



Assessing genetic diversity patterns at neutral and adaptive loci to inform population reinforcement of an endangered migratory vulture

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Abstract

One of the primary goals of conservation translocation programs should be the maintenance of both population demographic stability and genetic diversity. Here, we provide genetic management recommendations to inform a population reinforcement of the declining Egyptian Vulture population in the Balkans. Specifically, we examined whether the number of released individuals is sufficient to prevent genetic diversity loss due to random genetic drift and what the origin of the individuals should be that comprise the captive breeding pool. To this aim, we estimated and assessed genetic diversity levels and genetic structure of Egyptian Vulture populations across much of the species' range using both neutral and non-neutral candidate loci involved in migration. We then evaluated the effects of the currently proposed population management scheme and candidate source populations on retaining allelic diversity. Our results show low differentiation values among populations and absence of genetic structure which point to past high gene flow. Furthermore, there was no predicted significant impact of different source populations on the genetic diversity of the recipient Balkan population. We also found that the declining Egyptian Vulture population in the Balkans still retains high levels of genetic diversity and therefore genetic diversity restoration is not currently needed. However, without any management, diversity is likely to decrease fast because of increased genetic drift as the population size continues to decline. Population reinforcement with nine birds per year for 20 years would provide sufficient demographic support for the population to retain > 85% of rare allelic diversity. Birds originating from the Balkans would ensure ecological and behavioral similarity and thus would be the best option for reinforcement. Nevertheless, our results demonstrate that to prevent further population contraction and loss of adaptive alleles, releasing individuals of different origin would also be appropriate.

Keywords Captive-breeding · Conservation translocation · Egyptian vulture · *Neophron percnopterus* · Non-neutral markers · Population supplementation

Zusammenfassung

Untersuchung der genetischen Vielfalt an neutralen und adaptiven Loci, um die Auswilderungsstrategie einer bedrohten, ziehenden Geierart zu begleiten.

Eines der Hauptziele von Auswilderungsprogrammen sollte die Erhaltung der demografischen Stabilität und der genetischen Vielfalt einer Population sein. Wir präsentieren Empfehlungen aus genetischer Sichtweise, um die bedrohte Schmutzgeierpopulation auf der Balkanhalbinsel mit in Gefangenschaft aufgezogenen Individuen zu verstärken. Insbesondere untersuchten wir, ob die Zahl der jährlich freigelassenen Individuen ausreicht, um den Verlust der genetischen Vielfalt

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aufgrund zufälliger genetischer Drift zu verhindern, und aus welcher Region die in Gefangenschaft bestehende Brutpopulation stammen sollte. Zu diesem Zweck haben wir die genetische Vielfalt und die genetische Struktur der Schmutzgeierpopulationen über einen Großteil des Verbreitungsgebiets der Art quantifiziert und bewertet, wobei sowohl neutrale als auch nicht neutrale Kandidatenloci verwendet wurden, die das Zugverhalten regulieren. Anschließend bewerteten wir die Auswirkungen der derzeit vorgeschlagenen Auswanderungsstrategie und der potentiellen Herkunftspopulationen auf die Erhaltung der allelischen Vielfalt. Unsere Ergebnisse zeigen geringe Differenzierung zwischen Populationen und das Fehlen einer genetischen Struktur, was auf einen hohen Genfluss in der Vergangenheit hinweist. Darüber hinaus sagen wir keine signifikanten Auswirkungen verschiedener Herkunftspopulationen auf die genetische Vielfalt der verstärkten Population auf dem Balkan voraus. Wir stellten fest, dass die rückläufige Schmutzgeierpopulation auf dem Balkan immer noch ein hohes Maß an genetischer Vielfalt aufweist und daher eine Wiederherstellung der genetischen Vielfalt derzeit nicht erforderlich ist. Ohne jegliches Management wird die Vielfalt jedoch aufgrund der zunehmenden genetischen Drift bei weiter abnehmender Populationsgröße wahrscheinlich schnell abnehmen. Eine Populationsverstärkung mit neun Vögeln pro Jahr über einen Zeitraum von 20 Jahren würde eine ausreichende demografische Unterstützung für die Population bieten, um > 85% der seltenen Allelvielfalt zu erhalten. Vom Balkan stammende Vögel würden eine ökologische und verhaltensmäßige Ähnlichkeit gewährleisten und wären daher die beste Wahl, um die bestehende Population zu verstärken. Dennoch zeigen unsere Ergebnisse, dass auch die Auswanderung von Individuen unterschiedlicher Herkunft angemessen wäre, um einen weiteren Populationsrückgang und den Verlust adaptiver Allele zu verhindern.

Introduction

In many animal species, the patterns of genetic differentiation and gene flow are highly influenced by the ecological characteristics of their habitats as well as their migratory behavior (Willoughby et al. 2017). Although birds, and particularly raptors, can show long-distance migratory behavior, they can also exhibit natal and breeding-site fidelity (Grande et al. 2009; Newton 2010; Bounas et al. 2016). As a general pattern, migratory populations of raptors have been found to have weaker genetic structure and higher genetic diversity than resident populations (Webster et al. 2002; Willoughby et al. 2017). However, due to the natal and breeding-site fidelity, demographically reduced populations can exhibit fine-scale genetic structure (Di Maggio et al. 2015; Bounas et al. 2017). Furthermore, patchily distributed and locally isolated populations as those typical of threatened species are susceptible to a greater influence of genetic drift that will eventually lead to a reduced genetic diversity, and to an inbred population which could further result in reduced fitness. This would compromise the species' ability to adapt to a changing environment and consequently increase its extinction risk (Frankham 1996; Amos and Balmford 2001; Reed and Frankham 2003). Therefore, one of the most important steps to consider before implementing a translocation strategy on a species is to understand its genetic diversity and population structure along with the identification of the underlying processes shaping them.

The Egyptian Vulture (*Neophron percnopterus*) is a long-lived migratory raptor that has suffered a steep decline throughout most of its range due to variety of threats along its flyway (Oppel et al. 2021a). The remaining Balkan population has shown a dramatic decline in numbers and range during the last decades likely due to higher mortality compared to other populations (Buechley et al. 2021; Oppel

et al. 2021b), and as a result is becoming more and more fragmented and thus prone to extinction (Veleviski et al. 2015; Birdlife International 2021). Such ongoing population declines require the development and implementation of conservation management strategies to stop or even reverse them. Therefore, many actions have been carried out in the last 20 years in the Balkans to mitigate threats. However, despite all these efforts, the Egyptian Vulture's Balkan population shows only local and weak signs of stabilization, that do not guarantee the species' survival in the long term (Oppel et al. 2016; Arkumarev et al. 2018). A feasibility study assessing the reinforcement of the Egyptian Vulture in the Balkans was developed recently (Saravia et al. 2020) determining that the implementation of a captive-breeding program for the Egyptian Vulture in Bulgaria and Greece could significantly reduce the population's risk of extinction. Furthermore, a population viability analysis of the Balkan population showed that releasing nine birds per year for the next 20 years combined with the implementation of a range of conservation management actions to increase the species survival rates in the wild (e.g., reduce the use of poison baits in the breeding grounds, insulate dangerous electricity infrastructure and improve survival of immature birds through food provisioning) would be an appropriate strategy to stabilize the population (Oppel et al. 2021b).

Translocations of raptors to peripheral or newly founded colonies can be used as an effective conservation action that can result in population stabilization or even increase in the short term (Seddon et al. 2014; Morandini et al. 2017). However, such programs should consider maximizing both the genetic and the adaptive similarity between native and translocated populations to minimize any unforeseen adaptive and evolutionary consequences (Holderegger et al. 2006). Thus, one of the primary goals of conservation translocation programs should be the

maintenance of both demographic stability and genetic diversity. Most studies on the genetic impacts of translocations investigate diversity at putatively neutral markers such as microsatellites, providing population structure and differentiation patterns shaped by neutral evolutionary forces, such as genetic drift, mutation, or dispersal and gene flow (Kirk and Freeland 2011). However, genetic variation can be partitioned into 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 genetically inherited and could be shaped by natural selection (Pulido 2007; Liedvogel et al. 2011). A few candidate gene markers (e.g., ADCYAP1, CREB1, CLOCK and NPAS2), have been identified by mining circadian molecular pathways in the avian genome for tandem repeats (Steinmeyer et al. 2009). Studying variation in such genes may offer new insights into local adaptation of populations and their evolutionary and ecological implications.

Here, we provide genetic management recommendations to inform a population reinforcement that will preserve unique allelic diversity of the declining Egyptian Vulture population in the Balkans. Population viability modeling has already provided insight on how often and how many individuals should be released to stabilize the population (Oppel et al. 2021b) and the viability of captive-bred Egyptian vultures has been demonstrated (Efrat et al. 2022). This study aims to resolve two further questions, namely: (1) is the number of released individuals sufficient to prevent genetic diversity loss due to random genetic drift?, and (2) what should be the origin of the individuals that comprise the captive breeding pool? Such questions are often faced in different species conservation management programs and the integration of genetic data should be considered as an essential task in the design and implementation of a successful population reinforcement strategy (Caughley 1994). To address these questions, we (a) estimate and assess genetic diversity levels and genetic structure of Egyptian Vulture populations across much of the species range using both neutral and non-neutral (i.e., putatively adaptive) candidate loci involved in migration, (b) evaluate the effects of the currently proposed population management scheme on retaining allelic diversity, and (c) identify the best candidate source populations to be used for reinforcement by comparing their effect on the recipient population's genetic diversity. This approach can provide an adaptive framework with which to manage the selection of captive-bred individuals and improve the chances of success of the reinforcement program.

Methods

Sample collection

The samples included in the analyses ($n = 89$) were collected from seven sites throughout the part of the species' distribution that performs regular long-distance migrations (Fig. 1a): the Iberian Peninsula ($n = 11$), the Balkan Peninsula ($n = 23$), Central Turkey ($n = 9$), Israel ($n = 22$), Eastern Anatolia and Caucasus ($n = 10$), Central Asia ($n = 5$). These samples were supplemented by 10 samples from the Canary Islands belonging to the non-migratory subspecies *Neophron percnopterus majorensis*. Additionally, we included samples from nine captive-bred individuals that were released in the Balkans as part of a pilot reinforcement program (Arkumarev et al. 2021). To minimize biases associated with relatedness, only a single fledgling per brood was sampled. Two drops of blood ($\approx 50 \mu\text{l}$), plucked feathers and, in one case, a talon, were obtained from each individual and immediately stored in blood storage cards (NucleoCards, Macherey-Nagel) or absolute ethanol at room temperature until DNA extraction.

Loci amplification and genotyping

DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's protocol. Each individual was genotyped at a total of 12 microsatellite (putatively neutral) and four non-neutral (putatively adaptive) loci. Four microsatellite loci (BV6, BV13, BV14, BV20) were originally isolated from the Bearded Vulture (*Gypaetus barbatus*; (Gautschi et al. 2000)), whereas eight (Np51, Np163, Np166, Np229, Np249, Np257, Np259, and Np296) were developed specifically for the Egyptian Vulture (Agudo et al. 2008). To examine adaptive genetic variation, we amplified microsatellite-containing parts of ADCYAP1, CREB1, CLOCK, and NPAS2 genes (Steinmeyer et al. 2009). Details on loci properties and primers used for their amplification are presented in Table S1. Samples from Central Turkey were not genotyped for the candidate genes due to a lack of extracted DNA. Microsatellite loci were amplified in two multiplex reactions, whereas the four candidate genes were amplified in a single multiplex reaction (Chakarov et al. 2013) using forward 5'-fluorescent labeled primers and the KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems). Each 12.5- μl reaction contained ~ 20 ng template DNA, 2 pM of each primer and 1 \times KAPA2G Mix and was carried out using the following profile: an initial denaturation step of 3 min at 95 °C, 30 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension step of 10 min at

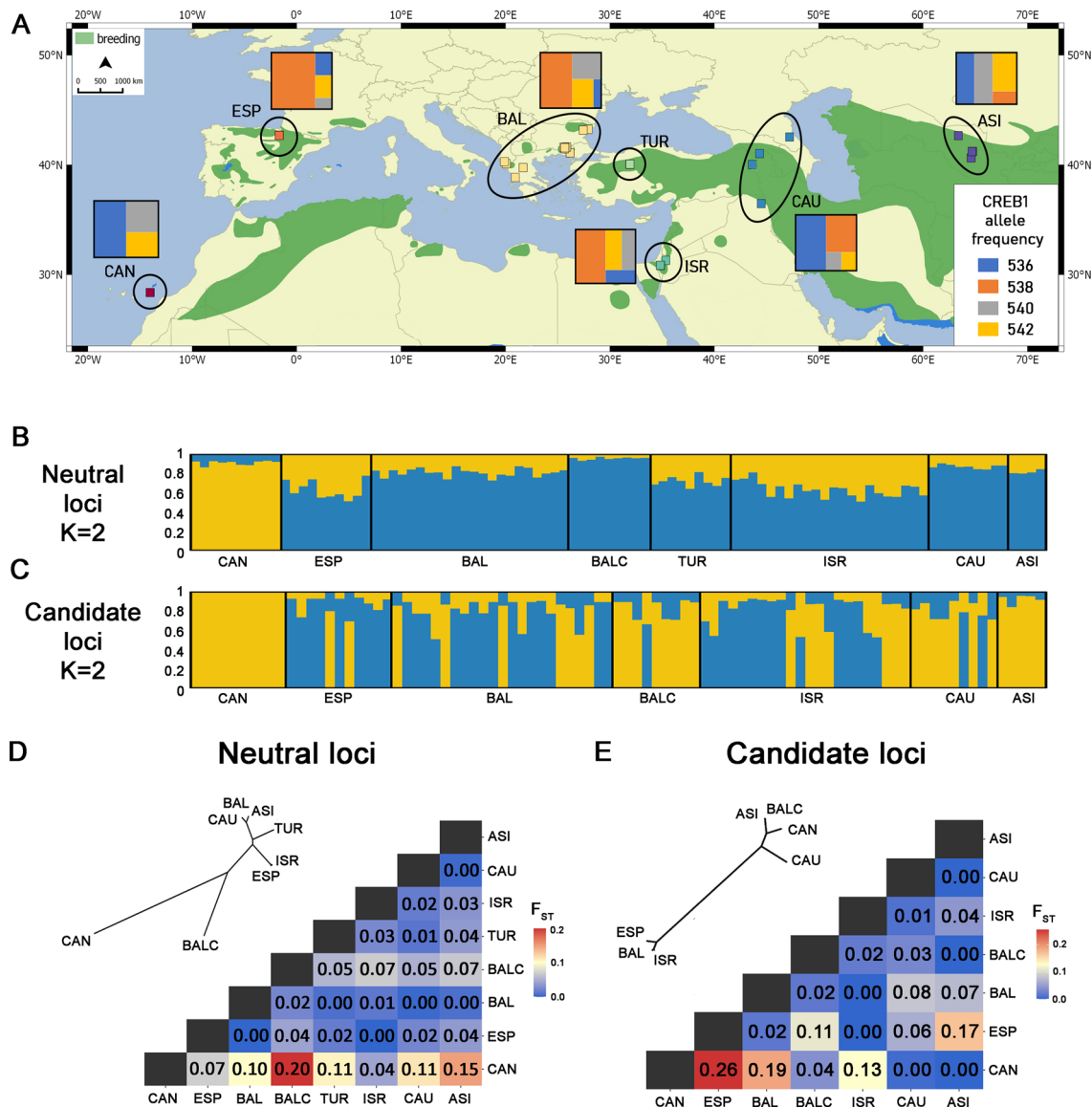


Fig. 1 **a** Map of the sampling locations of Egyptian Vulture individuals included in the study along with shaded species distribution and with allele frequency treepLOTS for locus CREB1 in each sampled population. Proportions of membership to each of the two inferred clusters ($K=2$) of individual Egyptian Vultures using STRUCTURE for both the neutral (**b**) and the non-neutral dataset (**c**). UPGMA

tree using pairwise Jost's D as genetic distance measure and matrix of pairwise F_{ST} values for all population comparisons, based on the neutral (**d**) and non-neutral (**e**) dataset. Shading reflects degree of divergence and corresponds to F_{ST} values indicated in legend (right). Populations are in geographic order from Canary Islands to Central Asia

72 °C. PCR products were separated and visualized using an ABI 3730xl capillary sequencer (Applied Biosystems) and genotypes were scored by eye with STRand v.2.4.59 (Toonen and Hughes 2001). Randomization of samples was used throughout lab processes to avoid any plate/gel-specific errors that might lead to population-specific biases (Meirmans 2015). Genotyping errors, due to null alleles and stuttering, were examined for all loci and sampled populations using MICROCHECKER (Van Oosterhout et al. 2004). No locus was found to harbor any null alleles.

Genetic diversity and population differentiation

We explored patterns of genetic diversity by calculating the observed (H_o) and unbiased expected (uH_E) heterozygosity, inbreeding coefficient (F_{IS}), and rarefied allelic richness (A_R) for each studied population separately, based on the neutral dataset using the R package “hierfstat” (Goudet 2005). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and calculation of allele frequencies in

each population were performed in the “PopGenReport” R package (Adamack and Gruber 2014) for both datasets.

To characterize the strength and pattern of genetic structure within both datasets, first we calculated pairwise fixation indices (F_{ST}) among the sampling localities and calculated their respective 95% confidence intervals with 1000 bootstraps in “hierfstat”. As a complimentary measure of genetic differentiation, Jost’s D was calculated for all pairs of sampling localities using the R package “mmod” (Winter 2012). Furthermore, we subjected the data to a principal component analysis (PCA) within the R package “adeget” (Jombart 2008). Population structure was further evaluated through the Bayesian clustering software Structure 2.3.4 (Pritchard et al. 2000) to infer the number of genetically homogeneous clusters (K) present in both the neutral and the non-neutral datasets. We assumed 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). The most likely number of genetic clusters, K , was evaluated following the ΔK method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl and Vonholdt 2012), as well as by calculating the posterior probability for each K . The analysis was conducted both on the full dataset as well as only with individuals belonging in the *N. p. percnopterus* subspecies. Using the neutral dataset, a Mantel test was performed to obtain any statistically significant associations between pairwise genetic ($F_{ST}/1 - F_{ST}$) and geographic (Euclidian) distance matrices using the R package “ade4” (Dray and Dufour 2007). Regarding the non-neutral dataset, we additionally examined any associations between latitude, longitude and both mean allele length (MAL) for each individual and the frequencies of the most common allele (MCA) for each locus using linear regressions. All analyses were performed in R 4.0.5 (R Core Team 2021).

Simulation of genetic diversity under population reinforcement scenarios

We used the R package “AlleleRetain” (Weiser et al. 2012) to calculate the probability that the Balkan population retains rare allelic diversity (an allele with a starting frequency of 0.05) with and without reinforcement actions. “AlleleRetain” simulates neutral allelic diversity, so results reflect diversity loss through genetic drift. Models were parameterized based on values from an integrated population model developed for the species that was used to estimate the optimal number of individuals to be released to stabilize the Egyptian Vulture population in the Balkans (Oppel et al. 2021b). Full details of parameters used for “AlleleRetain” simulations can be found in Table S2. Simulations were run for 20 years, conforming to the Reinforcement Strategy developed for the

species (Arkumarev et al. 2022) and we modeled two scenarios: one where no reinforcement would take place (baseline) and one where the population would be reinforced with nine birds every year for 20 years (Saravia et al. 2020; Oppel et al. 2021b; Arkumarev et al. 2022). To evaluate any changes in the genetic diversity of the Balkan population after reinforcement with individuals from alternative candidate source populations, we followed the approach used by Robinson et al. (2021). Briefly, we simulated sampling nine individuals by randomly drawing two alleles per locus based on the allele frequencies for each population using the randomization feature in PopTools 3.2.5 (Hood 2010). These nine genotypes from each candidate source population were then added into the extant Balkan population and changes in heterozygosity and effective number of alleles were evaluated by means of a Kruskal–Wallis test. Since the majority of the currently established pairs in captivity that can produce juveniles readily available for release under the framework of the Balkan restocking program are of Spanish and Balkan origin (Saravia et al. 2020) and to examine if releasing individuals from multiple populations provides any benefit, we considered the following practically feasible scenarios: (a) all nine released individuals originate from the Balkan captive breeding pool, (b) all nine released individuals originate from Spain, (c) multiple sourcing with five individuals from Spain and four from the Balkans, and (d) multiple sourcing with three individuals from Spain, three from the Balkans and three from Central Turkey. Although many other combinations of individuals are theoretically possible, we considered these scenarios as the most relevant for future management because more diverse and balanced sourcing of individuals from a larger geographic range is unlikely to be practically feasible.

Results

Genetic diversity

The Balkan population (BAL) showed high levels of genetic diversity exhibiting the highest observed heterozygosity values followed by the Eastern Anatolia and Caucasus (CAU) natural populations, whereas high heterozygosity was also apparent in the captive-bred birds released in the Balkan Peninsula ($H_o = 0.61, 0.58, 0.60$ respectively; Table 1). In fact, this was the sole group that showed a high and significantly negative F_{IS} value implying an excess of heterozygotes. On the other hand, the lowest heterozygosity levels were observed in the Canary Island subspecies (CAN), the Iberian (ESP) and the Central Asian (ASI) populations ($H_o = 0.37, 0.44$ and 0.48 respectively). Central Turkey (TUR) and Israel (ISR) populations showed intermediate levels of heterozygosity

Table 1 Measures of neutral genetic variation of all sampled Egyptian Vulture populations

Population	<i>N</i>	π	<i>A</i>	<i>A_r</i>	Ho	uHe	<i>F_{is}</i>
Canary Isl. (CAN)	10	0.06	2.8±0.5	2.2±0.3	0.37±0.09	0.39±0.09	-0.007±0.067
Iberia (ESP)	11	0.08	3.8±0.5	2.7±0.3	0.44±0.07	0.52±0.07	0.102±0.064
Balkans (BAL)	23	0.11	4.8±0.7	3.0±0.3	0.61±0.08	0.57±0.07	-0.073±0.038
Captive released birds (BALC)	9	0.13	3.5±0.5	2.8±0.3	0.60±0.06	0.58±0.07	-0.114±0.09
Central Turkey (TUR)	9	0.02	3.1±0.4	2.6±0.3	0.53±0.08	0.55±0.07	-0.020±0.083
Israel (ISR)	22	0.10	4.8±0.7	3.0±0.3	0.52±0.07	0.56±0.07	0.012±0.045
Eastern Anatolia and Caucasus (CAU)	10	0.17	4.3±0.6	2.9±0.3	0.58±0.06	0.60±0.06	-0.040±0.059
Central Asia (ASI)	5	0.10	2.8±0.3	2.6±0.3	0.48±0.08	0.55±0.08	-0.027±0.081

Number of genotyped individuals (*N*), private allelic richness (π) based on a subsample of 10 alleles, mean number of alleles per locus (*A*), mean allelic richness (*A_r*) based on a subsample of 10 alleles, observed (Ho) and unbiased expected (uHe) heterozygosity, and inbreeding coefficient (*F_{is}*). Values are presented as means ± standard error. Significant *F_{is}* is given in bold

(Ho = 0.53 and 0.52 respectively). Private alleles were rare but present in some sampled populations with higher frequencies in Israel and the captive-bred Balkan population. Only locus BV6 was found to depart from HWE in the case of the Central Asian (ASI) population. Genetic diversity estimates for neutral loci are summarized in Table 1. Regarding the putatively adaptive loci, CLOCK was found to be monomorphic and thus not included in further analyses. We found greater variation in ADCYAP1 and CREB1 with four different alleles across populations. Observed heterozygosity for candidate genes was in general fairly low (Table 2), with the highest heterozygosity levels observed in ESP for ADCYAP1 (Ho = 0.27), in the Balkan population and the captive-bred individuals in locus CREB1 (Ho = 0.46 and 0.74, respectively) and in CAU and ISR for NPAS2 (Ho = 0.33 and 0.32 respectively). All populations in ADCYAP1 and NPAS2 showed no significant differences between observed and expected heterozygosity, but in locus CREB1, observed heterozygosity was consistently lower than expected in all wild populations, except for the Canary Island one.

Genetic structure

The PCA did not exhibit any evidence of genetic structure among populations both for the neutral and non-neutral dataset. For the neutral dataset, although individuals formed a rather dispersed group in space, individuals from the Canary Island population seem to form a cluster along PC1 and PC2 (Fig. S1). All individuals from the rest of the populations overlapped with each other although some captive-bred individuals seemed to differentiate in space. In the PCA performed on the non-neutral loci, all individuals were dispersed in space, forming one inseparable cloud (Fig. S1). The Bayesian clustering method implemented in STRUCTURE, suggested a weak population structuring. When all individuals were modeled for the neutral loci, the ΔK -method suggested two clusters (*K* = 2) as the most likely population structure (in accordance with the posterior probability that was also higher for *K* = 2; Fig. S2, Table S3). For *K* = 2, the admixture model indicated two homogeneous gene pools corresponding to the two different subspecies (*N. p. percnopterus* vs *N. p. majorensis*; Fig. 1b). No further substructure was indicated among the *N. p. percnopterus* subspecies; in fact, the posterior probability value suggested

Table 2 Summary statistics for each candidate gene (except for CLOCK that was found to be monomorphic), for all Egyptian Vulture populations

Population	ADCYAP1			CREB1			NPAS2		
	Ho	He	A	Ho	He	A	Ho	He	A
Canary Isl. (CAN)	0.000	0.000	1	0.444	0.623	3	0.180	0.189	2
Iberia (ESP)	0.273	0.244	3	0.222	0.451	4	0.273	0.236	2
Balkans (BAL)	0.043	0.043	2	0.455	0.637	4	0.130	0.122	2
Captive released birds (BALC)	0.111	0.105	2	0.750	0.742	4	0.000	0.000	1
Israel (ISR)	0.136	0.129	3	0.150	0.664	4	0.318	0.325	2
Eastern Anatolia and Caucasus (CAU)	0.111	0.105	2	0.167	0.625	4	0.333	0.278	2
Central Asia (ASI)	0.000	0.000	1	0.400	0.720	4	0.000	0.000	1

Observed (Ho) and expected (He) heterozygosity and the number of alleles per locus (A). Deviations from HWE are given in bold

$K=1$, indicating a lack of any structure among populations. Similarly, when the model was run for the non-neutral dataset, the ΔK -method suggested two clusters ($K=2$) as the most likely population structure followed by $K=1$, based on the posterior probability (Table S4, Fig. S3). For $K=2$ the admixture model indicated two gene pools with all individuals from the Canary Islands subspecies showing high membership coefficients in the first cluster, whereas the rest of the individuals showed a mixed ancestry from a second cluster (Fig. 1c). Evaluation of the scenario of $K=7$ with a high ΔK value, showed that all individuals present the same membership proportions to seven clusters, indicative of a lack of any genetic structure. Pairwise population differentiation patterns were similar for both F_{ST} and Jost's D estimates (Table S5). Based on the neutral loci, high values were observed between the Canary Island subspecies and the rest of the populations, as expected. The highest differentiation value was observed between the Canary Island and the Central Asian populations (CAN-ASI; F_{ST} : 0.15). Subspecies aside, low and non-significant values were observed among the rest of the populations indicating weak differentiation (Fig. 1d, Table S5). In fact, the Balkan population was not significantly differentiated from any other population with pairwise F_{ST} values being almost zero between the Balkan and both the Spanish (ESP) and the eastern Anatolia and Caucasus (CAU) populations. On the other hand, examination of the candidate loci dataset provided contrasting results. High differentiation values were observed between the Mediterranean populations (ESP, BAL, and ISR) and all the rest (Table S6, Fig. 1e). The highest value was observed between the Canary Island and the Spanish populations (CAN-ESP; F_{ST} : 0.26). The Mantel test exploring any isolation by distance effects, showed that genetic distance is not significantly correlated with geographical distance across all sampled *N. p. percnopterus* populations ($R^2=0.15$, $p=0.16$). When examining allele clines in non-neutral loci we detected a longitudinal cline for CREB1 gene. We did not find any significant association between allele length and longitude ($R^2=0.005$, $p=0.55$), but rather the frequency of the most common allele (538) was found to be decreasing with increasing longitude (eastwards; Fig. 1a). The specific clinal allele was absent in genotyped individuals from the Canary Island subspecies population.

Simulation of reinforcement scenarios

Without management, the Balkan Egyptian Vulture population showed a 35% probability of retaining low-frequency alleles after 20 years. Reinforcing the population with nine individuals per year for 20 years would result in retaining 85% of alleles at a frequency of 0.05 (Fig. 2a). The simulation-based evaluation of changes in the genetic variability of the Balkan population after reinforcement using alternative

candidate source populations did not reveal any significant patterns (Fig. 2b). Expected heterozygosity showed only a slight increase with the addition of individuals from Spain and with individuals from multiple sources (Balkans, Spain, and Central Turkey), but was not significantly different compared to other sources (Kruskal–Wallis test; $H=0.68$, $P=0.88$). Similarly, post-reinforcement effective number of alleles was only marginally higher (and not statistically significant) when individuals of Spanish or mixed origin were added ($H=0.71$, $P=0.8$).

Discussion

The current study was developed to further inform the reinforcement strategy of the Egyptian Vulture's Balkan population with individuals bred in captivity that aims to halt the population's decline (Arkumarev et al. 2022). We demonstrate that the declining Egyptian Vulture population in the Balkans still retains high levels of genetic diversity and is therefore not in need of any management actions aiming to restore genetic diversity (i.e., genetic rescue). However, without any management, it is likely that diversity will decrease because of increased genetic drift as the population size diminishes. The planned population reinforcement with nine birds per year for 20 years will provide enough demographic support allowing the population to retain > 85% of rare allelic diversity. Overall, genetic diversity patterns were found to be similar to those found in other vulture species (Kretzmann et al. 2003; Le Gouar et al. 2008; Poulakakis et al. 2008). Low differentiation values among populations point to the existence of high gene flow, a similar pattern to the one shown in the Griffon Vulture (*Gyps fulvus*) in Europe (Le Gouar et al. 2008). Indeed, until recently the species was widely distributed across Europe forming a rather continuous population whereas gene flow could be further facilitated by the wide range movements of Egyptian vultures especially immature birds that explore many potential breeding areas before settling to breed (Phipps et al. 2019; Opperl et al. 2022). As expected, we observed lower levels of genetic diversity in the insular subspecies population (Frankham 1997; Agudo et al. 2011) and all results regarding genetic structure were congruent with its delineation as a subspecies (Donazar et al. 2002). Reinforcement of any population of the nominate subspecies with birds coming from Canary Islands is therefore not recommended as our study confirms the genetic differentiation of Canarian Egyptian Vultures compared with continental populations and furthermore highlights differentiation at candidate migration loci.

The stochastic simulations applied in this study come with certain limitations, especially our simulations of releasing birds from different source populations. This approach explores changes in genetic diversity just after the release

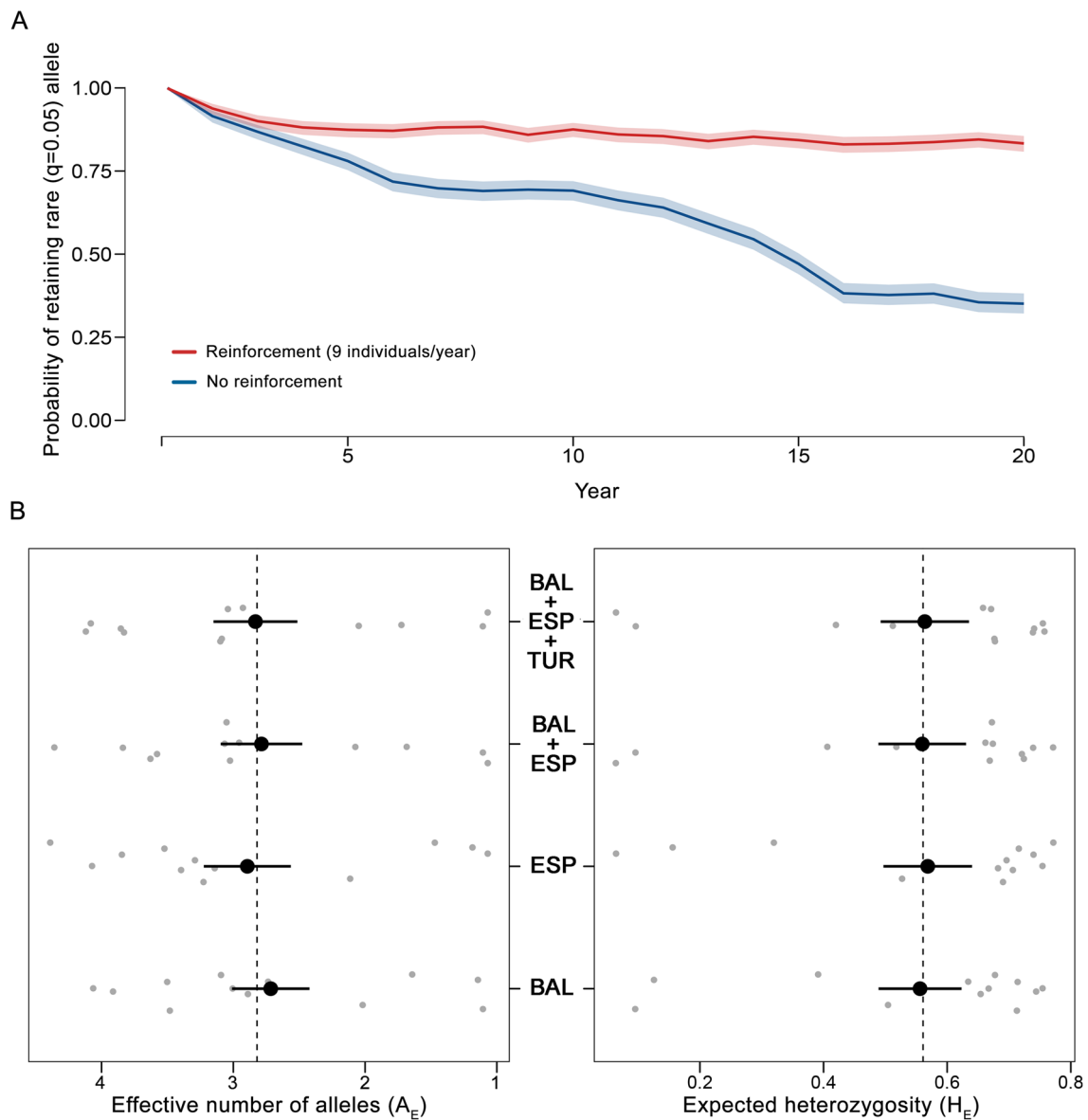


Fig. 2 **a** Probability of retaining a selectively neutral allele at a starting population frequency of 0.05 after 20 calendar years for the Egyptian Vulture population in the Balkans, with (red) and without (blue) reinforcement actions. Solid lines indicate the mean estimate over 1000 iterations and shaded ribbons represent 95% confidence intervals. **b** Mean (\pm SE, black bar) effective number of alleles (left)

and the expected heterozygosity (right) when 9 randomly simulated individuals from each source population denoted on the Y-axis are released into the extant Egyptian Vulture Balkan population every year for 20 years. Dashed line shows the value of the extant population in each parameter and jitter shows the value for each locus (color figure online)

of individuals in the population, only at that specific point in time and making the simplifying assumption that mating is random (Robinson et al. 2021). However, not all released birds will manage to breed, so the effect of different source populations on genetic diversity will be even lower than calculated. In the long term, retaining genetic variation could be subject to demographic changes as well as any underlying environmental processes and ultimately to random genetic drift. Thus, our models consider rare and neutral alleles. Such alleles may be easily lost by genetic drift in

small populations, but there is also increasing evidence that they provide adaptive potential in future and conservation strategies should retain the largest possible number of them (Petit et al. 1998; Weiser et al. 2013). Maximizing the number of alleles in a few markers has been found to lead to higher response to selection than maximizing their heterozygosity (Vilas et al. 2015). We have not examined the effects of population reinforcement on putatively adaptive loci. Based on the allele frequency cline, we observed it could be possible that some genotypes may indeed provide

a selective advantage, however measurable data on fitness differences do not exist. Monitoring of the released birds and their migration outcome should be implemented to further assess any fitness-related costs. In any case, any such disadvantage could be avoided if populations with similar ecological requirements are used as a source.

Non-neutral pairwise F_{ST} values based on candidate loci were not associated with those based on microsatellites, showing that they do not reflect similar patterns of population structure and thus history. Such a discrepancy could reflect the migratory behavior of the species that could in turn shape the underlying genetic structure of the species. Genes putatively involved in migration are expected to evolve under natural selection and may provide signs of local adaptation. In fact, analysis of putatively adaptive loci revealed a clinal pattern of allele frequencies for CREB1, a transcription factor involved in the light-induced clock induction (Tischkau et al. 2003). Polymorphisms at CREB1 have been previously implicated with juvenile dispersal behavior in Buzzards (*Buteo buteo*): earlier dispersing individuals carried shorter CREB1 alleles than later dispersers (Chakarov et al. 2013). In addition, CREB1 allele size significantly predicted male molt speed, with longer alleles being associated with faster molt (Bazzi et al. 2017). In our case, we did not detect any association based on allele size, but rather a longitudinal distribution of the most common allele frequency. Despite the fact that our sampling was limited in the eastern part of the species' distribution and could confound the results, this clinal allele was not detected in the non-migratory subspecies located in the Canary Islands. Our result is therefore in line with the recent evidence that CREB genes have a key role in influencing the distance of migratory flights via co-regulation of the capacity for long-term memory (Gu et al. 2021).

Our results also provide an indirect evaluation of the species captive breeding stock by including samples of captive-bred individuals released so far in the Balkans. When all captive-bred birds were handled as a single population, they displayed significant deviation from HWE with the F_{IS} values indicating a heterozygote excess. Furthermore, F_{ST} values between the captive-bred individuals and the rest of the populations was higher than among the rest of the groups. Such an observation is rather expected, as captive-bred individuals form a very heterogeneous group due to their diverse origin (Arkumarev et al. 2021). Besides, genetic diversity of the source population and the existent genetic diversity of the recipient population are major concerns that can determine the success of conservation translocations (Armstrong and Seddon 2008). Our genetic structure analyses showed low levels of differentiation among populations and is therefore consistent with our simulation-based approach which did not show any significant impact of different sources on the genetic diversity of the recipient Balkan population.

Conclusions

Our findings provide the Egyptian Vulture reinforcement program with enough flexibility regarding selection of individuals to be released. The genetic diversity of an Egyptian Vulture captive breeding pool that consists of individuals from any population (of the nominate subspecies) would be effective in retaining high levels of genetic variability of the native Balkan population. Birds originating from the Balkans would ensure ecological and behavioral similarity and thus would be the most sensible choice for the Egyptian Vulture reinforcement actions (Frankham et al. 2002; IUCN 2013; Whiteley et al. 2015). Nevertheless, our study indicates that releasing individuals of different ancestry (originating from Iberia and Central Turkey) would also be feasible. In fact, as the Balkan captive breeding pool is still small and under development (Saravia et al. 2020), releasing birds that originate from well-established pairs albeit of different origins, would be the most pragmatic approach to prevent population contraction and further loss of adaptive alleles, as these pairs have higher chances of producing offspring, thus ensuring enough birds are available for release.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics statement Egyptian Vulture samples were collected under field research permits issued by every relevant national authority and were

obtained. This study was carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of the Council of Europe (<http://conventions.coe.int/Treaty/EN/Treaties/Html/123.htm>).

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