based on sequence data, were included in all SSCP gels to facilitate allele identification and augment quality control standards.

Unique alleles identified in the SSCP analysis were reamplified in an independent PCR and resequenced. For quality control purposes, we extracted, amplified and sequenced in duplicate 20% of the individuals typed using SSCP.

We verified that resulting SSCP allele configurations and quality control tests were consistent with sequence data; no inconsistencies between the SSCP and the sequence scores were identified, and there were no failures in quality control comparisons. Similar to the MHC class II locus, the high frequency of homozygous individuals for a particular MHC class I allele and the high sequence similarity between the alleles isolated from the same species (see Table 1), and verification using SSCP procedures, made cloning of individual alleles unnecessary.

The phylogenetic relationships among MHC sequences were visualized through neighbour-net networks built using SplitsTree 4.0 (Huson & Bryant, 2006) and based on Kimura 2-parameter distances (Kimura, 1980). Rates of positive diversifying selection at putative peptide-binding regions (PBR) and non-PBR codons were inferred by comparing nonsynonymous (dN) and synonymous (dS) substitution rates. Codons were classified as PBR and non-PBR in accordance with the predicted PBR of humans (see Bjorkman *et al.*, 1987; Brown *et al.*, 1993) and previous analyses of positive selection in birds, including kestrel MHC genes (see Alcaide *et al.*, 2007, 2009; Balakrishnan *et al.*, 2010). For the MHC class I, codons 4, 6–8, 22, 24, 37, 59–61, 64–65, 67 and 72 (exon 3) were labelled as PBR sites. For the MHC class II, codons 5, 7, 9, 13, 24, 26, 28, 32–35, 40, 43, 49, 52–54, 56, 57, 61, 63, 64, 66, 67, 70, 74, 77, 78, 81, 82, 84, 85 and 88 (exon 2) were labelled as PBR sites. Calculations were made in MEGA 5.0 (Tamura *et al.*, 2011) using the modified distance-based Nei & Gojobori (1986) method with Jukes & Cantor (1969) correction and 10 000 bootstrap replicates. Two set of analyses were performed, one including all kestrel sequences and the other including all falcon sequences (see Fig. 1).

Gene expression analyses

We used the one-step RT-PCR kit (Qiagen, Valencia, CA) to obtain PCR amplicons from expressed MHC sequences. We used the primers MhcI-Faelex3F (5′-GGCTGAGGAAATACGTGAG-3′) and MHCIex4Rv to target expressed MHC class I sequences. Specific primers MhcII-Faelex2F (5′-TGCCGGCACAACTACGAG-3′) and MhcII-Faelex3R (5′-ACCATTTCACCTCGATCTCC-3′) were used to amplify expressed MHC class II B sequences. Note here that the application of these primers using genomic DNA would amplify intron 3 and intron 2 for the MHC class I and the MHC class II B gene, respectively. Cycling conditions consisted of 30 min at 50 °C for the reverse transcription step followed by an initial PCR activation step of 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 55 °C and 72 °C, and a final extension step of 2 min at 72 °C.

Results and discussion

We observed a remarkably low level of inter- and intraspecific MHC variation in falcons that strongly contrasts with the extensive polymorphism previously documented in congeneric kestrels (Alcaide *et al.*, 2010). Inspection of the phylogenetic network (Fig. 1) shows that the degree of MHC polymorphism within a single kestrel species is much larger than the polymorphism detected across several species of falcons. In addition, the intermingling of kestrel alleles across species (Fig. 1) supports evidence for the trans-species evolution of the polymorphism (i.e. the retention of MHC motifs during periods of time exceeding the evolutionary split between species: Klein, 1987). By contrast, this pattern is not observed in falcons: MHC sequences are more likely to cluster according to species and in a distribution that mirrors the mitochondrial DNA-based phylogeny of the falcon clade (Fig. S1). The number of highly similar amino acid sequences found at the MHC class II B (exon 2) of falcons ranged from one to three per species (see Table 1). Unlike falcons, lesser kestrels have shown more than 100 different alleles at the same locus, as well as high allelic divergence (Alcaide *et al.*, 2008; Table 1). Likewise, the diversity of the MHC class I repertoire (exon 3) of falcons was very low, with alleles differing in their amino acid sequence ranging from just one to four per species, again in contrast with lesser kestrels,

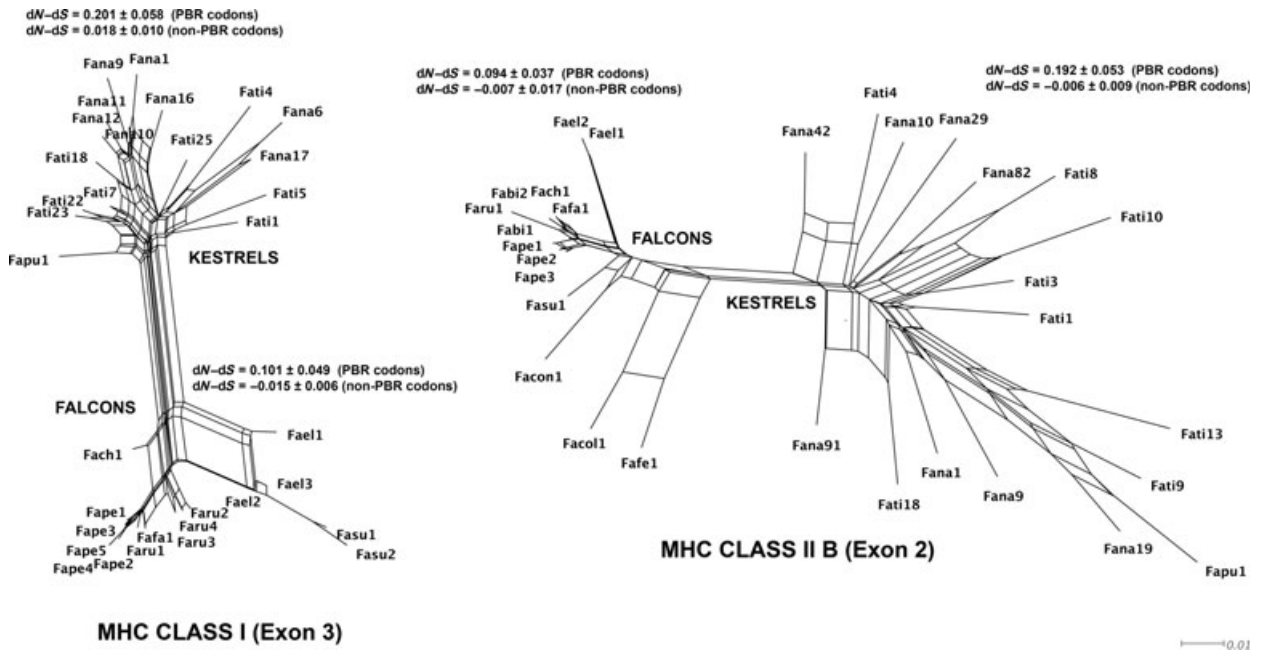

Fig. 1. Phylogenetic network of MHC class I and MHC class II alleles from different species of falcons and kestrels. Rates of diversifying selection (± SD) at putative PBR and non-PBR codons are also indicated. Species codes are as follows: Fana (*Falco naumanni*, Lesser kestrel); Fati (*Falco tinnunculus*, Eurasian kestrel); Fapu (*Falco punctatus*, Mauritius kestrel); Fafe (*Falco femoralis*, Aplomado falcon); Facol (*Falco columbarius*, Merlin); Facon (*Falco concolor*, Sooty falcon); Fasu (*Falco subbuteo*, Eurasian hobby); Fape (*Falco peregrinus*, Peregrine falcon); Faru (*Falco rusticolus*, Gyrfalcon); Fabi (*Falco biarmicus*, Lanner falcon); Fach (*Falco cherrug*, Saker falcon); Fafa (*Falco fasciinucha*, Taita falcon); Fael (*Falco eleonorae*, Eleonora's falcon). Numbers reflect allele codes (see also Table S1 for GenBank accession numbers).

in which more than 80 different alleles have been isolated (Table 1).

Low MHC variability in falcons is not due to the survey of paralogous genes or pseudogenes

In agreement with Alcaide *et al.* (2007), our analyses did not detect more than two alleles per MHC locus per individual. This finding, independently obtained in different laboratories [the Estación Biológica de Doñana (EBD-LEM) and USGS Alaska Science Center Molecular Ecology (ASC-MEL)], supports the putative occurrence of single MHC class I and MHC class II B genes within the genus *Falco* (see similar results in parrots: Hughes *et al.*, 2008). By contrast, PCR-based approaches have uncovered multiformity in MHC loci in other avian species, including raptors (e.g. Alcaide *et al.*, 2007, 2009; Canal *et al.*, 2010; Miller *et al.*, 2011). That said, only Southern blot experiments or the genome characterization of one of the members of the genus would yield conclusive evidence for the extreme MHC simplicity inferred by PCR-based methods. Notwithstanding, hybridization experiments have mirrored quite well the actual number of functional MHC class II B genes inferred using PCR-based approaches, especially in those avian species with low numbers of duplicates (e.g. Burri *et al.*, 2008; Strandh *et al.*, 2011). The apparently simple MHC architecture within *Falco*, coupled with comparisons of coding and noncoding sequences across species (see Fig. S2), argues against confounding effects deriving from the inadvertent survey of paralogous genes. Studies in owls have clearly demonstrated that two ancient MHC class II B lineages can be easily distinguished by the accumulation of adaptive mutations following gene duplication (Burri *et al.*, 2010). Finally, we also confirmed that the MHC genes investigated here are functional, as indicated by the lack of stop codons or altered reading frames in coding sequences and the retrieval of intronless MHC sequences from the cDNA obtained in the spleen (see Fig. S3). Intronless sequences were never obtained from genomic DNA in falcons (e.g. Alcaide *et al.*, 2007, 2009).

Genetic drift and demographic events are not sufficient to explain reduced MHC variation in falcons

Genetic drift following population decline can deplete genetic variation in natural populations. Empirical data indicate that both MHC and neutral diversity are usually lost during these demographic episodes (reviewed in Radwan *et al.*, 2010), albeit these estimates are not

always directly correlated. Some studies suggest that balancing selection can counteract genetic drift and retain MHC polymorphism even in severely bottlenecked populations (e.g. Aguilar *et al.*, 2004). On the other hand, some studies have shown that genetic drift may override selection, especially in small and isolated populations (e.g. Miller & Lambert, 2004). Recent papers incorporating both empirical data and simulations suggest that the simultaneous action of genetic drift and natural selection may drive a more rapid loss of MHC variability relative to neutral variation (e.g. Ejsmond & Radwan, 2011; Sutton *et al.*, 2011). Despite these contrasting results regarding the relative roles of different evolutionary forces in the determination of MHC variation, it seems clear that if genetic drift was the primary mechanism responsible for the low MHC variability found in falcons, its erosive effect would be generalized throughout the genome (e.g. Babik *et al.*, 2005, 2009). This is effectively the case of peregrine falcons from the Fiji archipelago (see Talbot *et al.*, 2011), where genetic drift has led to monomorphism at neutral nuclear microsatellite and mtDNA loci within this insular population (Table S2). This phenomenon could similarly apply to the highly endangered Mauritius kestrel (Nichols *et al.*, 2001).

However, throughout their global range, data from neutral markers do not support the hypothesis that low MHC polymorphism observed in falcons is due to recent population declines (see a summary in Table S2). Indeed, previous studies have reported levels of variation in microsatellite loci in falcons that are not different from those found in kestrels (Table S2). In addition, data from mtDNA control region sequences show considerable levels of genetic variability (Table S2). For example, the saker falcon, *F. cherrug*, exhibits high mtDNA and microsatellite variation, but low MHC diversity. Although microsatellite estimates cannot be easily compared among species due to the application of different loci and variable sample sizes, we can extract important conclusions at the species level. For instance, Jacobsen *et al.* (2008) and Brown *et al.* (2007) showed that Scandinavian and North American populations of peregrine falcons, respectively, have not experienced significant loss of genetic variability after recent contaminant-induced population bottlenecks (see Table S2). It has been argued (Jacobsen *et al.*, 2008) that the current levels of genetic diversity in recovered Scandinavian and some North American populations are unexpectedly high due to introgression following the introduction of captive-bred peregrine falcons, some of which were not native subspecies (Enderson *et al.*, 1995; Heinrich, 2009). Nevertheless, populations of peregrine falcons in Alaska, which were not augmented subsequent to population declines (Enderson *et al.*, 1995; Heinrich, 2009), show levels of variation at neutral genetic markers (microsatellite loci, mtDNA control region) that are similar to levels observed in augmented populations (Table S2).

It is important to point out that comparisons of diversity estimates between microsatellite and MHC markers are not straightforward, mainly due to differences in their mutational and evolutionary mechanisms (Ellegren, 2000). Ideally, patterns of nucleotide variation at MHC markers should be compared with sequence data from other functional nuclear markers. We therefore compared data from a set of recombination-activating gene (RAG-1) nuclear sequences, deposited in GenBank (Table S2, see Wink *et al.*, 2010 for details), between two falcon (peregrine and saker falcons) and two kestrel species (lesser *F. naumanni* and Eurasian kestrels *F. tinnunculus*). Once again, we found no indication that reduced MHC diversity in falcons is accompanied by low levels of genome-wide genetic variability (Table S2). For example, 13 haplotypes with 17 polymorphic sites across 1774 bp at RAG-1 are found within peregrine falcon (Table S2).

The observed pattern of low MHC variability could also be the consequence of a strong bottleneck in a common ancestor of falcons. However, although overall divergence times of falcons are still unresolved, falcons and kestrels diverged as far back as 10.2 million years ago (Hedges *et al.*, 2006). It seems highly unlikely that MHC variation in falcons has not recovered since then, unless natural selection has played a determinant role in the evolution of these adaptive genes. Closely related species, including the peregrine falcon, saker falcon, taita falcon *F. fasciinucha*, lanner falcon *F. biarmicus* and gyrfalcon *F. rusticolus*, which radiated much more recently (Nittinger *et al.*, 2005), present remarkably high similarity among the MHC alleles isolated. This suggests that falcons derive from a common ancestor with already-depleted and slow-evolving MHC. Microsatellite and mtDNA loci may have mutated faster than the MHC loci, recovering genetic variation in a shorter period of time, but this would not explain the retention of considerable levels of genetic diversity at the RAG-1 nuclear gene found in some of these species (Table S2). Moreover, MHC genes are also known to respond rapidly to pathogen-mediated selection (reviewed by Spurging & Richardson, 2010) and are subjected to elevated recombination rates that generate high genetic polymorphism (e.g. Mikko & Andersson, 1995; Richman *et al.*, 2003). In fact, a recent study in the Berthelot's Pipit *Anthus berthelotii* exemplified how gene conversion can rapidly restore MHC variability in a bottlenecked bird population (Spurgin *et al.*, 2011). Collectively, all these lines of evidence lead us to conclude that even if a strong bottleneck was initially responsible for low MHC variation in a common ancestor of falcons, genetic drift exclusively, or even primarily, cannot explain the continued maintenance of low levels of MHC variation in the face of apparently 'normal' levels of genome-wide variation across falcon species and within their populations.

Natural selection is the most plausible explanation for reduced MHC variation in falcons

We cannot completely reject the hypothesis that genetic drift has played some role in the initial generation of low MHC variability in the common ancestor of extant falcons. Likewise, we do not suggest that genetic drift does not continue to act on the genome of extant falcons, including at MHC loci. However, we suggest that the most parsimonious explanation for the maintenance of the low levels of MHC variation observed across falcon species (and within their populations) is the action of natural selection. Low MHC variation was not only observed within and among closely related falcons, as mentioned above, but extends to other species more deeply rooted in the phylogeny of the genus (e.g. the Merlin or the Aplomado falcon *F. femoralis*, Fig. 1). It is plausible that a selection event dramatically reduced MHC variation in a common ancestor of all these species. Strong selective regimes mediated by particularly abundant and/or harmful pathogens could have driven the depletion of MHC variation via directional selection in a common ancestor. Local adaptations against specific pathogens would promote the observed pattern, especially if falcons were confined to a restricted range where these pathogens occur. Although low pathogen exposure could have also contributed to the maintenance of low MHC variability through relaxed selection pressure (Slade, 1992), we would expect that, in the absence of selection, MHC variability would be more similar to neutral genetic expectations (reviewed in Spurging & Richardson, 2010).

Further, this group of raptorial birds encompasses species with contrasting life-history traits that have successfully colonized most continents and environments worldwide (Table S1). This characteristic alone would presumably have exposed colonizers to a series of novel pathogens (Abi-Rachad *et al.*, 2011), likely repeatedly generating variation at MHC loci, given adherence to theoretical expectations of adaptive immune response at MHC loci. Further, species such as the truly cosmopolitan peregrine falcon, as well as the more geographically restricted Eleonora's falcon, are highly migratory and therefore exposed to at least two different pathogen faunas during their annual migratory cycle (e.g. Gangoso *et al.*, 2010), in addition to those found at the stopovers during migration. Moreover, Eleonora's falcons are exposed to high horizontal transmission rates of pathogens as a result of colonial breeding (e.g. Tella, 2002). Thus, a combination of low and very specific pathogen pressure is therefore an unlikely explanation of the maintenance of low MHC diversity during the diversification of this avian group.

The data we present indeed suggest a slower and more conservative evolution of the MHC of falcons than that of their kestrel counterparts. Slow-evolving MHC genes under purifying selection have already been characterized in birds in previous studies (e.g. Jarvi *et al.*, 2004; Strand *et al.*, 2007). It is not only surprising that there is high resemblance of allele sequence among closely related species, but that there is also the synonymous translation of several intraspecific alleles (see Table 1 and Fig. 1). These findings suggest the existence of strong selective constraints against nonsilent mutants, perhaps due to the need to preserve a very specific biological function. However, small variations at functionally important sites within and between species could be species or environmentally specific, demonstrating slight differences in the response to pathogens. For instance, the resident peregrines from Neotropical South America, Patagonia and Falkland Islands (*F. p. cassini*) are fixed for a different MHC class II allele (differing in just one amino acid position) than northern populations of subspecies (*F. p. tundrius* and *F. p. anatum*) that migrate to South America during the winter (Table 1). Even though low intraspecific variability points towards a major role of stabilizing selection, we found compelling evidence for an important role of diversifying selection at larger evolutionary time scales. For both the MHC class I and class II loci, nonsynonymous substitution significantly exceeded synonymous substitution rates specifically at PBR codons after analysing the entire set of falcon sequences (*Z*-test, $P < 0.001$, Fig. 1). It is important to notice that the vast majority of amino acid polymorphisms found within and among species at MHC class I and class II map those regions expected to play a vital role during antigen recognition (see Fig. S4 and Alcaide *et al.*, 2009). However, for both classes of MHC loci again, the signal of diversifying selection is significantly weaker across several species of falcons than across just three closely related species of kestrels (two-tailed *t*-test, $P < 0.001$ for both MHC loci). Indeed, diversifying selection rates at the putative PBR of either the lesser or the Eurasian kestrels alone (data for individual species not shown) and other avian species (e.g. Ekblom *et al.*, 2003) are much more pronounced than across all falcon species we investigated.

Taken together, these findings suggest that there may have been a radical and complex reinvention of how falcons respond immunologically to pathogens in general. We propose that the highly polymorphic and fast-evolving MHC genes found in kestrels represent the ancestral state and that the subsequent depletion of MHC variation and stability of alleles in falcons illustrate an evolutionary transition towards low polymorphic, but functional and expressed genes, with a very specific immunological function. This radical transition should have been accompanied by the evolution of compensatory mechanisms at either the innate or the adaptive branches of the immune system (e.g. Lenz *et al.*, 2009; Star *et al.*, 2011) and is testable. For instance, genetic variation at other immune genes may orchestrate the coevolutionary arms race with

pathogens (e.g. Acevedo-Whitehouse & Cunningham, 2006) or, alternatively, these species may deploy powerful innate defences that override the need to trigger a costly adaptive response. Indeed, some studies have shown that the level of expression of MHC genes is directly related to the strength of diversifying selection acting upon them (e.g. Worley *et al.*, 2008). Here, low MHC variability and relaxed diversifying selection might reflect low expression levels, probably associated with a high efficiency of the innate immunity in falcons (e.g. Wegner *et al.*, 2007).

Conclusions and further considerations

This study brings into focus one of the most compelling instances thus far challenging the relative importance of MHC variability in evolutionary potential and long-term persistence of natural populations. Reduced MHC variation in falcons has not precluded the ability of apparently healthy populations of these birds of prey to flourish globally (Table S1). Although we cannot presently be absolutely certain that there are other, polymorphic MHC genes in the genome of *Falco* species, our striking results prompt compelling questions about the importance of maintaining specific rather than extensive MHC repertoires and about the trade-offs between innate and acquired immunity and fitness that led to the proposed radical transition. We encourage further research on potential compensatory mechanisms through which these species respond to diverse and continuously evolving pathogens. In short, this study opens new and fruitful avenues in the fields of evolution and immunity, as well as conservation biology.

Acknowledgments

We thank R. Burri, A. Roulin, J. Hull and N. Salamin for critical discussions and reading of the manuscript; F. Hiraldo for support; C. Anderson, J. Frick, T. Swem, T. Booms and C. White for kindly providing samples; and J.J. Moreno, J.L. Barroso and E. Luque for help with field work. This study was partially funded by CGL2009-11445 Project and the U. S. Geological Survey's Alaska Regional Executive DOI on the Landscape initiative. During the writing, L.G. was supported by the FP7-REGPOT 2010-1 EcoGenes Project (Grant No. 264125). Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Author contributions

L.G., M.A., J.M.G. and J.F. designed the research; L.G., M.A., J.M.G., J.M., S.T., G.K.S. and S.S. performed the research; M.A., J.M., S.T., G.K.S. and S.S. analysed the data; and L.G., M.A. and J.F. wrote the paper. L.G. and M.A. contributed equally to the work. All authors discussed the results and commented on the manuscript.

References

Abi-Rachad, L., Jobin, M.J., Kulkarni, S., McWhinnie, A., Dalva, K., Gragert, L. *et al.* 2011. The shaping of modern human immune systems by multiregional admixture with archaic humans. *Science* **334**: 89–94.

Acevedo-Whitehouse, K. & Cunningham, A.A. 2006. Is MHC enough for understanding wildlife immunogenetics? *Trends Ecol. Evol.* **21**: 433–438.

Aguilar, A., Roemer, G., Debenham, S., Binns, M., Garcelon, D. & Wayne, R.K. 2004. High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. *Proc. Natl Acad. Sci. USA* **101**: 3490–3494.

Alcaide, M., Edwards, S.V. & Negro, J.J. 2007. Characterization, polymorphism and evolution of MHC class II B genes in birds of prey. *J. Mol. Evol.* **65**: 541–554.

Alcaide, M., Edwards, S.V., Negro, J.J., Serrano, D. & Tella, J.L. 2008. Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the lesser kestrel (*Falco naumanni*). *Mol. Ecol.* **17**: 2652–2665.

Alcaide, M., Edwards, S.V., Cadahía, L. & Negro, J.J. 2009. MHC class I genes of birds of prey: isolation, polymorphism and diversifying selection. *Conserv. Genet.* **10**: 1349–1355.

Alcaide, M., Lemus, J.A., Blanco, G., Tella, J.L., Serrano, D., Negro, J.J. *et al.* 2010. MHC diversity and differential exposure to pathogens in kestrels (Aves: Falconidae). *Mol. Ecol.* **19**: 691–705.

Allendorf, F.W. & Luikart, G. 2007. *Conservation and the Genetics of Populations*. Blackwell Publishing, Oxford.

Babik, W., Durka, W. & Radwan, J. 2005. Sequence diversity of the MHC DRB gene in the Eurasian beaver (*Castor fiber*). *Mol. Ecol.* **14**: 4249–4257.

Babik, W., Pabijan, M., Arntzen, J., Cogalniceanu, D., Durka, W. & Radwan, J. 2009. Long-term survival of a urodele amphibian despite depleted major histocompatibility complex variation. *Mol. Ecol.* **18**: 769–781.

Balakrishnan, C.N., Ekblom, R., Völker, M., Westerdahl, H., Godinez, R., Kotkiewicz, H. *et al.* 2010. Gene duplication and fragmentation in the zebra finch major histocompatibility complex. *BMC Biol.* **8**: 29.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. & Wiley, D.C. 1987. Structure of the Human Class-I Histocompatibility Antigen, Hla-A2. *Nature* **329**: 506–512.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. *et al.* 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364**: 33–39.

Brown, J.W., Van Coeverden de Groot, P.J., Birt, T.P., Seutin, G., Boag, P.T. & Friesen, V.L. 2007. Appraisal of the consequences of the DDT-induced bottleneck on the level and geographic distribution of neutral genetic variation in Canadian peregrine falcons, *Falco peregrinus*. *Mol. Ecol.* **16**: 327–343.

Burri, R., Niculita-Hirzel, H., Roulin, A. & Fumagalli, L. 2008. Isolation and characterization of major histocompatibility complex (MHC) class II B genes in the Barn owl (Aves: *Tyto alba*). *Immunogenetics* **60**: 543–550.

Burri, R., Salamin, N., Studer, R.A., Roulin, A. & Fumagalli, L. 2010. Adaptive divergence of ancient gene duplicates in the avian MHC class II. *Mol. Biol. Evol.* **27**: 2360.

Canal, D., Alcaide, M., Anmarkrud, J.A. & Potti, J. 2010. Towards the simplification of MHC typing protocols: targeting

classical MHC class II genes in a passerine, the pied flycatcher *Ficedula hypoleuca*. *BMC Res. Notes* **3**: 236.

Castro-Prieto, A., Wachter, B. & Sommer, S. 2011. Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. *Mol. Biol. Evol.* **28**: 1455–1468.

Chomczynski, P. & Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.

Cramp, S. & Simmons, K.L. 1980. *The birds of the Western Palearctic*, Vol. II. Oxford University Press, Oxford.

Dahse, R., Fiedler, W., Riese, U., Eggeling, F. & Ernst, G. 1998. A semi-automated highly sensitive approach for mutation detection. *Technical Tips Online* **3**: 138–140.

Ejsmond, M. & Radwan, J. 2011. MHC diversity in bottlenecked populations: a simulation model. *Conserv. Genet.* **12**: 129–137.

Ekblom, R., Grahn, M. & Höglund, J. 2003. Patterns of polymorphism in the MHC class II of a non-passerine bird, the great snipe (*Gallinago media*). *Immunogenetics* **54**: 734–741.

Ellegren, H. 2000. Heterogeneous mutation processes in human microsatellite DNA sequences. *Nat. Genet.* **24**: 400–402.

Enderson, J.H., Heinrich, W., Kiff, L. & White, C.M. 1995. Population changes in North American peregrines. Transactions of the North American Wildlife and Natural Resources Conference **60**: 142–161.

Gangoso, L., Grande, J.M., Llorente, F., Jiménez-Clavero, M.A., Pérez, J.M. & Figuerola, J. 2010. Prevalence of neutralizing antibodies to West Nile virus in Eleonora's falcons in the Canary Islands. *J. Wildlife Dis.* **46**: 1321–1324.

Hedges, S.B., Dudley, J. & Kumar, S. 2006. TimeTree: a public knowledge-base of divergence times among organisms. *Bioinformatics* **22**: 2971–2972.

Heinrich, W. 2009. Peregrine falcon recovery in the continental United States, 1974–1999, with notes on related programs of the Peregrine Fund. In: *Peregrine Falcon Populations in the 21st Century* (J. Sielicki & T. Mizera, eds), pp. 431–444. Turul Publications and Poznań University of Life Sciences Press, Warsaw-Poznan, Poland.

del Hoyo, J., Elliott, A. & Sargatal, J. 1994. *Handbook of the Birds of the World, Vol II: New World Vultures to Guinea Fowls*. Lynx Editions, Barcelona, Spain.

Hughes, C.R., Miles, S. & Walbroehl, J.M. 2008. Support for the minimal essential MHC hypothesis: a parrot with a single, highly polymorphic MHC class II B gene. *Immunogenetics* **60**: 219–231.

Huson, D.H. & Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* **23**: 254–267.

Jacobsen, F., Nesje, M., Bachmann, L. & Litjeld, J.T. 2008. Significant genetic admixture after reintroduction of peregrine falcon (*Falco peregrinus*) in southern Scandinavia. *Conserv. Genet.* **9**: 581–591.

Jarvi, S.I., Tarr, C.L., McIntosh, C.E., Atkinson, C.T. & Fleischer, R.C. 2004. Natural selection of the major histocompatibility complex (MHC) in Hawaiian honeycreepers (*Drepanidinae*). *Mol. Ecol.* **13**: 2157–2168.

Jukes, T.H. & Cantor, C.R. 1969. Evolution of protein molecules. In: *Mammalian Protein Metabolism* (H.N. Munro, ed), pp. 21–132. Academic Press, New York.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120.

Klein, J. 1986. *Natural History of the Major Histocompatibility Complex*. John Willey & Sons, New York.

Klein, J. 1987. Origin of major histocompatibility complex polymorphism: the trans-species hypothesis. *Hum. Immunol.* **19**: 155–162.

Lenz, T.L., Eizaguirre, C., Scharsack, J.P., Kalbe, M. & Milinski, M. 2009. Disentangling the role of MHC-dependent 'good genes' and 'compatible genes' in mate-choice decisions of three-spined sticklebacks *Gasterosteus aculeatus* under semi-natural conditions. *J. Fish Biol.* **75**: 2122–2142.

LI-COR, Inc. 1999. *DNA Sequencing Manual, Global Edition, IR2 System*. LI-COR, Inc., Nebraska.

Meyer-Lucht, Y. & Sommer, S. 2009. Number of MHC alleles is related to parasite loads in natural populations of yellow necked mice, *Apodemus flavicollis*. *Evol. Ecol. Res.* **11**: 1085–1097.

Mikko, S. & Andersson, L. 1995. Low major histocompatibility complex class-II diversity in European and North-American moose. *Proc. Natl Acad. Sci. USA* **92**: 4259–4263.

Miller, H.C. & Lambert, D.M. 2004. Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (*Petroicidae*). *Mol. Ecol.* **13**: 3709–3721.

Miller, H.C., Bowker-Wright, G., Kharkrang, M. & Ramstad, K. 2011. Characterisation of class II B MHC genes from a ratite bird, the little spotted kiwi (*Apteryx owenii*). *Immunogenetics* **63**: 223–233.

Nei, M. & Gojobori, T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* **3**: 418–426.

Nichols, R.A., Bruford, M.W. & Groombridge, J.J. 2001. Sustaining genetic variation in a small population: evidence from the Mauritius kestrel. *Mol. Ecol.* **10**: 593–602.

Nittinger, F., Haring, E., Pinsker, W., Wink, M. & Gamauf, A. 2005. Out of Africa? phylogenetic relationships between *Falco biarmicus* and the other Hierofalcons (Aves: *Falconidae*). *J. Zool. Syst. Evol. Res.* **43**: 321–331.

Oetting, W.S., Lee, H.K., Flanders, D.J., Wiesner, G.L., Sellers, T.A. & King, R.A. 1995. Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. *Genomics* **30**: 450–458.

Radwan, J., Biedrzycka, A. & Babik, W. 2010. Does reduced MHC diversity decrease viability of vertebrate populations? *Biol. Cons.* **143**: 537–544.

Richman, A.D., Herrera, L.G., Nash, D. & Schierup, M.H. 2003. Relative roles of mutation and recombination in generating allelic polymorphism at an MHC class II locus in *Peromyscus maniculatus*. *Genet. Res. Camb.* **82**: 89–99.

Roulin, A. & Wink, M. 2004. Predator-prey relationships and the evolution of colour polymorphism: a comparative analysis in diurnal raptors. *Biol. J. Linn. Soc.* **81**: 565–578.

Slade, R.W. 1992. Limited Mhc polymorphism in the southern elephant seal -implications for Mhc evolution and marine mammal population biology. *P. Roy. Soc. Lond. B* **249**: 163–171.

Sommer, S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Front. Zool.* **2**: 16.

Spurgin, L.G., Van Oosterhout, C., Illera, J.C., Bridgett, S., Gharbi, K., Emerson, B.C. *et al.* 2011. Gene conversion rapidly generates major histocompatibility complex diversity in recently founded bird populations. *Mol. Ecol.* **20**: 5213–5225.

Spurging, L.G. & Richardson, D.S. 2010. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proc. R. Soc. B* **277**: 979–988.

Star, B., Nederbragt, A.J., Jentoft, S., Grimholt, U., MalmstrØm, M., Gregers, T.F. *et al.* 2011. The genome sequence of Atlantic cod reveals a unique immune system. *Nature* **477**: 207–210.

Steffens, D.L., Sutter, S.L. & Roemer, S.C. 1993. An alternate universal forward primer for improved automated DNA sequencing of M13. *Biotechniques* **15**: 580–581.

Strand, T., Westerdahl, H., Höglund, J., Alatalo, R.V. & Siitari, H. 2007. The MHC class II of the black grouse (*Tetrao tetrix*) consists of low numbers of B and Y genes with variable diversity and expression. *Immunogenetics* **59**: 725–734.

Strandh, M., Lannefors, M., Bonadonna, F. & Westerdahl, H. 2011. Characterization of MHC class I and II genes in a subantarctic seabird, the blue petrel, *Halobaena caerulea* (Procellariiformes). *Immunogenetics* **63**: 653–666.

Sunnucks, P., Wilson, A.C.C., Beheregaray, L.B., Zenger, K., French, J. & Taylor, A.C. 2000. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol. Ecol.* **9**: 1699–1710.

Sutton, J.T., Nakagawa, S., Robertson, B.C. & Jamieson, I.G. 2011. Disentangling the roles of natural selection and genetic drift in shaping variation at MHC immunity genes. *Mol. Ecol.* **20**: 4408–4420.

Talbot, S., Palmer, A.G., Sage, G.K., Sonsthagen, S.A., Swem, T., Brimm, D.J. *et al.* 2011. Lack of genetic polymorphism among peregrine falcons of Fiji. *J. Av. Biol.* **42**: 1–14.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum parsimony likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–2739.

Tella, J.L. 2002. The evolutionary transition to coloniality promotes higher blood parasitism in birds. *J. Evol. Biol.* **15**: 32–34.

Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A. & Warman, M.L. 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* **29**: 52–54.

Wegner, K.M., Kalbe, M. & Reusch, T.B.H. 2007. Innate versus adaptive immunity in sticklebacks: evidence for trade-offs from a selection experiment. *J. Evol. Ecol.* **21**: 473–483.

Wikelski, M., Foufopoulos, J., Vargas, H. & Snell, H. 2004. Galápagos birds and diseases: invasive pathogens as threats for island species. *Ecol. Soc.* **9**: 5.

Wink, M., El-Sayed, A.A. & Gonzalez, J. 2010. Fortschritte in Erforschung der Phylogenie der Greifvögel und der Fakten. *50 Jahre Orden Deutscher Falkoniere*: 22–28.

Worley, K., Gillingham, M., Jensen, P., Kennedy, L.J., Pizzari, T., Kaufman, J. *et al.* 2008. Single locus typing of MHC class I and class II B loci in a population of red jungle fowl. *Immunogenetics* **60**: 233–247.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 General information on ecology and distribution of the 12 species of kestrels and falcons analysed.

Table S2 Indices of neutral markers diversity at: (A) microsatellite, (B) mitochondrial DNA, and (C) RAG-1 for several species and populations-within-species of falcons and kestrels.

Figure S1 Phylogenetic relationships among *Falco* species inferred from nucleotide sequences of the cytochrome b gene.

Figure S2 (A) Alignment of the nucleotide sequences of intron 3 (for the MHC class I) and intron 2 plus exon 3 (for the MHC class II B). (B) Neighbour-joining tree of MHC class I and MHC class II B gene fragments.

Figure S3 Amplification of MHC loci from cDNA in the Eleonora's falcon.

Figure S4 Alignment of the nucleotide and amino acid sequences of MHC class I and MHC class II alleles in falcons.

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Received 3 February 2012; revised 22 March 2012; accepted 6 April 2012