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Population structure, diversity, and phylogeography in the near-threatened Eurasian black vultures *Aegypius monachus* (Falconiformes; Accipitridae) in Europe: insights from microsatellite and mitochondrial DNA variation

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The Eurasian black vulture (*Aegypius monachus*) has experienced a severe decline during the last two centuries and is globally classified as near-threatened. This has led to the extinction of many traditional breeding areas in Europe and resulted in the present patchy distribution (Iberian and Balkan peninsulas) in the Western Palearctic. In the present study, we describe the current genetic status of the European populations using both mitochondrial cytochrome *b* sequences and nuclear microsatellite markers, comparing with those found in Asia (Mongolia and Caucasus region). Although, mitochondrial (mt)DNA revealed a relatively low genetic variability (haplotype diversity), no evidence of genome-wide genetic erosion exists because nuclear diversity exhibits normal levels and strong differentiation. A highly philopatric dispersal behaviour must be invoked to explain the existence of a clear pattern that revealed by the phylogeographic analysis, which indicates a sharp East–West clinal distribution and an allopatric differentiation. The distribution of mtDNA haplotypes one in the Iberian population and two in Balkan population and the significance divergence at nuclear loci fulfill the definitions of those populations as evolutionary significant units. We discuss how management strategies should aim at the maintenance (or increase) of current genetic variability levels, suggesting that independent conservation plans are urgently required to protect these two breeding European populations from extinction. *Biological Journal of the Linnean Society*, 2008, **95**, 859–872.

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INTRODUCTION

The Eurasian black vulture (*Aegypius monachus*) is the largest bird of prey (Falconiformes) in the world (i.e. the unrelated, slightly larger Andean Condor is now affiliated with the Ciconiiformes). By being mainly a scavenger or carrion eater bird with a long life-span and an extended home-range, vast areas of suitable habitats are considered indispensable for the species' well being (Cramp & Simmons, 1980; Carrete & Donazar, 2005). Its global range extends from the Iberian Peninsula across southern Europe and through the central Asian plateau to Mongolia and China.

Listed in Appendix I of the CITES (Convention on International Trade of Endangered Species of Wild Fauna and Flora) and considered near-threatened by the IUCN (2006 IUCN Red List of Threatened Species) the Eurasian black vulture has attracted some conservation attention. It is classified as vulnerable at European level (BirdLife International, 2006) and endangered in Greece (Handrinos, 1992). Despite the fact that in some regions (e.g. Spain) its populations are now recovering (Sánchez, 2004), the species has suffered a serious demographic decline during the last century, mainly as a result of human pressure (i.e. the degradation and destruction of its breeding habitats, direct persecution and poisoning, the abandoning of extensive livestock economy, and the rarefaction of wild ungulate populations) (Hiraldo, 1974; Donazar *et al.*, 2002). Many traditional breeding areas were lost and the species is now extinct in France, Italy, Poland, Slovakia, Austria, Croatia, Yugoslavia, Romania, Moldova, and Cyprus (Cramp & Simmons, 1980; Meyburg & Meyburg, 1984), resulting in the present patchy distribution of breeding nuclei in Europe (Fig. 1). Extant European populations are confined to Spain, the Balkans (Rhodope Mountain), and the Caucasus mountains (Russia, Georgia, Armenia, and Azerbaijan). It is worth noting that there is no evidence (field observations) of contact between Balkan and Iberian populations subsequent to the extinction of the intermediate population(s) in 19th Century. Much less information is available regarding the status and population trends of the species in Asia, where the bulk of the global population resides. It appears that breeding populations are more or less stable in Mongolia (where the species is described as common) and Pakistan (where it is described as scarce), although fluctuations in distribution and breeding success occur. In Kazakhstan, however, populations of all vulture species are in severe decline. This trend may be mirrored in a number of other central Asian countries where populations of both domesticated livestock and wild ungulates have declined greatly in recent years (BirdLife International, 2006).

Faced with the growing challenge of deriving strategies for salvaging diminishing flora and fauna, conservation biologists and ecologists continue to search for methods that can distinguish unambiguous units for conservation purposes (Fraser & Bernatchez, 2001). An understanding of the genetic diversity and the spatial structure of populations is important for establishing the appropriate scale and subunits for conservation management and minimizing genetic erosion (Moritz, 1999). Current genetic patterns in a species are shaped both by historical and contemporary factors that affect its biogeography and its demography. The relative contributions of historic

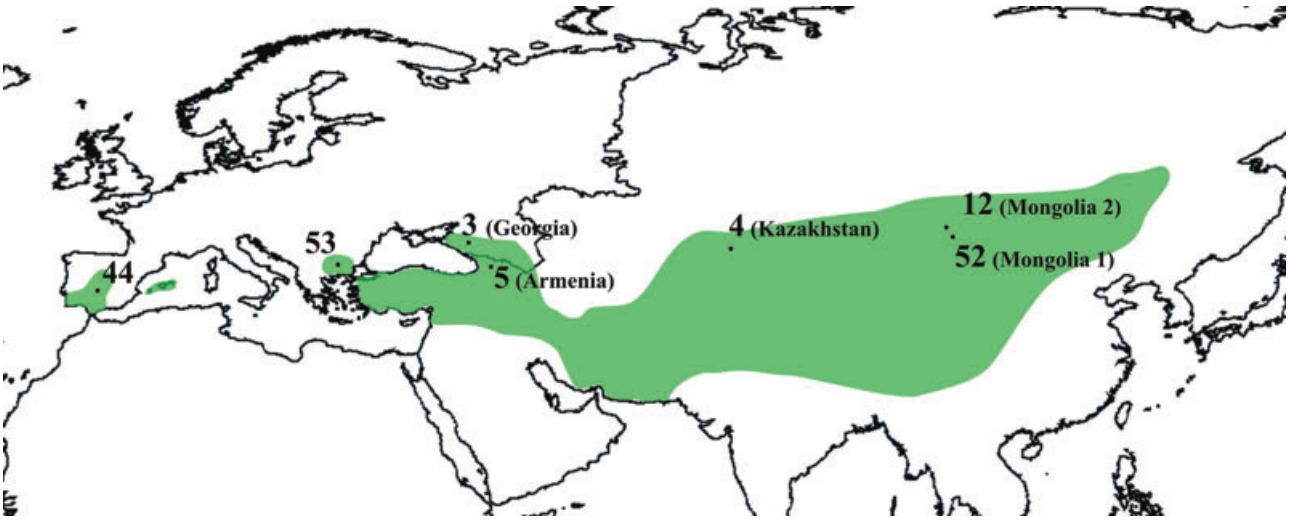

Figure 1. A map of Eurasia showing the location of the sampling areas, the number of samples from each locality, and the present distribution of the species *Aegypius monachus*.

and contemporary factors in shaping the genetic makeup of the species are not easy to unravel, and a combination of several analyses at different temporal scales (i.e. haplotype relatedness, demographic history, and population genetics) might be necessary to describe the geographical structure and investigate the historic or contemporary processes that determine its origin (Bernatchez, 2001; Godoy *et al.*, 2004).

However, there has been no attempt to describe the geographical distribution of genetic diversity or the evolutionary history of *A. monachus*. Like many large-sized raptors, *A. monachus* is difficult to study because: (1) it is an endangered species with large home ranges; (2) it keeps small or limited populations, whereas some of them are not well monitored and the available information are limited; (3) its nests are located in inaccessible places; and (4) males and females are indistinguishable visually, and adults are sometimes difficult to capture and mark.

To date, few genetic studies have been carried out for large-sized vultures, including bearded vultures (*Gypaetus barbatus*) (Negro & Torres, 1999; Gautschi *et al.*, 2003; Godoy *et al.*, 2004), griffon vultures (*Gyps fulvus*) (Le Gouar *et al.*, 2006), Egyptian vulture (*Neophron percnopterus*) (Kretzmann *et al.*, 2003), Andean condors (*Vultur gryphus*) (Hendrickson *et al.*, 2003), and Old World vultures (Lerner & Mindell, 2005). These studies have shown low levels of genetic diversity (in *G. fulvus*, this was quite high compared to the other species of vultures), strong differentiation between indigenous and captive population, and the existence of discernable evolutionary lineage in Europe.

In the present study, we have applied nuclear multilocus genotypes (eight microsatellites) and mitochondrial cytochrome *b* (cyt *b*) sequences to the two major remaining breeding populations of Europe (Iberian and Balkan Peninsula). The study aimed to evaluate whether current genetic diversity of these populations could have been affected by the species decline by comparing current estimates with those found in Asia (Mongolia), in which the breeding populations are more or less stable and the species is described as common. As well as providing insights into the past and current processes that have shaped the evolution and genetic structure of *A. monachus*, our objectives are also designed to identify management units for conservation of this large bird of prey.

It known that the ability to genetically profile non-invasively collected samples has proven particularly important for conservation, allowing researchers to genetically 'tag' individuals of threatened or endangered species without capture. Accordingly, we also used non-invasively collected samples (e.g. feathers and bones) to demonstrate how non-invasive genetic tagging can be of particular use when studying vultures. Although hair and scat (the most common non-invasive material) have been used extensively for genetic tagging (Taberlet *et al.*, 1997), large-scale studies utilizing feathers for individual identification remain rare (Rudnick *et al.*, 2005).

MATERIAL AND METHODS

Sampling and DNA extraction, amplification, and sequencing

In total, 173 samples of *A. monachus* were collected from wild-caught individuals captured during field ecology projects (Fig. 1). DNA from blood or muscle tissue ($N = 135$) was extracted with DNeasy Tissue Kit (Qiagen) following the manufacturer's instruction. DNA from dry skins/bones ($N = 12$) and naturally molted adult feathers ($N = 26$) was isolated using DNA techniques for museum specimens. The feathers and skins were washed three times in 1 mL of 10 mM Tris-HCl (pH 8.0) on a rotary mixer for 24 h per wash to re-hydrate (Austin & Melville, 2006), whereas 40 mg of powder bones were incubated in 1 mL of proteinase K digestion buffer composed of 10 mM Tris (pH 8.0), 10 mM NaCl, 50 mM ethylenediaminetetraacetic acid (pH 8.0), 0.5% sodium dodecyl sulphate and 1 mg mL^{-1} proteinase K for 1 h at 56 °C under agitation (Rohland & Hofreiter, 2007). In both cases, after centrifugation and washing of the 'pellet' with 1 mL of ddH_2O, we followed used the DNeasy Tissue Kit in accordance with the manufacturer's instructions. A partial sequence of mitochondrial cyt *b* gene was amplified using the universal primers L14841 and H15149 (Palumbi, 1996) in a polymerase chain reaction (PCR) protocol of denaturation 94 °C for 5 min, and 35 amplification cycles of 94 °C for 30 s, 47 °C for 30 s, and 72 °C for 45 s, followed by an extension at 72 °C for 10 min. A negative control was run with each round of PCR. PCR products were purified with the NucleoSpin Extract II purification kit (Macherey-Nagel). Single stranded sequencing was performed with the primers of PCR, using the Big-Dye Terminator (version 3.1) Cycle Sequencing kit on an ABI 377 sequencer. PCR fragments were sequenced in both directions to assure sequence accuracy. Mitochondrial DNA sequences were edited using SEQUENCHER, version 4.2 (Gene Codes Corporation) and deposited in GenBank (EF426498–537).

Genotyping

We tested 19 microsatellite primer pairs, developed for the Griffon Vulture *G. fulvus* (Mira *et al.*, 2002) and the bearded vulture *G. barbatus* (Gautschi *et al.*, 2000). Eight loci proved polymorphic and showed clearly scorable bands in *A. monachus*. One hundred and seventy-three individuals were successfully

genotyped at these eight microsatellite loci. Thermal cycling was performed under the following conditions: 5 min at 94 °C; a touchdown of 15 cycles of 94 °C for 1 min, 62 °C for 30 s (with a stepwise decrease of 1 °C at each cycle), 72 °C for 45 s; then 30 amplification cycles of 94 °C for 1 min, 47 °C for 30 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. PCR and loading multiplexes were developed to reduce the time and cost of genetic analyses (eight loci in four PCR reactions and loading into two gel-lanes). PCR products were analysed in an ABI-377 sequencer. GENESCAN and GENOTYPER, version 2.0 (Applied Biosystems) were used for the determination of allele sizes.

Mitochondtial DNA statistical analyses

The alignment of the cyt *b* sequences was performed with CLUSTAL X (Thompson *et al.*, 1997). Cytochrome *b* sequences were translated into amino acids prior to analysis to check for spurious gaps or stop codons. Sequence divergences were estimated using MEGA, version 3.1 (Kumar, Tamura & Nei, 2004). The settings for the DNA substitution model that best fitted the data were selected by the Akaike Information Criterion (Akaike, 1974) using the programs MODELTEST, version 3.7 (Posada & Crandall, 1998).

Phylogenetic relationships among cyt *b* sequences were estimated using Neighbour-joining (NJ) and Bayesian inference (BI) methods implemented in PAUP*4.0b10 (Swofford, 2002) and MRBAYES, version 3.1 (Ronquist & Huelsenbeck, 2003), respectively. BI analysis was run with four chains for 10^7 generations and the current tree was saved to file every 100 generations. The robustness of these analyses was assessed using bootstrap method with 1000 replications for NJ and the posterior probabilities for BI as the percentage of samples recovering any particular clade, where probabilities $\geq 95\%$ indicate significant support. In all phylogenetic analyses, individuals from two closely-related species of the same family (Accipitridae) were used as outgroup taxa: *G. fulvus* (X86752) (Seibold & Helbig, 1995) and *G. barbatus* (U08943) (Avise, Nelson & Sibley, 1994). Additionally, a haplotype network, which represents a set of statistically parsimonious ($P \geq 0.95$) connections between haplotypes, was constructed using the program TCS, version 1.21 (Clement, Posada & Crandall, 2000).

We attempted to gain some insight into the past demographic history of *A. monachus* by calculating the mismatch distribution on the pooled populations in ARLEQUIN, version 3.1 (Excoffier, Laval & Schneider, 2005). An irregular and multimodal distribution of pairwise differences between sequences is expected in stationary or shrinking populations, whereas a smooth, unimodal shape is typical of expanding populations (Excoffier & Schneider, 1999).

Microsatellite analyses

Departures from Hardy–Weinberg equilibrium and linkage equilibrium were tested using GENEPOP, version 3.4 (Raymond & Rousset, 1995), where the significance was evaluated by Fisher exact test *P*-values, applying the Markov chain method (10 000 dememorization). We determined levels of genetic differentiation among populations using F_{ST} (Weir & Cockerham, 1984) in ARLEQUIN, version 3.1 (Excoffier *et al.*, 2005), where the statistical significance was assessed by permutation test (10 000 permutations).

Genotyping errors, such as non-amplified alleles, short allele dominance, and scoring of stutter peaks, were assessed statistically using MICROCHECKER, version 2.2.3 (Van Oosterhout *et al.*, 2004). The probability of full-sib or unrelated pairs of black vultures, $P_{(ID)}$, bearing an identical multilocus genotype, was estimated using the software GIMLET, version 1.3.3 (Valiere, 2002) to explore the discrimination power of the microsatellite locus combination. For each microsatellite locus, we assessed genetic polymorphism by calculating the observed number of alleles (N_a), allele richness (R_S), average observed heterozygosity (H_o), gene diversity (H_S) across populations, and total gene diversity (H_T) in FSTAT, version 2.9.3 (Goudet, 2001). Commonly-used population genetic summary statistics, such as the mean number of alleles per locus (N_a), the number of unique alleles (N_{ua}), the observed heterozygosity (H_o), and the unbiased expected heterozygosity (H_E), were computed for each locus and population using GENETIX, version 4.05.2 (Belkhir *et al.*, 2001).

Each population was tested for heterozygosity excess to detect recent population bottlenecks. The program BOTTLENECK (Cornuet & Luikart, 1996; Piry, Luikart & Cornuet, 1999) was run under the two-phase model of microsatellite evolution (Dirienzo *et al.*, 1994) with 10% of the infinite allele model and 90% of the stepwise mutation model.

The genetic structure was further examined with a Bayesian clustering method and the admixture analysis implemented in STRUCTURE, verison 2.2 (Pritchard, Stephens & Donnelly, 2000). Markov chain Monte Carlo parameters were set on burn-in period of 30 000 and run length of 10^6 iterations and each run was repeated five times to ensure convergence among estimated parameters. The true number of clusters (K) was estimated by: (1) using the model choice criterion implemented in STRUCTURE that is the maximal value estimate of posterior probability of the data for a given K, $\Pr(X|K)$ (Pritchard *et al.*,

Table 1. Alignment of variable positions found in seven unique haplotypes of cytochrome *b* sequences and their distribution across seven sampling sites

	Nucleotide position	Sampling locality						
	00000000112223						Mongolia	
	00013467280560							
Haplotype	36963200604251	Spain	Greece	Armenia	Kazakhstan	Georgia	1	2
Hap_1	CCGCTCCTACCATT	8	–	–	–	–	–	–
Hap_2	...T..GG..G...	–	7	–	–	–	–	–
Hap_3	.T.T..GG..G...	–	3	–	–	–	–	–
Hap_4	..ATCT.GG...C.	–	–	5	4	3	–	–
Hap_5	..ATC..GGT.CCC	–	–	–	–	–	4	2
Hap_6	..ATC..GGT.CC.	–	–	–	–	–	10	–
Hap_7	T.ATC..GGT.CC.	–	–	–	–	–	2	–

Mongolia 1, Erdenesant (Mongolia); Mongolia 2, Khustai (Mongolia).
Dots indicate identity with haplotype 1. The total number of individuals from each population with the corresponding haplotype is given.

2000) and (2) using another ad hoc quantity that is based on the second order rate of change of the likelihood function with respect to K (ΔK) as proposed by (Evanno, Regnaut & Goudet, 2005).

Analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) was performed using ARLEQUIN. *F*-statistics were used to estimate the proportion of genetic variability found among populations (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). An AMOVA was run with populations grouped according to the genetic clusters found in the structure analysis, and with populations grouped according to the clades found in the phylogenetic analysis of mitochondrial (mt)DNA structure analysis. If the fixation index over all loci (Weir & Cockerham, 1984) among populations within a group (F_{SC}) departed significantly from zero, then we considered that populations in the group should be further subdivided. Significance associated with the fixation index was evaluated through random allelic permutation procedures (10 000 permutations).

Correlation analysis between genetic and geographical distances (Mantel test) was calculated with the ISODLE Program implemented in GENEPOP as proposed by Rousset (1997). The statistical significance of the correlation of these matrices was assessed with a Mantel randomization test (10 000 permutations).

RESULTS

mtDNA data

Overall, the 48 sequences defined seven unique haplotypes (Table 1). Of the 311 sites examined, there were 14 variable cyt *b* sites. The model of nucleotide substitution chosen as best fit for our data was the Tamura–Nei model (Tamura & Nei, 1993) with an estimate of invariable sites ($I = 0.93$). Under this model of evolution, the sequence divergence among the specimens of *A. monachus* were in the range 0–3.3%, whereas the NJ and Bayesian trees have similar topologies. The Bayesian 50% majority rule consensus tree ($lnL = -866.935$) is presented in Figure 2A. The seven different haplotypes form four very well supported allopatric lineages, corresponding to different geographic regions throughout the Palearctic region, where the European populations are more closely related to each other. These lineages are the lineage A (Balkan Peninsula, Greek population), the lineage B (Iberian Peninsula, Spain population), the lineage C (Caucasian region, Armenia, Georgia, Kazakhstan populations), and the lineage D [(Far Eastern region, Mongolian populations (Khustai and Erdenesant)].

The shape of the mismatch distribution function for the whole data set is multimodal, reflecting the deep divergence between the four mitochondrial clades. A bimodal mismatch distribution is observed in the European populations. However, when each clade is analysed separately, the shape of the function is unimodal, conforming to the theoretical expectation for a growing population (data not shown).

The network of mtDNA haplotypes of *A. monachus* was presented in Figure 2B, where the topology is in accordance with the phylogenetic relationships obtained in BI and NJ analyses.

Microsatellite data

Two loci (BV5, BV12) deviated significantly from Hardy–Weinberg proportions due to heterozygote

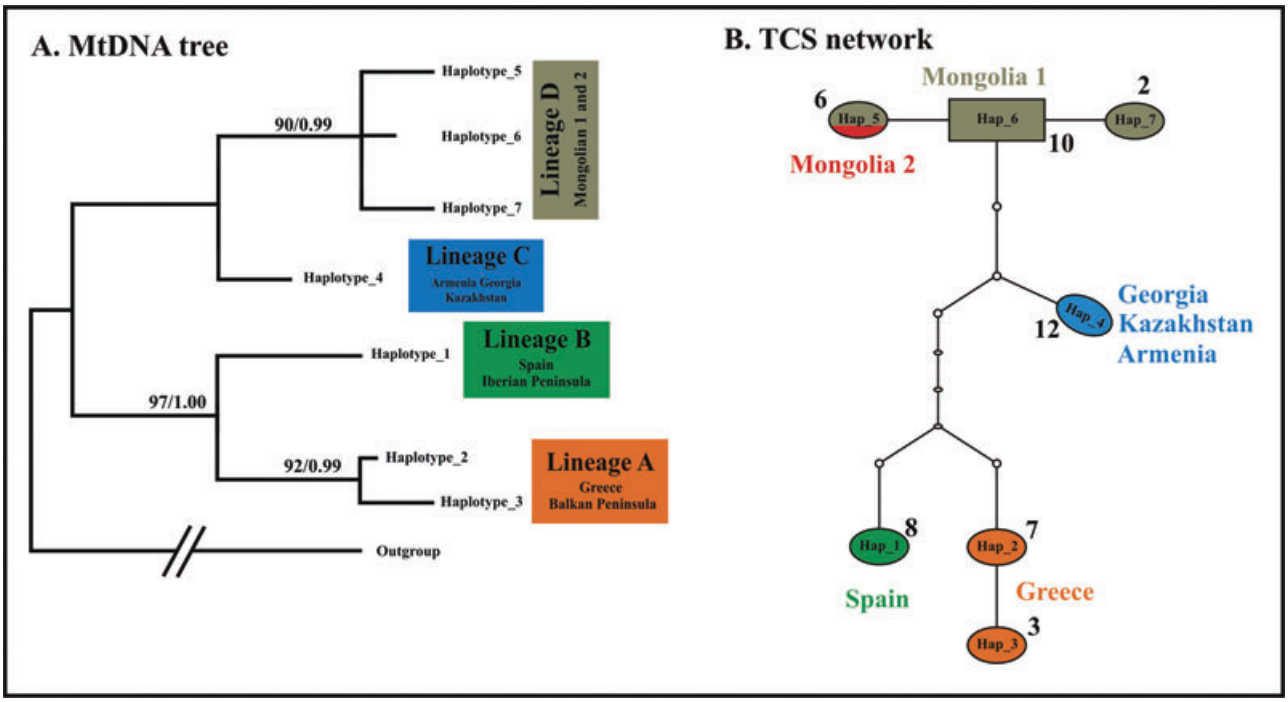

Figure 2. A, Bayesian 50% majority rule tree of mitochondrial (mt)DNA data based on Tamura–Nei + I model of evolution. Two sequences of *Gyps fulvus* and *Gypaetus barbatus* were used as outgroups. Numbers above branches are bootstrap values on Neighbour-joining and posterior probabilities values on Bayesian inference. B, unrooted statistical parsimony network of Eurasian black vulture mtDNA cytochrome *b* haplotypes. The numbers refer to the numbers of individuals that shared the same haplotype. Haplotypes colours correspond to those in (A).

deficit ($P < 0.05$). This was consistent across populations. Heterozygote deficit could be a sign of the population sub-structuring segregating or sampling artefacts. We assume that the patchy distribution of the populations is an important factor contributing to the lack of heterozygosity. However, null alleles, stuttering signals, or large allelic dropouts could also contribute to 'false positive' homozygous patterns. We examined the latter explanation using MICRO-CHECKER. Evidence for scoring error due to stuttering and large allelic dropouts or null alleles was not found. Three pairs of loci (BV5-BV13, BV6-BV20, BV12-Gf3H3) showed some evidence of linkage disequilibrium. However, no one pair of loci was consistently linked across all samples and therefore normal segregation of the eight loci may be assumed. Thus, this locus combination of microsatellite markers is currently being used to investigate the genetic population structure of Eurasian black vultures.

Analysis using GIMLET showed that these loci combined would only produce an identical genotype by chance in the case of full sibs with a probability of < 0.005 (i.e. these markers can even distinguish siblings with 99.9% probability). General guidelines for genetic tagging studies suggest using a suite of markers that achieve a reasonably low $P_{(ID)}$ and $P_{(IDsib)}$ bounded between 0.01 and 0.0001 (Waits, Luikart & Taberlet, 2001). The four most informative loci are BV5, Gf11A4, BV16, and BV11.

Diversity estimates varied among microsatellite loci (Table 2) and among populations (Table 3). The total number of alleles per locus in our sample of 173 individuals was in the range 3–20 at the eight loci. Average gene diversity (H_S) among the loci was 0.64 (range 0.11–0.89), whereas the gene diversity (H_T) estimates among all populations were in the range 0.18–0.91. Mean observed heterozygosity (H_o) ranged from 0.36 (Spain) to 0.56 (Greece). The lowest mean expected heterozygosity (H_E) was found in the population from Armenia (0.52), whereas the highest was in the population from Greece (0.72).

Two main clusters of populations were identified using STRUCTURE by both ad hoc quantities used in this study. Plotting the membership coefficient for each pre-defined population gave us the structure imprinted in Figure 3. The clusters that comprised the uppermost hierarchical level of population structure (Evanno *et al.*, 2005) were the Far Eastern cluster, consisting of the populations of Mongolia (Erdenesant and Khustai), and a West Asia – Europe cluster, consisting of Iberian, Balkan, and Caucasian (Armenia, Kazakhstan, and Georgia) populations.

Table 2. A comparison of genetic diversity at eight microsatellite loci in *Aegypius monachus*

Locus	N_a	R_s	H_o	H_s	H_T	F_{ST}	R_{ST}
BV5	18	3.484	0.377	0.846	0.910	0.117	0.579
BV11	14	3.319	0.844	0.838	0.879	0.053	0.316
BV12	5	2.108	0.051	0.296	0.367	0.188	0.060
BV13	9	2.659	0.696	0.667	0.693	0.079	0.025
BV16	20	3.338	0.140	0.891	0.848	0.003	0.000
BV20	8	2.868	0.602	0.662	0.792	0.234	0.103
Gf3H3	3	1.611	0.026	0.116	0.184	0.442	0.368
Gf4A4	20	3.382	0.776	0.816	0.895	0.138	0.310

F_{ST} and R_{ST} values were estimated according to Weir & Cockerham (1984) and Goodman (1997), respectively.
N_a, observed allele number; R_s, allelic richness; H_O, observed heterozygosity; H_S, gene diversity; H_T, overall gene diversity; F_{ST}, among population genetic differentiation; R_{ST}, gene differentiation accounting for variance in allele size.

Table 3. Sample size (N), the mean number of alleles per locus (N_a), unique alleles (N_{ua}), observed heterozygosity (H_O), and Nei's unbiased expected heterozygosity (H_E) for each population based on the eight microsatellite loci

Population	N	N_a	N_{ua}	H_O	H_E
Spain	44	4.00	3	0.3636	0.5950
Greece	53	7.625	7	0.5589	0.7259
Georgia	3	2.375	0	0.3750	0.5250
Armenia	5	3.375	1	0.4000	0.5944
Kazakhstan	4	3.25	0	0.4375	0.5833
Mongolia 1 (Erdenesant)	52	8.75	12	0.5589	0.6621
Mongolia 2 (Khustai)	12	5.5	0	0.5072	0.6520

Table 4. Pairwise comparison matrix of θ values among *Aegypius monachus* populations

	Spain	Greece	Georgia	Armenia	Kazakhstan	Mongolia 1
Spain	–					
Greece	0.107*	–				
Georgia	0.144*	0.093*	–			
Armenia	0.093*	0.065*	0.000	–		
Kazakhstan	0.120*	0.113*	0.011	0.000	–	
Mongolia 1	0.176*	0.148*	0.103*	0.091*	0.095*	–
Mongolia 2	0.163*	0.134*	0.079*	0.075*	0.063*	0.000

*Statistically significant at $P = 0.05$.

Overall, 94% of the samples are assigned with a high degree of certainty (membership coefficient for the population of origin: $P > 0.8$). Among the remaining individuals that display lower membership coefficients, only three present an assignment score lower than 0.74, probably indicating low levels of gene flow and thus high level of genetic diversity among the four clusters.

Genetic differentiation was examined using mean F_{ST} values (0–17.6%; Table 4), where 17 out of 21 comparisons deviate significantly from zero. There was no genetic evidence of recent bottlenecks in any of the *Aegypius* populations.

The results of the AMOVA (Table 5) confirmed the presence of phylogeographic structure in our data. The results of this analysis considering all populations as one demonstrated that most of the molecular variation was distributed within populations (89.68%) rather than among populations (10.32%) (Table 6), suggesting that there are some genetic structures within the group. The AMOVA of the two genetic clusters of structure analysis showed that only 2.86% of the

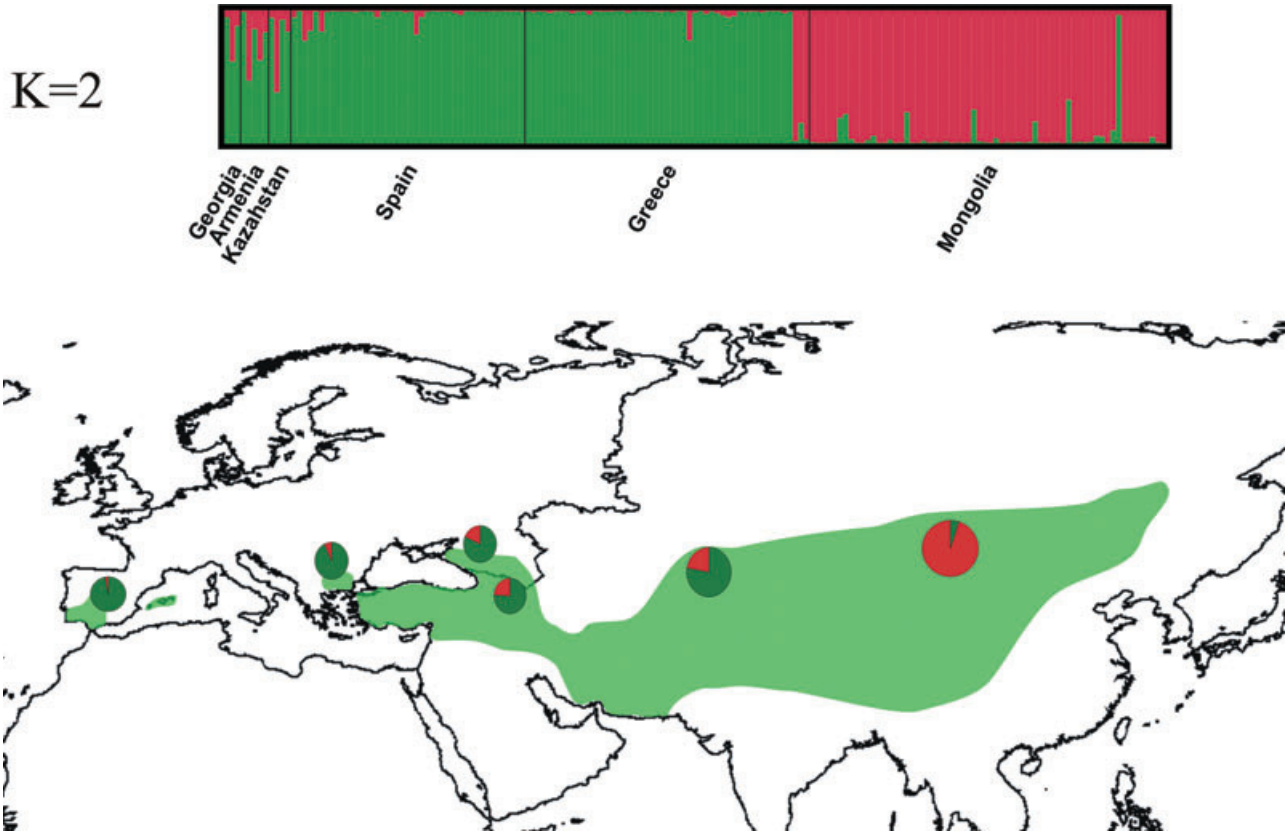

Figure 3. Bayesian analysis of the nuclear genetic structure of Aegypius populations based on eight microsatellite loci. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that indicate the individual's membership in two groups. The populations of individuals are indicated.

Table 5. Analysis of molecular variance (AMOVA) for *A. monachus* using eight microsatellite loci between (1) two groups (Far Eastern and West Asia-Europe groups) identified by STRUCTURE (2) four groups (Balkan Peninsula: lineage A, Iberian Peninsula: lineage B, Caucasian region: lineage C, and the Far Eastern: lineage D) identified by phylogenetic analysis of mtDNA and (3) all populations.

Source of variation		d.f.	Sum of Squares	Percentage of variation	Statistics	*P*-value
(1)	Among groups	1	13.435	2.86	$F_{ST} = 0.112$	0
	Among populations within groups	5	19.338	8.42*	$F_{SC} = 0.086$	0
	Within populations	339	311.709	88.72*	$F_{CT} = 0.028$	0.068
(2)	Among groups	3	30.627	12.05*	$F_{ST} = 0.109$	0
	Among populations within groups	3	2.147	0	$F_{SC} = 0.017$	0.695
	Within populations	339	311.709	89.03*	$F_{CT} = 0.12$	0.005
(3)	Among populations	6	32.773	10.32*	$F_{ST} = 0.103$	0
	Within populations	339	311.709	89.68*		

*Significant values ($P < 0.01$).

variance was attributed to differences among these clusters and 8.42% of the variance to differences among populations within these clusters. In this case, the fixation index among groups (F_{CT}) was not significant ($F_{CT} = 0.028$, $P = 0.068$), whereas it was significant among populations within groups ($F_{SC} = 0.086$, $P < 0.001$). These results suggested that populations were still structured within at least one group. An

Table 6. Analysis of molecular variance (AMOVA) for *Aegypius monachus* using eight microsatellite loci between: (1) two groups (Far Eastern and West Asia-Europe groups) identified by STRUCTURE; (2) four groups (Balkan Peninsula: lineage A, Iberian Peninsula: lineage B, Caucasian region: lineage C, and the Far Eastern: lineage D) identified by phylogenetic analysis of mtDNA; and (3) all populations

Source of variation	d.f.	Sum of squares	Percentage of variation	Statistics	*P*
(1) Among groups	1	13.435	2.86	$F_{ST} = 0.112$	0
Among populations within groups	5	19.338	8.42*	$F_{SC} = 0.086$	0
Within populations	339	311.709	88.72*	$F_{CT} = 0.028$	0.068
(2) Among groups	3	30.627	12.05*	$F_{ST} = 0.109$	0
Among populations within groups	3	2.147	0	$F_{SC} = 0.017$	0.695
Within populations	339	311.709	89.03*	$F_{CT} = 0.12$	0.005
(3) Among populations	6	32.773	10.32*	$F_{ST} = 0.103$	0
Within populations	339	311.709	89.68*		

*Significant values ($P < 0.01$).

AMOVA was run where the groups were defined based on the phylogenetic lineages of the mtDNA data (Fig. 2A, Balkan Peninsula: Lineage A; Iberian Peninsula: Lineage B; Caucasian region: Lineage C; and Mongolia: Lineage D). This time, the variance among groups increased to 12.05% and the variance among populations within groups decreased to 0%, indicating that none of the populations within each group was structured. Based on this group definition, the fixation index among groups was significant ($F_{CT} = 0.12$, $P < 0.001$), whereas it was not significant among populations within groups ($F_{SC} = 0.017$, $P = 0.69$). Consequently, according to the results of the AMOVA tests, we considered that the seven sampling populations could be assigned into four groups (i.e. that we described above), which are consistent with the produced lineages of BI analysis of mtDNA.

DISCUSSION

The results obatined in the present study are unique in that they comprise the first population genetic analysis of the Eurasian black vultures (*A. monachus*) in Europe. The analysis of both mitochondrial and microsatellite data allowed us to characterize the genetic composition of the current European breeding populations in terms of both diversity and structure. The number genetic studies of Eurasian raptors, most of them involving critically endangered or threatened species, have increased in the last decade, inferring important issues for the biodiversity and conservation status of those species (Negro & Torres, 1999; Nesje *et al.*, 2000; Cardia *et al.*, 2002; Vali, 2002; Kretzmann *et al.*, 2003; Godoy *et al.*, 2004; Martinez-Cruz, Godoy & Negro, 2004; Helbig *et al.*, 2005a, b; Roques & Negro, 2005; Hailer *et al.*, 2006; Hailer *et al.*, 2007).

At the evolutionary level, the phylogenetic analysis of cyt *b* sequences reveals the existence of two evolutionary lineages in Europe that correspond to the two different breeding populations (Iberian and Balkan peninsulas). Haplotypes clustered in two distinct haplogroups with a predominantly eastern or western distribution. This geographical pattern of haplotype distribution (Fig. 2) indicates a sharp East–West clinal distribution of clade proportions in populations ranging from Iberian Peninsula to Asia, which has been observed in bearded vultures (*G. barbatus*) (Godoy *et al.*, 2004) and the white-tailed eagle (*Haliaeetus albicilla*) (Hailer *et al.*, 2006, 2007).

With only seven haplotypes detected (Table 1), the Eurasian black vulture display a relatively low mitochondrial variability (haplotype diversity), a phenomenon that has been already reported in other raptor species, generally associated with recent demographic crashes: the Mauritius kestrel, *Falco punctatus* (Groombridge *et al.*, 2000); the Norwegian peregrine falcon, *Falco peregrinus* (Lifjeld *et al.*, 2002); the bearded vultures, *G. barbatus* (Godoy *et al.*, 2004); the critically endangered Spanish imperial eagle, *Aquila adalberti* (Martinez-Cruz *et al.*, 2004); the red kite (*Milvus milvus*) (Roques & Negro, 2005); and the white-tailed eagle (*Haliaeetus albicilla*) (Hailer *et al.*, 2006, 2007).

Wide-ranging species with high dispersal capabilities should have relatively low genetic structuring (Jones *et al.*, 2004). However, in the present study, significant structuring of genetic diversity was found among most black vulture populations, indicating a low level of gene flow. Although an analogue pattern was observed in bearded vultures (*G. barbatus*) (Godoy *et al.*, 2004) and the white-tailed eagle (*Haliaeetus albicilla*) (Hailer *et al.*, 2007), these results contrast with the general pattern of low levels of genetic

differentiation among populations of birds (Crochet, 2000) and with the results obtained for its sister species, the Egyptian vulture (Kretzmann *et al.*, 2003).

Despite the low mtDNA variability (haplotype diversity), the small number of substitutions (Table 1) revealed a clear geographic pattern of population structure because each population constitutes a distinct lineage (with the exception of the Caucasian region in which the three populations share the same haplotype). Moreover, the two European populations (Iberian and Balkan) form a sister group, whereas the populations from Caucasus are closer to Mongolian ones, comprising a group that appear to be more distinct from the European populations (Fig. 2). Much of the current haplotype diversity in *A. monachus* is probably a consequence of this strong within species phylogeographic structure. One to three different mtDNA haplotypes were obtained from each of the different populations of *A. monachus* (Table 1), suggesting a strong degree of spatial genetic structure within the species, limited exchange of individuals among these populations, and deep divergence between the lineages of *A. monachus*. In addition, the shape of the mismatch distribution for the whole data set (multimodal) and for the European subset (bimodal) lead to same conclusion. This pattern might have originated by divergence of these lineages in allopatry. However, such a high level of population differentiation is specially striking for a bird that usually flies long distances in a single day. A highly philopatric dispersal behaviour needs to be invoked to clarify this pattern and to explain the existence of a clear phylogeographical pattern, as in the case of Bearded Vultures (Godoy *et al.*, 2004).

Although the results from mitochondrial DNA refer only to female dispersal, similar results obtained with the eight nuclear microsatellite markers used in the present study suggest that this is not a sex-specific pattern. The use of eight variable microsatellites was sufficient to reveal a strong genetic structure at a large spatial scale (Table 4). The levels of genetic diversity found in *A. monachus* populations ($H_o = 0.36–0.56$) were comparable to values observed in a number of species of endangered vertebrates (Ciofi *et al.*, 2002) and were similar to those found in other raptor species (Nesje *et al.*, 2000; Kretzmann *et al.*, 2003; Rudnick *et al.*, 2005).

Beside the fact that the structure analysis indicates the presence of two main clusters of populations (Fig. 3) (Far Eastern and West Asia–Europe clusters), the significant hierarchical analyses of genetic substructure (AMOVA) supports four distinct groups throughout Eurasia. The significance of Mantel test ($P = 0.003$) for correlation between genetic distance and geographical distance, indicates that the genetic differentiation observed in nuclear DNA was linked to the phylogenetic groups defined by the mtDNA analysis. Consequently, our results support the hypothesis that gene flow between the phylogenetic groups is restricted and that the observed genetic differentiation of *A. monachus* is due to population history and isolation by distance. Thus, nuclear DNA genetic differentiation, as measured by microsatellite loci, is consistent with mtDNA phylogeographical groupings.

Birds of prey are vulnerable to extinction because most have traditionally been persecuted by humans. Based on BirdLife International (2006), *A. monachus* has small populations that appear to be suffering ongoing decline, despite the fact that, in parts of Europe, numbers are now increasing (see Introduction). Focusing on the two major breeding populations of Europe, the analysis of mtDNA revealed a low degree of genetic variability (haplotype diversity) (Table 1). Although the level of variability in the mitochondrial genome is low, no evidence of genome-wide genetic erosion exists because nuclear diversity exhibits normal levels. Signatures of a genetic bottleneck in the nuclear genome were not detected, indicating that the demographic bottleneck suffered during the 20th Century was neither critical nor lasting enough to have an impact on nuclear genetic variation at the species level. This could be probably due to the long generation time of the raptors, confirming the argument of Hailer *et al.* (2006) suggesting that the long generation time of several species (i.e. eagles, turtles, large mammals) has acted as an intrinsic buffer against loss of genetic diversity, leading to a shorter effective time of the experienced bottleneck.

Conservation implications for the European breeding populations

The conservation status of *A. monachus* has recently been a cause for concern. The two main threats to the species are direct mortality caused by humans (either accidentally or deliberately) and decreasing availability of food. The information obtained in the present analysis will be instrumental in refining conservation strategies to protect what remains in Iberian and Balkan breeding populations of Eurasian black vulture. Currently, it is a generally accepted conclusion that the loss of genetic diversity is not desirable because it reduces the ability of species to cope with environmental changes (Frankham, Ballou & Briscoe, 2002).

Phylogenetic analyses can identify genetically distinct lineages worthy of conservation and can also aid in setting priorities for conservation efforts. When population distinctiveness is evaluated on the basis of genetic criteria, it is usually accepted that distinct genetic populations are those showing reciprocally monophyletic mtDNA alleles and significant diver-

gence for allele frequencies at nuclear loci (Moritz, 1994). Based on these criteria, Moritz (1994, 1999) operationally defined evolutionarily significant units (ESUs) and management units (MUs) for conservation, whereas Crandall *et al.* (2000) argue that the assessment of distinctiveness should appropriately incorporate adaptive differences, as evidenced by genetic and ecological data.

In Europe, where only two mainly populations exist, the fixation of different mtDNA haplotypes in each population (one in Iberian population and two in Balkan population) and the significance divergence at nuclear loci fulfill the definitions of ESUs, even if fixation were to be an epiphenomenon resulting from anthropogenic reduction and fragmentation of the species' distribution.

It must be noted that even though there is no evidence (field observations) of contact between Balkan and Iberian populations, the two populations must have been indirectly connected in the past by gene flow through the now extinct Central European populations. Thus, the hypothesis of recent and historical ecological exchangeability (Crandall *et al.*, 2000) can neither be supported nor rejected due to lack of data. Based on the observation that varying circumstances require differing approaches, Fraser & Bernatchez (2001) provide a framework for management decisions when ecological data are not available or insufficient. Our microsatellite and mitochondrial data provide strong evidence that historical isolation has led to the differential accumulation of mutations in both populations, and this, together with the importance of genetic drift and geographic subdivision, leads us to conclude that the two breeding populations represent distinct ESUs. Maximum priority for conservation should be given because on the basis of these, and due to its genetic divergence from the Eastern populations, they comprise a major contribution to the total species' genetic diversity. It is worth notng that, although we sampled only 12 individuals from the 'Caucasus' region (Armenia and Georgia, Kazakhstan), these birds were distinct from the other European or Mongolian populations, whereas the same results were obtained when the analyses were repeated after removing the specimens from Caucasus.

Genetic factors, including loss of diversity and inbreeding depression, will increase the extinction risks by reducing adaptive potential and decreasing average fitness, respectively (Brook *et al.*, 2002). As we describe above, the levels of variability in mtDNA are relatively low but there was no evidence of genome-wide genetic erosion in the European blank vulture populations. Consequently, management strategies should aim to preserve the extant diversity of the Balkan and Iberian populations.

If the goal of a conservation program is to preserve genetic distinctiveness and ongoing evolutionary processes, introductions of birds should be discouraged. A number of studies have demonstrated that artificially restoring gene flow between isolated populations can counteract the effects of inbreeding depression (Hedrick, 1995; Madsen *et al.*, 1999; Mansfield & Land, 2002; Robertson, Karika & Saul, 2006). Translocations have been frequently used in the management of threatened birds (Komdeur, 1994; Armstrong & McLean, 1995). Despite the success of many conducted programs, translocations are fraught with potential dangers. These include the introduction of exotic pathogens and a host of difficulties associated with acclimating captive raised animals to natural environments (Snyder *et al.*, 1996). Additionally, rescue effect gene flow or captive propagation could be used to raise a population above a demographically critical size, but subsequent reduction or cessation of these factors might be required to enable further adaptation and greater population productivity (Stockwell, Hendry & Kinnison, 2003). Guidelines for translocations emphasize the need for a multidisciplinary approach by taking into account biological, socio-economic and legal requirements (Robertson *et al.*, 2006).

The future of the species in Europe depends on the effective implementation of conservation strategies both *in situ* and *ex situ* that must necessarily consider genetic issues such as those described in the present study. Consequently, the aim of the conservation effort would not only be to maintain the habitats that these ESUs exploit, but also to preserve and/or increase the genetic variation of the European breeding populations.

In summary, the study of genetic variability and population structure should be a key factor in management and recovery programmes to enable the retention of genetic diversity in endangered or threatened species. The present study shows that mtDNA and microsatellite markers are useful tools for performing such investigations in Eurasian black vultures, and that single, non-invasively collected vultures feathers and bones yield sufficient quantities of DNA for both mtDNA and microsatellite genotyping.

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