Using major histocompatibility complex markers to assign the geographic origin of migratory birds: examples from the threatened lesser kestrel

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Abstract

Gathering knowledge about the migratory routes and wintering areas of threatened populations is fundamental for their successful conservation. Here, we used a non-invasive approach that relies on major histocompatibility complex (MHC) polymorphism to infer the breeding origin of a long-distance migratory bird, the lesser kestrel Falco naumanni, in its most important wintering quarters in the Sub-Saharan Africa (Senegal and South Africa). Private alleles support a strong connectivity between wintering Senegalese and western European breeding populations. On the other hand, birds wintering in South Africa were genetically differentiated with respect to western European breeding populations and might therefore gather individuals from the eastern distribution range. This study demonstrates that, at least at wide continental scales, MHC genes can be powerful intrinsic markers to study migration and migration connectivity, thus adding value to its role in conservation and management.

Introduction

Despite intensive ringing efforts during the last decades and the increasing number of studies in recent years using modern tracking techniques or intrinsic markers such as stable isotopes, trace elements or genetic markers (Webster et al., 2002; Hobson, 2005; Wink, 2006; Coiffait et al., 2009), little is known about wintering and stopover sites of many migratory species (Marra et al., 2006; Faaborg et al., 2010). Given that limiting factors may act on migratory animals in both the breeding and wintering grounds, as well as through the migration routes (Newton, 2004), information on population connectivity is crucial for the effective development of conservation and management initiatives of threatened migratory species (Webster et al., 2002; Marra et al., 2006).

Among popular genetic markers, mitochondrial DNA has been extensively used in phylogeographic studies to unravel spatial patterns of genetic differentiation in the wild. Compared with nuclear DNA, mutations in mitochondrial DNA markers become more rapidly fixed because of a four-times smaller effective population size and the possibility of being more effectively affected by selective sweeps (Ballard & Whitlock, 2004). Thus, the utility of mitochondrial DNA markers to resolve evolutionarily significant units and decipher migratory routes is widely recognized (e.g. Banguera-Hinestroza et al., 2002; Stefanni & Thorley, 2003; Lopes, Hortas & Wennerberg, 2008; Perego et al., 2009). Nevertheless, a single locus approach that can be affected by the co-amplification of nuclear insertions of the mitochondrial genome (i.e. numts; Mindell, 1997), genetic introgression and sex-biased dispersal may sometimes complicate and even confound analyses (e.g. Hurst & Jiggins, 2005; Rubinoff & Holland, 2005). Although mtDNA markers are greatly useful at vast geographical scales, their resolution power at smaller geographical scales has proven unsuccessful in several studies as well (e.g. Lovette, Clegg & Smith, 2004; Wink, Sauer-Gurth & Pepler, 2004; Lopes et al., 2008). Multilocus genotypes based on polymorphic microsatellite markers have become a popular alternative during the last two decades (e.g. Piry et al., 2004; Manel, Gaggiotti & Waples, 2005). Limited genetic differentiation, mostly attributed to homoplasy and back-mutation of microsatellites, has arisen, however, as an important shortcoming (Queney et al., 2001; Boulet & Norris, 2006). In fact, several studies have documented the low occurrence of private alleles, even at vast geographical scales (e.g. Mank & Avise, 2003; Alcaide et al., 2008).

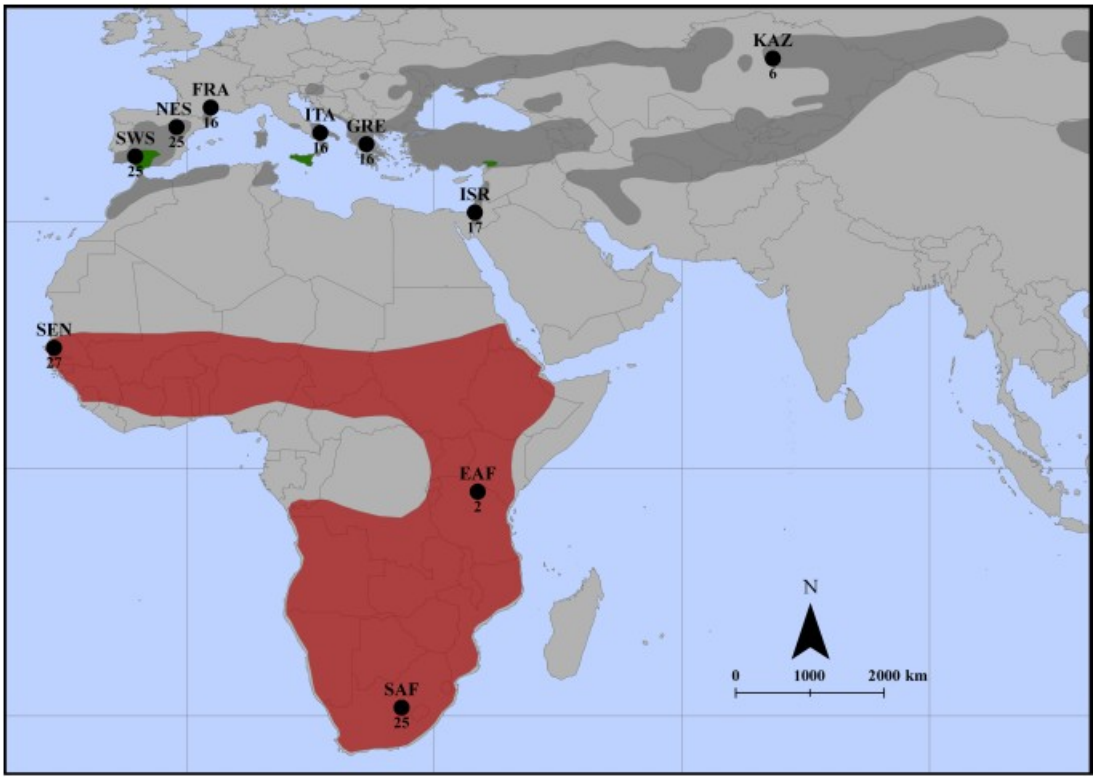

Figure 1 Distribution range of lesser kestrel Falco naumanni and sampled locations. Dark grey; red and green areas indicate breeding and wintering ranges and partially resident populations of lesser kestrels; respectively (modified from BirdLife International, 2010). SWS, south-west Spain; NES, north-east Spain; FRA, France; ITA, Italy; GRE, Greece; ISR, Israel; KAZ, Kazakhstan; SEN, Senegal; SAF, South Africa; EAF = east Africa (specimens collected in Tanzania and Kenya and deposited at the Museum of Comparative Zoology Harvard University; IDs 133154 and 78921, respectively; see 'Discussion'). Numbers indicate individuals sampled.

Despite being widely considered as a classic candidate to reflect local adaptations, studies testing the suitability of the major histocompatibility complex (MHC) to identify the origin of captive or vagrant individuals are surprisingly scarce in the literature. As far as we know, MHC markers have only been used for genetic stock identification of salmons to take appropriate fishery management decisions (Beacham et al., 2001, 2004). The MHC is a multigene family that plays a crucial role during pathogen confrontation and clearance in jawed vertebrates. MHC genes code for cell-surface glycoproteins that bind and present short foreign peptides (antigens) to specialized CD4+ and CD8+ lymphocytes, thus, initiating the development of the adaptive immune response. Extraordinarily high levels of genetic polymorphisms are commonly found within those exons comprising the antigen-binding sites, being large repertoires of alleles maintained by some form of balancing selection (Sommer, 2005; Piertney & Oliver, 2006; Spurgin & Richardson, 2010). The spatio-temporal distribution of MHC variation is expected to reflect pathogen–host co-evolutionary dynamics. Different populations may therefore exhibit contrasting frequencies of the fittest alleles to cope with local pathogen communities. The relative role of neutral evolutionary forces and natural selection on the distribution of MHC diversity nevertheless remains difficult to disentangle in detail (Alcaide, 2010).

In this study, we have tested the suitability of MHC markers to infer migratory connectivity in the globally vulnerable lesser kestrel Falco naumanni (BirdLife International, 2010). This long-distance migratory and colonial falcon breeds in mid-latitudes, from the Iberian Peninsula to China, and winters mainly in the Sub-Saharan Africa (Fig. 1). It has been suggested that populations from different parts of the breeding range tend to remain separated during the winter season, western breeding populations migrating to west Africa and eastern breeding populations heading to South Africa (Moreau, 1972). Although band recoveries, preliminary genetic analyses and tracking of kestrels seem to support this pattern, no conclusive information has been provided so far (see Wink et al., 2004; Rodríguez et al., 2009; Mihoub et al., 2010). Previous analyses of genetic variation at a single MHC class II B gene of the lesser kestrel have revealed extensive genetic polymorphism (>100 alleles) and remarkable patterns of genetic differentiation between European and Asian breeding populations, including a considerable occurrence of private alleles (Alcaide et al., 2008, see supporting information Table S1). This pattern contrasted with relatively

homogenous distributions of microsatellite alleles but was in agreement with geographic variation at fast-evolving mitochondrial DNA sequences (Alcaide et al., 2008; see also Wink et al., 2004).

Profiting from previous research, our main objective is to infer the breeding origin of the African wintering quarters of lesser kestrels. To this aim, we sampled and MHC-typed naturally shed feathers from two African countries (Senegal and South Africa) known to host thousands of wintering lesser kestrels in large communal roosts (up to 28 600 and 1 18 000, respectively; LPO, 2010; MKP, 2010). These numbers roughly represent the estimated population size of the species in its breeding range (about 1 40 000 individuals; BirdLife International, 2010), and consequently, elucidating its breeding origin is a priority for the conservation of the lesser kestrel.

Materials and methods

Non-invasive sampling of wintering grounds

Moulted feathers were collected on the ground of two roost sites during a single visit in daylight hours (around 20 January 2007–2009) to avoid disturbing the birds. Feathers were kept in paper envelopes at room temperature until genetic analyses were carried out during the boreal winter of 2009/2010. Sampled roosts were located in Kaolack (14108^{0}N, 16105^{0}W, Senegal), and in Phillipstown (30126^{0}S, 24128^{0}E, South Africa). These roosts are known to harbour more than 35 000 wintering lesser kestrels (around 28 000 and 7000 individuals, respectively). Lesser kestrels shared the Senegalese roost with swallow-tailed kites Chelictinia riocourii and the South African roost with con-generic falcons (red-footed falcon Falco vespertinus and Amur falcon Falco amurensis) (for more details, see LPO, 2010; MKP, 2010).

DNA extraction, MHC amplification and sequence analyses

DNA extracts were obtained from tips and blood clots of moulted feathers (Horvath et al., 2005) according to the HotSHOT protocol (Truett, 2006). Information on sampling and DNA extraction from breeding locations is available in Alcaide et al. (2008). The second exon of a single and highly polymorphic MHC class II B gene (thereafter referred as Fana-DAB locus) was PCR-amplified and sequenced following Alcaide et al. (2008). Direct sequencing chromatograms were carefully inspected by eye and edited in BIOEDIT v7.0.5.3 (Hall, 1999), and International Union of Pure and Applied Chemistry nucleotide degenerate codes were introduced for each heterozygous site. MHC diploid genotypes were then resolved into individual haplotypes using the Bayesian PHASE platform (Stephens & Donnelly, 2003) implemented in DNASP v5 (Librado & Rozas, 2009). For this purpose, we ran unphased genotypes jointly with a database containing more than 100 MHC class II alleles inferred through traditional cloning methods (Alcaide et al., 2007, 2008) and also through the investigation of allele segregation patterns from parents to offspring (M. Alcaide, Unpubl. data). Calculations were carried out over 1000 iterations, 10 thinning interval and 1000 burn-in iterations. The information provided by PHASE is valuable because it permits us to assess the presence or the absence of informative alleles from western or eastern breeding populations (see Table S1). In order to rule out the possibility of sampling the same individual more than once, we discarded those feathers reporting the same MHC genotype (14 cases corresponding to four individuals, see Table S2).

Estimates of genetic differentiation between breeding and wintering populations

Genetic differentiation between breeding and wintering grounds was calculated using the nucleotide-sequence-based estimate of genetic differentiation K_{ST} in DNASP (Hudson, Slatkin & Maddison, 1992). Furthermore, we calculated an additional genetic measure based on allelic composition between sampling locations (D_{est} – Jost, 2008), using the online program SMOGD v2.6 (Crawford, 2010). Both indexes range between 0 (no genetic differentiation; negative values should be treated as 0) and 1 (complete genetic differentiation). Statistical significance was only evaluated for K_{ST} index by permutating haplotypes among samples (9999 permutations). Given the extraordinary extent of genetic polymorphism at the Fana-DAB locus, the occurrence of identical alleles in different populations and the very low frequencies of the vast majority of alleles (see Alcaide et al., 2008; Tables S1 and S2), we did not calculate assignment probabilities for individual birds. Instead, we evaluated whether wintering populations were more genetically related to either European or Asian breeding populations.

Results

Out of the 174 feathers collected in the wintering roosts, 111 (64%) yielded no or weak PCR amplification, ruling out the sequencing of these samples. Feathers collected in 2007 and 2008 showed a lower amplification rate than the feathers collected in 2009 (Likelihood ratio test: $G_2 = 9.32$, $P < 0.009$). No differences in PCR amplification success were detected between wintering roosts (Likelihood ratio test: $G = 1.44$, $P = 0.23$).

Our PHASE-based inferences revealed 41 alleles unreported in the breeding areas. Overall, MHC genotypes permitted us to discriminate up to 27 and 25 genetically distinct individuals in the Senegalese and South African roost, respectively (Table S2). All but three birds (94%) were heterozygous at the Fana-DAB locus. The inferring of the gametic phase in these individuals was highly useful to elucidate the breeding origin of the birds wintering in both geographical areas. Senegalese genotypes reported a high occurrence of alleles isolated previously from European breeding populations (Fig. 2 & Table S2). Twenty-two out of the 34 alleles (64.7%) inferred from the Senegalese roost

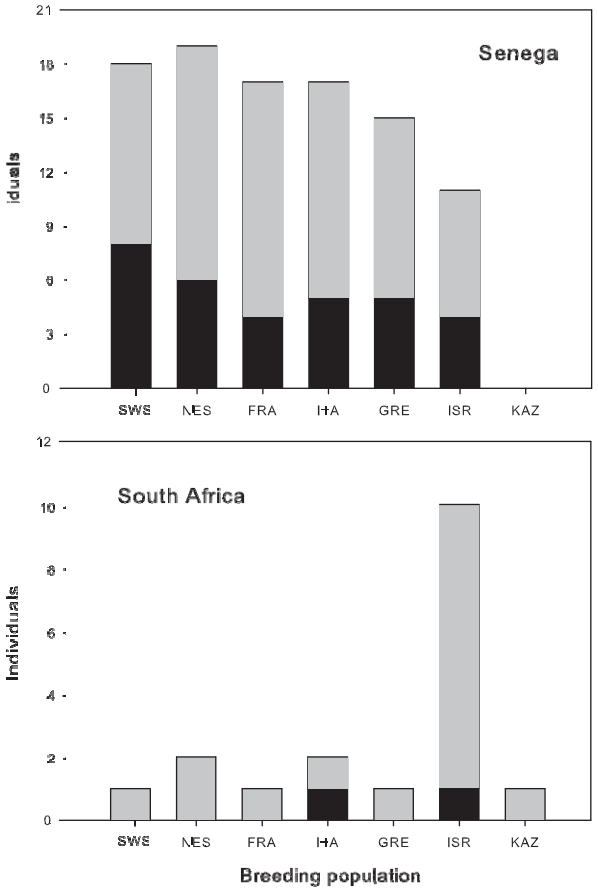

Figure 2 Number of individuals sampled in the Senegalese and South African roosts showing one (grey) or two alleles (black) present in each breeding populations. SWS, south-west Spain; NES, north-east Spain; FRA, France; ITA, Italy; GRE, Greece; ISR, Israel; KAZ, Kazakhstan.

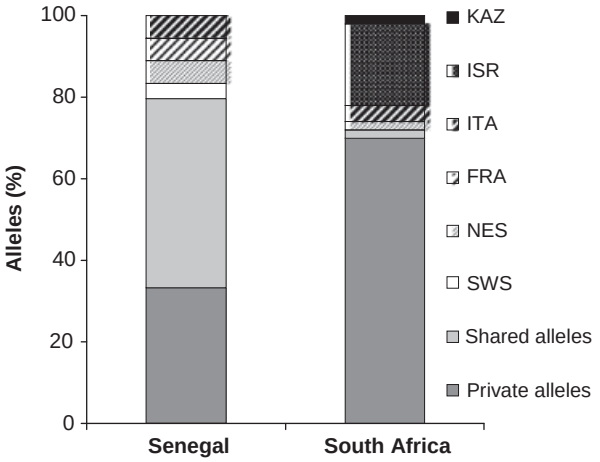

Figure 3 Origin of alleles found in the wintering areas of lesser kestrels Falco naumanni (54 and 50 alleles in Senegal and South Africa, respectively). SWS; NES; FRA; ITA; ISR and KAZ indicate private alleles found in south-west Spain; north-east Spain; France; Italy; Israel and Kazakhstan; respectively. 'Shared alleles' and 'Private alleles' correspond to alleles present in several breeding areas and alleles not isolated previously in any breeding area, respectively (see Table S1).

have been isolated previously in European breeding populations (Fig. 3). In addition, the most abundant alleles within the Senegalese roost (Fana2 = 16.6%, Fana1 = 7.4% and Fana19 = 5.56%) were also among the most abundant alleles in Europe (see Tables S1 and S2). None of the alleles isolated in Senegal corresponded to private alleles from the eastern breeding distribution range. In contrast, only three out 37 South African alleles (8.1%) were isolated previously in European breeding populations. We found no trace of the commonest European alleles, but a high incidence of alleles unreported at the breeding grounds. An important fraction of the alleles isolated in South Africa (five out 37 different alleles, 13.5%) were exclusively found in breeding populations from the eastern distribution range of the species (Fig. 3).

According to our estimates of genetic differentiation (K_{ST} and D_{est}), western and central Mediterranean breeding populations were not significantly differentiated with respect to the Senegalese roost but were remarkably differentiated with respect to the South African roost (Table 1). On the other hand, Israeli and Kazakhstani populations showed the highest degree of genetic differentiation when compared with Senegal and the lowest, although still significant, when compared with South Africa (Table 1). Genetic differentiation between the Senegalese and South African roosts was relatively high and significant ($K_{ST} = 0.0216$, $P = 0.0013$; $D_{est} = 0.928$).

Discussion

Our genetic analyses show a compelling genetic resemblance between European breeding populations of lesser kestrels and wintering ones in Senegal. Moreover, our results support previous findings that pinpointed South Africa as an important wintering ground of breeding birds from the eastern distribution rather than European birds (Wink et al., 2004). Besides its relevance for the conservation of the globally vulnerable lesser kestrel, the present study is one of the very few using MHC markers to decipher the breeding origin of migrating organisms (Beacham et al., 2001, 2004) and the first study that has relied on MHC polymorphism to unravel migratory connectivity in birds. It demonstrates the suitability of MHC markers to achieve or complement molecular studies aimed at tracking wildlife in the future. The use of MHC markers in combination with other intrinsic markers (trace elements, stable isotopes or other genetic markers) might thus significantly enhance our ability to infer migratory routes and trace the origin of captive stock or illegal trade (see Beacham et al., 2001, 2004; for similar studies in salmons).

MHC as a genetic marker

The Fana-DAB locus worked relatively well for non-invasive samples. Importantly, the collection of naturally shed

Table 1 Estimates of genetic differentiation (K_{ST} and D_{est}) between breeding and wintering populations of lesser kestrels Falco naumanni

	Senegal			South Africa		
Population	K_{ST}	P-value	D_{est}	K_{ST}	P-value	D_{est}
South-west Spain	0.00227	0.25	–0.04427	0.03406	**<0.001**	0.98819
North-east Spain	0.00005	0.40	0.04160	0.03724	**<0.001**	0.96769
France	0.00997	0.07	0.06251	0.05489	**<0.001**	0.92743
Italy	–0.00166	0.54	–0.01332	0.03916	**<0.001**	0.90249
Greece	0.00461	0.19	0.03169	0.03373	**<0.001**	0.97332
Israel	0.01847	0.010	0.65273	0.02683	0.003	0.60196
Kazakhstan	0.03262	0.002	1.00000	0.02310	0.018	0.88918

P-values for K_{ST} estimates are showed. Statistically significant values are indicated in bold.

feathers during day hours allowed us to avoid disturbing the birds resting in overcrowded roosts at night. Furthermore, feather collection is a very straightforward task that provides a higher number of samples than non-destructive procedures involving capture and handling of birds (Taberlet, Waits & Luikart, 1999). As an important pitfall, non-invasive samples usually contain degraded DNA that may result in an increased risk of genotyping errors and allele dropout (Taberlet et al., 1999; Segelbacher, 2002). From the battery of individuals that we successfully genotyped, heterozygosity levels (0.94) fell in the range documented during previous studies based on the analysis of fresh blood samples (Alcaide et al., 2008, 2010). Consequently, we do not expect our results be affected by high rates of allele dropout. Contrary to microsatellites, MHC alleles are identical in size and preferential amplification of small against large alleles is not expected. Furthermore, the relatively low size of polymorphic MHC exons (~300 bp) make these markers well suited for genetic approaches based on DNA degraded into short fragments (Taberlet et al., 1999). The high rates of PCR failure can be nonetheless associated with a high proportion of low-quality samples (we could not even observe the blood clot in many of the sampled feathers) and poor storage conditions (Taberlet et al., 1999; Segelbacher, 2002). In fact, we found significant evidence of higher amplification success for feathers collected in 2009 (with a shorter storage period at room temperature) than those gathered during previous years.

Compared with other nuclear markers, the Fana-DAB locus displays higher occurrence of private alleles than microsatellites and patterns of MHC structuring has shown to be as sharp as those revealed by fast-evolving mitochondrial DNA sequences (see Wink et al., 2004; Alcaide et al., 2008). We also experienced higher yields of MHC amplification over mitochondrial markers (authors' unpublished data). Mitochondrial markers have also exhibited methodological problems related to the co-amplification of numts in this and other species (Mindell, 1997; Alcaide et al., 2008). Although we cannot discard that this fact is due to intrinsic characteristics of the PCR profile, it is known that avian blood is a tissue rich in nuclear DNA (avian erythrocytes are nucleated) but relatively depleted of mitochondrial DNA. Higher yields during PCR amplification can therefore be expected when targeting nuclear markers on DNA extracts obtained from blood clots. However, working with MHC markers is not easy and several caveats must be highlighted. The characterization of MHC genes in non-model species, the co-amplification of different fragments of the multigene family during single PCR experiments and extensive levels of genetic polymorphism require a considerable set-up before using these markers (reviewed by Babik, 2010). However, we have to note that important advances for the amplification and genotyping of MHC genes in non-model species have been developed (Babik, 2010) and several studies have successfully reported the cross-amplification of MHC fragments across a wide diversity of avian species (e.g. Alcaide et al., 2007, 2009; Burri et al., 2008; Canal et al., 2010). Even though several studies have documented that MHC markers might be more genetically structured than other nuclear markers in some bird species (see for instance Ekblom et al., 2007; Alcaide et al., 2008; Loiseau et al., 2009), we cannot rule out the lack of spatial patterns of genetic differentiation in others. It is well known that balancing selection can mitigate the effects of genetic drift (e.g. van Oosterhout et al., 2006) and the retention of ancestral polymorphism has been documented, for instance, in passerines (Anmarkrud et al., 2010).

The segregation of lesser kestrel breeding populations at wintering range

The genetic data provided in this study agree with previous assumptions and findings regarding patterns of migratory connectivity in the lesser kestrel (Moreau, 1972; Wink et al., 2004; Rodríguez et al., 2009). A loop migration for western European breeding kestrels has been hypothesized to cross the Sahara desert in a wide front during the post-nuptial migration and to return through the western Sahara and/or coastal Africa (Heim de Balsac & Mayaud, 1962). Both population size estimates (LPO, 2010) and our MHC inferences suggest that an admixture of individuals coming from different European breeding populations may compose the Senegalese roost. This hypothesis would be in agreement with five recent ring recoveries as well (Mihoub et al., 2010). On the other hand, the lack of common European MHC alleles in the South African roost, the identification of private alleles from Asian populations, previous research relying on mitochondrial cytb sequences

(Wink et al., 2004) and several ring recoveries (Rodrıguez et al., 2009) point towards a connection between South African roosts and breeding populations from the eastern distribution range. The relatively high levels of genetic differentiation between the South African roost and the Asian populations from which we had MHC data could be explained by the congregation of wintering birds from non-sampled and maybe genetically structured breeding populations from the eastern distribution range. In fact, the number of birds wintering in South Africa is fivefold that wintering in west Africa (LPO, 2010; MKP, 2010). Therefore, it would be crucial to elucidate whether kestrels of different roosting flocks originate from the same eastern breeding areas. In this respect, it is important to observe that an exclusive amino acid motif found at high frequencies in Kazakhstani birds by Alcaide et al. (2008), but lacking in European populations, was not found in any sampled feather from the South African roost. Interestingly, this highly informative amino acid motif was found in the two alleles isolated from a museum specimen collected in Kenya in 1915 (deposited at the MCZ collection hosted by Harvard University; ID 78921; authors' unpublished data), suggesting an Asian origin. More research in this region should be encouraged to clarify whether east African regions represent only migratory stopovers or if they are likely to occasionally host large populations of wintering Asiatic birds, especially during those years when the South African wintering population decrease considerably (MKP, 2010).

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Supporting information

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

Table S1. Allelic frequencies in breeding and wintering populations of lesser kestrels. Genbank accession numbers are shown. SWS = South West Spain; NES = North East Spain; FRA = France; ITA = Italy; GRE = Greece; ISR = Israel; KAZ = Kazakhstan; SEN = Senegal; SAF = South Africa.

Table S2. Genotypes of individuals from Senegal and South Africa and presence of alleles in the breeding populations. + = a shared allele with the breeding population; ++ = two shared alleles; SWS = South West Spain; NES = North East Spain; FRA = France; ITA = Italy; GRE = Greece; ISR = Israel; KAZ = Kazakhstan.