Scientific Papers. Series D. Animal Science. Vol. LXVIII, No. 1, 2025
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

GENETIC INSIGHTS FROM NEUTRAL AND ADAPTIVE MARKERS GUIDE CONSERVATION OF LESSER KESTREL POPULATIONS IN BULGARIA

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Abstract

The genetic diversity and population structure of the Lesser Kestrel (Falco naumanni), a threatened migratory falcon, were assessed to inform conservation strategies under the LIFE project for the species' reinforcement in Bulgaria, by means of neutral microsatellite markers and non-neutral candidate gene markers associated with migratory behavior. The Core European populations (Spain, Italy, Greece) exhibit high genetic diversity and low differentiation, making them suitable sources for conservation translocations. In contrast, peripheral populations (e.g., Mongolia, Limnos Island) are genetically distinct and less suited for reinforcement due to potential risks of outbreeding depression. Non-neutral markers showed minimal genetic differentiation among populations, suggesting a lack of disruptive adaptive divergence within the species' range. These findings emphasize the importance of genetic similarity and diversity in translocation strategies and highlight the potential for maintaining adaptive potential while avoiding maladaptive outcomes. This integrated approach offers a framework for enhancing the genetic resilience and long-term stability of Lesser Kestrel populations in Bulgaria.

Key words*: conservation translocation, genetic diversity, genetic management, microsatellite markers, population structure.*

INTRODUCTION

Genetic differentiation and gene flow patterns in several species are shaped by the ecological features of their habitats and whether they migrate or not (Willoughby et al., 2017). Birds can exhibit natal and breeding-site fidelity in addition to long-distance migratory behavior, especially raptors (Grande et al., 2009; Newton, 2010). In general, migratory raptor populations show higher genetic diversity compared to non-migratory populations; however, they tend to show low levels of genetic differentiation among geographically isolated groups, resulting in a weak population structure. but at the same time population structure is weak (Webster et al., 2002). However, fine-scale genetic structure can be seen in demographically decreased populations because of the fidelity to the natal and breeding sites (Bounas et al., 2017; Di Maggio et al., 2015). Moreover, populations that are regionally isolated and patchily distributed, are more vulnerable to genetic drift, which will ultimately drive a population to genetic diversity loss. Additionally, a population that suffers from inbreeding may further exhibit lower fitness. This would increase species' risk of extinction by impairing its capacity to adapt to a changing environment (Amos & Balmford, 2001; Frankham, 1996; Reed & Frankham, 2003). Therefore, knowing a species' genetic diversity, population structure, and the processes that shape them is one of the most crucial things that need to be considered in order to inform a reintroduction strategy.

A species of particular conservation concern where such genetic considerations are critical is the Lesser Kestrel (*Falco naumanni*). This migratory falcon exhibits broad distribution, breeding in higher latitude from the Mediterranean across to Mongolia and China, and its wintering grounds are located in Sub-Saharan Africa (Cramp & Simmons, 1980). The species went through severe declines across its distribution, at least in Europe in the early ‘60s, mainly due to intensification of agriculture and

subsequent habitat degradation along with changes in land use (Inigo & Barov, 2010). Particularly in the Central and Eastern Mediterranean region the decrease was severe, resulting in local extinctions and thus causing range reduction and population fragmentation (Inigo & Barov, 2010). Today, the species still has a "patchy" distribution despite the fact that its extincrion risk is low (BirdLife International, 2017). However, because of the recent recolonization of several sites across its European distribution, it has been downlisted to 'Least Concern' by IUCN. Despite the encouraging signals of population recovery, still its abundance is low compared to the one described for the '60s. Therefore, actions that will foster the recovery and the stabilization of populations both in the core and the expanding peripheral populations are still needed.

Genetic management is particularly critical in conservation translocations, aiming to enhance demographic stability while addressing the challenges of maintaining genetic diversity and adaptive potential of populations. Translocating raptors to peripheral or newly established colonies can serve as an effective conservation action that may lead to short-term population stabilization or even growth (Morandini & Ferrer, 2017; Seddon et al., 2014). Nonetheless, these programs should aim at enhancing both the genetic and the adaptive similarity between native and introduced populations to reduce any adverse adaptive and evolutionary outcomes (Holderegger et al., 2006). Thus, a central aim of conservation translocation programs should be the preservation of both demographic stability and genetic variation. Off-site conservation breeding serves three main functions in a management or recovery program aimed at preserving a specific species: a) to offer demographic and/or genetic support for current wild populations, b) to create sources for founding new populations in the wild, and c) to prevent extinction of populations that lack an immediate chance of survival in the wild by keeping them in captivity (Allendorf & Luikart, 2009). Choosing the initial pool of the founding individuals for a captive breeding programme is a crucial and challenging task for many species. Individuals selected to provide demographic and genetic assistance for wild populations should be chosen to enhance genetic and ecological (adaptive) diversity. In contrast, newly introduced populations must possess sufficient genetic diversity to adapt to their new environment.

The majority of research regarding the genetic effects of translocations examines diversity at putatively neutral markers like microsatellites, revealing population structure and differentiation patterns influenced by neutral evolutionary forces, such as genetic drift, mutation, and gene flow or dispersal (Kirk & Freeland, 2011). Nonetheless, genetic variation encompasses another component that is the functional diversity; populations under the influence of different environmental factors may have evolved in response to different selective pressures. Migratory birds, such as the Egyptian Vulture, exhibit complex behavioral and physiological responses that are likely influenced by genetics and could be shaped by natural selection (Liedvogel et al., 2011; Pulido, 2007). A few candidate gene markers (e.g., ADCYAP1, CREB1, CLOCK and NPAS2), have been identified by analysing circadian molecular pathways in the avian genome for tandem repeats (Steinmeyer et al., 2009). Examining variations in these genes could provide fresh perspectives on how populations adapt locally and the evolutionary and ecological consequences of this adaptation.

This paper provides genetic management recommendations to inform strategies that will preserve the allelic diversity of the Lesser Kestrel and strengthen the Bulgarian population of the species, after its nesting was restored in the country by Green Balkans in 2014 (Gradev et al., 2016). To address this aim, this study provides an assessment of the genetic diversity levels and genetic structure of the Lesser Kestrel populations across most of the range of the species'. Specifically, we examine both neutral markers, which help assess overall genetic diversity and reveal patterns shaped by random processes like gene flow and genetic drift, and adaptive markers, which offer insights into how populations may respond to environmental pressures and selective forces. This distinction is crucial for understanding both the current genetic health of populations and their potential for long-term survival and adaptation. Then, the assessment of genetic similarity, the preservation of traits suited to local

environments, will help pinpoint the most suitable source populations that can be used for reinforcement actions thus safeguarding the Bulgarian population's genetic diversity, given that the population is still in low numbers, with 40-50 breeding pairs distributed just in 3-4 colonies (Gradev et al., 2021). Given these low numbers, this study aims to provide genetic insights to inform conservation strategies that can bolster the population's genetic diversity and contribute to its long-term survival.

MATERIALS AND METHODS

We used two genetic datasets that utilise samples from 15 sites throughout the species distribution, from the Iberian Peninsula to Mongolia (Figure 1): A set of 295 individual genotypes for 18 microsatellite loci after Bounas et al. (2017) and a set of 96 genotypes for repeat-rich regions within ADCYAP1, CREB1, CLOCK, and NPAS2 genes (Steinmeyer et al., 2009).

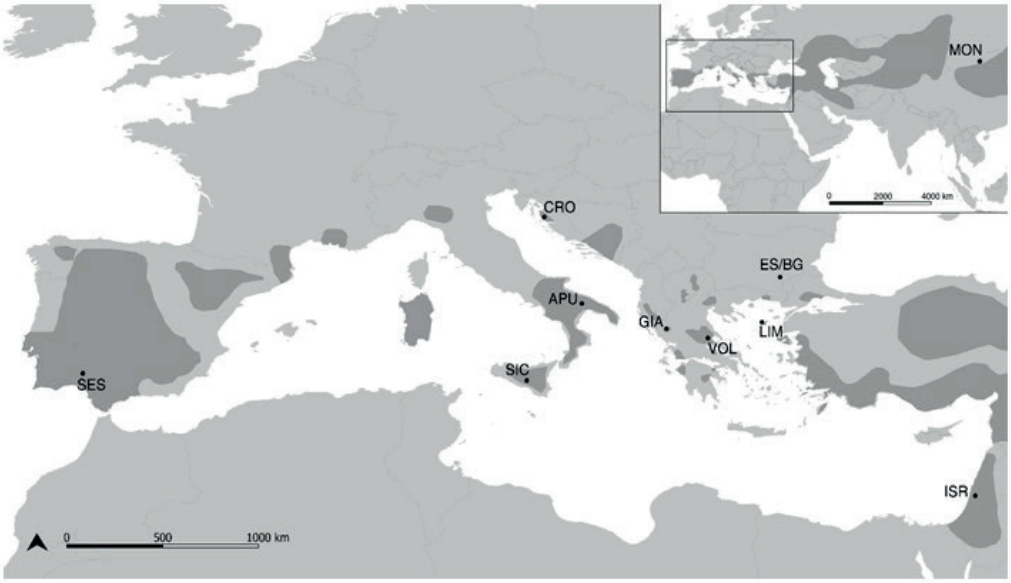

Figure 1. Map of the lesser kestrel populations used in the present study. SES: Andalucia, ES/BG: Bulgaria (Extremaduran origin), APU: Apulia, SIC: Sicily, CRO: Croatia, GIA: Ioannina, LES: Agrinio, TRI: Trikala, LAR: Larisa, VOL: Volos, KIL: Kilkis, KAL: Komotini, LIM: Limnos, ISR: Israel, MON: Mongolia. Shaded areas represent the breeding distribution of the species (modified from BirdLife International)

The four candidate genes were amplified in a single multiplex reaction (Chakarov et al., 2013) using the KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems). In each reaction, we inserted 20 ng template DNA, 2 pM of each primer and 1 × KAPA2G Mix.

The PCR settings were the following: first, an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 15 s at 95°C, then an annealing step of 30 s at 60°C and a final extension step of 30 s at 72°C, with a final extension step of 10 min at 72°C.

PCR products were run on an ABI 3730xl capillary sequencer (Applied Biosystems) and final genotypic data were obtained using STRand v.2.4.59 (Toonen & Hughes, 2001).

To assess the genetic diversity in each population we calculated standard genetic diversity indices including the number of alleles, heterozygosities (observed and expected) and private allele richness. For this we used GENALEX v.6.5 (Peakall & Smouse, 2012) and HP-RARE (Kalinowski, 2005). We used FSTAT 2.9.3.2 (Goudet, 2002) to calculate allele richness, corrected for population sample size, and further calculated inbreeding coefficient (Fis), tested for deviations from Hardy-Weinberg (HW) proportions at locus and population levels, and finally test loci for linkage disequilibrium (LD). For these we used 1000 randomizations and adjusted the significance for multiple comparisons.

To describe the pattern of genetic structure across the datasets, first we computed pairwise fixation indices (Fst) among populations and estimated 95% confidence intervals (1000 bootstraps) in "hierfstat". Jost's D was also calculated among populations using the package "mmod" (Winter, 2012) in R. In addition, we performed a principal component analysis (PCA) using "adegenet" (Jombart, 2008). Population structure was additionally assessed by subjecting all individuals in a Bayesian clustering analysis implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). This analysis allowed to infer the number of genetically homogeneous clusters (K) present in both the neutral and the non-neutral datasets. We used the admixture ancestry model and correlated allele frequencies (Falush et al., 2003), and runs were set with a burn-in period of 10^5 iterations followed by 5×10^5 MCMC steps with 10 replicates for each K value (1-9). Structure results were then further analysed to identify the number of genetic clusters formed by the sampled individuals, using the ΔK method (Evanno et al., 2005) from STRUCTURE HARVESTER (Earl & Vonholdt, 2012). Furthermore we calculated the posterior probability for each K. Finally isolation by distance patterns were explored by conducting a Mantel test between pairwise genetic (Fst/1 − Fst) and geographic distance matrices using the R package "ade4" (Dray & Dufour, 2007).

RESULTS AND DISCUSSIONS

Results on the populatin genetic diversity, i.e. number of alleles, observed and expected heterozygosity, allelic richness, private alleles and inbreeding coefficient, based on the neutral loci are shown in Table 1.

Table 1. Measures of genetic variation of all sampled lesser kestrel populations based on microsatellite markers

Location	Code	n	A	Ar	Ho	He	π	Fis
Croatia	CRO	14	5.625 (0.645)	4.7 (0.45)	0.654 (0.048)	0.65 (0.048)	0	-0.023 (0.043)
Bulgaria (Extremaduran, Spain origin)	ES/BG	25	8.250 (1.289)	5.6 (0.66)	0.674 (0.040)	0.699 (0.037)	6	0.029 (0.037)
Andalucia, Spain	SES	19	7.125 (0.930)	5.4 (0.55)	0.635 (0.037)	0.696 (0.037)	1	0.082 (0.041)
Central Greece	VOL	20	7.563 (1.194)	5.5 (0.68)	0.678 (0.051)	0.662 (0.048)	1	-0.027 (0.035)
Ioannina, Greece	GIA	24	7.563 (1.099)	5.2 (0.56)	0.634 (0.051)	0.654 (0.048)	2	0.036 (0.031)
Limnos, Greece	LIM	11	5.125 (0.562)	4.8 (0.49)	0.661 (0.060)	0.667 (0.041)	1	0.024 (0.060)
Israel	ISR	20	7.250 (1.097)	5.5 (0.66)	0.619 (0.062)	0.682 (0.042)	5	0.124 (0.055)
South Italy	APU	44	9.375 (1.463)	5.5 (0.55)	0.669 (0.038)	0.707 (0.036)	3	0.053 (0.025)
North Mongolia	MON	17	7.188 (0.963)	5.4 (0.59)	0.585 (0.057)	0.655 (0.049)	10	0.155 (0.046)
Sicily	SIC	12	5.938 (0.814)	5.1 (0.615)	0.682 (0.038)	0.667 (0.041)	2	-0.045 (0.045)

Note: number of sampled individuals (n), number of alleles (A), allelic richness (Ar), observed (Ho) and expected (He) heterozygosity (along with their respective standard errors, SE), rarefied private allelic richness (π) and inbreeding coefficient (Fis) along with 95% confidence intervals (CI).

Allelic richness was highest in the core European populations of Spain Greece and Italy but low in the Croatian and Limnos island populations (LIM, CRO). The highest heterozygosity values were observed in the Sicilian (SIC) population whereas the lowest levels were found in the Mongolian population (MON).

Regarding the putatively adaptive loci, all four genes showed low levels of genetic diversity with four, three, two and one alleles detected in ADCYAP1, CREB1, CLOCK and NPAS2 respectively (Table 2).

Table 2. Estimates of genetic diversity of the sampled Lesser Kestrel populations based on the candidate gene data

Population	locus	n	Na	Ho	He	π	Fis
ES/BG	*Adcyap1*	15	4	0.40	0.38	-	0.012
	Creb1	15	2	0.20	0.28	-	0.311
	Clock	15	1	0.00	0.00	-	-
	Npas2	15	2	0.07	0.06	-	0.000
APU	*Adcyap1*	15	3	0.47	0.44	-	-0.032
	Creb1	15	2	0.27	0.44	-	0.429
	Clock	15	2	0.13	0.12	-	-0.037
	Npas2	15	2	0.07	0.06	-	0
CRO	*Adcyap1*	12	3	0.42	0.50	2	0.203
	Creb1	12	3	0.33	0.56	-	**0.439**
	Clock	9	2	0.11	0.10	-	0.000
	Npas2	12	1	0.00	0.00	-	-
ISR	*Adcyap1*	15	3	0.53	0.58	-	0.122
	Creb1	15	2	0.40	0.44	-	0.134
	Clock	15	2	0.13	0.12	-	-0.037
	Npas2	15	1	0.00	0.00	-	-
LIM	*Adcyap1*	9	2	0.56	0.40	-	-0.333
	Creb1	3	2	1.00	0.50	-	-1.000
	Clock	5	1	0.00	0.00	-	-
	Npas2	10	1	0.00	0.00	-	-
MON	*Adcyap1*	14	2	0.36	0.44	-	0.217
	Creb1	14	2	0.14	0.34	-	**0.600**
	Clock	14	2	0.07	0.07	-	0.000
	Npas2	14	2	0.07	0.07	-	0.000
CGR	*Adcyap1*	15	3	0.60	0.53	-	-0.105
	Creb1	14	2	0.21	0.50	-	**0.594**
	Clock	14	2	0.07	0.07	-	0.000
	Npas2	15	2	0.07	0.06	-	0.000

Note: number of sampled individuals (n), Number of alleles (A), observed (Ho) and expected (He) heterozygosity and inbreeding coefficient (Fis). Statistically significant values are written in bold.

Observed heterozygosity for adaptive loci was generally low except for ADCYAP1 gene. For this gene, Central Greece population showed the highest heterozygosity levels (Ho = 0.60). Populations did not show any significant differences between observed and expected heterozygosity in ADCYAP1, CLOCK and NPAS2, but in locus CREB1, observed heterozygosity was significantly lower than expected in Central Greece, Mongolia and Croatia.

According to the neutral dataset, STRUCTURE results some population structure. When modelling all individuals, the ΔK-method indicated that two clusters (K = 2) represented the most likely population structure. In this scenario, all European populations were found to belong in the first cluster, and the MON and LIM populations exhibited high membership to the second (Figure 2). Lesser kestrels from Israel (ISR) were determined to be significantly admixed (Figure 2). In the scenario of K = 3, a third gene pool is mostly represented by the Croatian population. There were no signs of any further substructure in the core European populations of Spain, Italy and Central Greece (Figure 2). When the model was run for the non-neutral dataset, the posterior probability value suggested K = 1, suggesting there is no structure among populations.

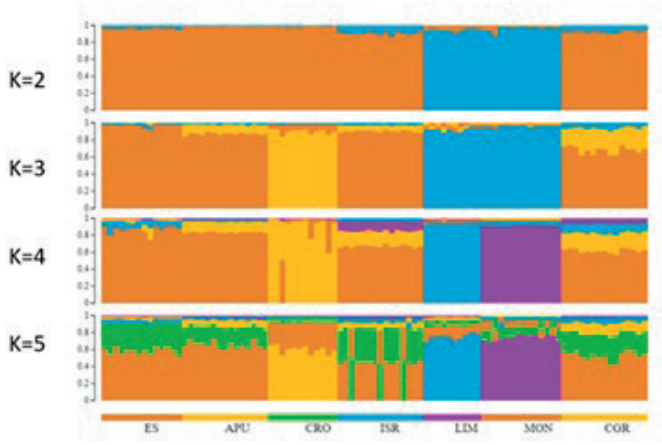

Figure 2. Admixture proportions (proportions of membership to each of K inferred clusters) of individual lesser kestrels for the neutral dataset (own source)

Similarly, Fst and D values among populations, did not show any statistically significant differences based on the non-neutral dataset. Using the microsatellite dataset, Fst values largely confirmed STRUCTURE results (Table 3). Finally, genetic distance did not show any association with geographical distance across all populations ($r^2 = 0.05$, $p = 0.9$).

Table 3. Pairwise Fst-values (below diagonal) and D values (above diagonal) among Lesser Kestrel populations based on the neutral microsatellite markers

	ES/BG	**APU**	**CRO**	**ISR**	**LIM**	**MON**	**CGR**
ES/BG	-	0..00	**0..04**	**0.02**	**0.03**	**0.04**	0.01
APU	0.00	-	0.03	0.01	**0.03**	**0.04**	0.01
CRO	**0.08**	**0.06**	-	**0.02**	**0.07**	**0.07**	0.01
ISR	**0.04**	0.02	**0.05**	-	**0.03**	**0.04**	0.01
LIM	**0.07**	**0.08**	**0.14**	**0.08**	-	**0.04**	**0.05**
MON	**0.09**	**0.10**	**0.14**	**0.08**	**0.08**	-	**0.05**
CGR	0.01	0.01	**0.03**	0.03	**0.10**	**0.10**	-

The analysis of neutral microsatellite data revealed that the core European populations - specifically those from Spain (already comprising the founder Bulgarian population), Greece, and Italy (APU) - exhibited the highest levels of allelic richness and genetic diversity. These populations also showed low genetic differentiation among each other, suggesting a high degree of gene flow and a shared genetic background. In contrast, the Croatian (CRO) and Limnos Island (LIM) populations displayed the lowest allelic richness and higher levels of genetic differentiation. The Mongolian population was also genetically distinct from the European populations, as indicated by the Bayesian clustering analysis and pairwise Fst values.

The lack of significant genetic structure among the core European populations implies that they constitute a relatively homogeneous genetic group. This homogeneity, coupled with their high genetic diversity, makes them suitable candidates for sourcing individuals for reinforcement efforts in Bulgaria. The genetic differentiation of the Mongolian and Limnos populations suggests that translocating individuals from these populations may not be advisable due to potential genetic incompatibilities and the risk of outbreeding depression.

The analysis of the putatively adaptive candidate genes (ADCYAP1, CREB1, CLOCK, and NPAS2) did not reveal significant genetic differentiation among populations. The low levels of genetic diversity and lack of structure in these genes may indicate that selective pressures on migratory behavior are similar across the species' range, or that these markers are not sufficiently variable to detect adaptive differences. This finding suggests that, from the perspective of the candidate genes studied, there may be minimal risk of disrupting local adaptations through translocations among European populations.

The absence of a significant isolation-by-distance pattern further supports the notion that geographic distance is not a major barrier to gene flow among Lesser Kestrel populations in Europe. This is consistent with the species' migratory behavior and capacity for long-distance dispersal, which can facilitate genetic exchange across broad spatial scales.

CONCLUSIONS

The present report provides a comprehensive assessment of the genetic diversity and population structure of Lesser Kestrel populations across a substantial portion of the species' range, utilizing both neutral microsatellite markers and putatively adaptive candidate genes associated with migratory behavior. The findings offer critical insights into the genetic landscape of the species, which are essential for informing conservation strategies aimed at reinforcing the Bulgarian population and preserving the overall genetic diversity of the species.

Given the current genetic composition of the Bulgarian population (founders of spanish origin) and the findings of this study, sourcing additional individuals from other genetically diverse core populations, such as Greece and Italy, could enhance the genetic diversity and adaptive potential of the Bulgarian population. Incorporating individuals from these populations may introduce novel alleles and promote heterozygosity, thereby strengthening the population's resilience to environmental changes and reducing the risks associated with inbreeding. Such an approach aligns with conservation best practices that advocate for maximizing genetic similarity and minimizing the introduction of maladaptive alleles.

However, caution is needed when considering translocations from genetically distinct populations, such as those from Mongolia or Limnos Island. Introducing individuals from these populations could lead to outbreeding depression if local adaptations are disrupted. Therefore, translocation efforts should prioritize populations that are genetically similar to the target population.

ACKNOWLEDGEMENTS

This research work was carried out under the framework of LIFE for Lesser Kestrel (LIFE19 NAT/BG/001017) implemented by Green Balkans – Stara Zagora NGO, with the support of Programme LIFE of the European Union

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