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Phylogeography, genetic structure and diversity in the endangered bearded vulture (*Gypaetus barbatus*, L.) as revealed by mitochondrial DNA

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Abstract

Bearded vulture populations in the Western Palearctic have experienced a severe decline during the last two centuries that has led to the near extinction of the species in Europe. In this study we analyse the sequence variation at the mitochondrial control region throughout the species range to infer its recent evolutionary history and to evaluate the current genetic status of the species. This study became possible through the extensive use of museum specimens to study populations now extinct. Phylogenetic analysis revealed the existence of two divergent mitochondrial lineages, lineage A occurring mainly in Western European populations and lineage B in African, Eastern European and Central Asian populations. The relative frequencies of haplotypes belonging to each lineage in the different populations show a steep East–West clinal distribution with maximal mixture of the two lineages in the Alps and Greece populations. A genealogical signature for population growth was found for lineage B, but not for lineage A; futhermore the Clade B haplotypes in western populations and clade A haplo-types in eastern populations are recently derived, as revealed by their peripheral location in median-joining haplotype networks. This phylogeographical pattern suggests allopatric differentiation of the two lineages in separate Mediterranean and African or Asian glacial refugia, followed by range expansion from the latter leading to two secondary contact suture zones in Central Europe and North Africa. High levels of among-population differentiation were observed, although these were not correlated with geographical distance. Due to the marked genetic structure, extinction of Central European populations in the last century re-sulted in the loss of a major portion of the genetic diversity of the species. We also found direct evidence for the effect of drift altering the genetic composition of the remnant Pyrenean population after the demographic bottleneck of the last century. Our results argue for the management of the species as a single population, given the apparent ecological exchangeability of extant stocks, and support the ongoing reintroduction of mixed ancestry birds in the Alps and planned reintroductions in Southern Spain.

Keywords: ancient DNA, conservation genetics, genetic structure, *Gypaetus barbatus*, mitochondrial DNA, phylogeography

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Introduction

The bearded vulture (*Gypaetus barbatus*) inhabits high-altitude mountain ranges in the Old World, from the Iberian Peninsula to China and south to the Drakensberg Mountains in South Africa. In Africa the species has a discontinuous and peripheral distribution with populations in South Africa and Lesotho, Ethiopia and Morocco (Mundy *et al*. 1992), the latter of which may be on the brink of extinction (Godino *et al*. 2003). Bearded vultures are widespread in Central Asian mountains, where the

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stronghold of the species is considered to be. In contrast, the species experienced severe demographic declines in Europe. Populations in the Alps, the Balkans, Greece and Southern Spain have run extinct during the 20th century, due mainly to human persecution, through hunting and poisoning. Currently, the only remnant European populations are in Corsica and Crete (less than 10 breeding pairs each; Thibault *et al*. 1992; Xirouchakis & Nikolakakis 2002) and in the Pyrenees (300–400 individuals, around 100 breeding pairs) (R. Heredia unpublished; Heredia & Heredia 1991). *In situ* conservation efforts were initiated during recent decades in several countries to preserve the remaining populations. In addition, a captive breeding programme has been implemented by a multinational coalition to repopulate the Alps. The captive stock is composed mainly of individuals of Eastern European or Asian origin. So far, more than 100 captive-bred individuals have been released to the wild and some have already mated and reproduced. Given the success of the Alps reintroduction project, a second release site has been chosen in the Sierra de Cazorla, where the last Southern Spanish population was extirpated in 1987.

The knowledge of the evolutionary history and genetic status of the species is deemed critical for the success of *ex situ* and *in situ* conservation programmes, because it allows the definition of management units and the design of management strategies aimed at minimizing genetic erosion while preserving subspecific distinctiveness (Haig 1998; Hedrick 2001). 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 and contemporary factors in shaping the genetic makeup of the species are not easy to disentangle, but several strategies have been proposed (Templeton 1998; Bernatchez 2001; Knowles & Maddison 2002). A combination of a series of analyses at different temporal scales, including haplotype relatedness, demographic history and population genetics, might be necessary to describe not only a geographical structure but to also investigate the historic or contemporary processes that originate it (Althoff & Pellmyr 2002; Bernatchez 2001).

Previous genetic studies of bearded vultures have shown low levels of genetic diversity in the Pyrenean population based on multilocus DNA fingerprinting (Negro & Torres 1999) and differentiation between the Pyrenean and the captive population based on microsatellite markers (Gautschi *et al*. 2003). More extensive analyses that include the now extinct European populations are now possible through the use of museum specimens and adequate molecular markers and DNA extraction techniques (Leeton *et al*. 1993; Roy *et al*. 1994; Mundy *et al*. 1997).

Here we report the variation in the mitochondrial control region found after a wide survey that includes a major portion of the current and past distribution range of the species. The use of museum specimens, at a scale seldom seen in previous studies, has allowed the analysis of now extinct populations and the direct addressing of changes in population genetic composition through time. The recent evolutionary history of the species is reconstructed by testing alternative phylogeographical hypotheses; the past population structure is described and discussed in relation to the biology of the species, and finally the genetic diversity of the different populations is estimated. In the case of the remnant Pyrenean population, the comparison of historic and contemporary samples enabled us to test the hypothesis that the bottleneck of the last century has affected its genetic composition and diversity. With this information the definition of a management strategy for the species becomes possible, and specific questions concerning the suitability of a mixed ancestry captive breeding programme, the reintroduction of birds of Asian ancestry in Europe, the expected gene flow between reintroduced and remnant population and the risks for genetic depauperation of remnant populations are addressed.

Materials and methods

Samples

A total of 172 specimens of bearded vulture (*Gypaetus barbatus*) have been analysed covering a major portion of the current and past distribution range of the species. Of these, 57 blood/tissue samples and three feathers are contemporary, whereas 104 skin/feather and eight footpad samples are from museum specimens, the only available source for extinct populations (Appendix I). Contemporary living or dead individuals were sampled by collecting blood or muscular tissue, respectively. Blood and tissue samples were stored in ethanol or modified lysis buffer (Seutin *et al*. 1991). Either the tip of a covert feather and surrounding skin or pieces of the footpad (Mundy *et al*. 1997) were used as the source of DNA from museum specimens. Several samples came from individuals in the Alps Captive Breeding Programme; in these cases, recorded pedigree information was taken into account to include all founding haplotypes and to avoid sampling the same mitochondrial lineage more than once (Frey *et al*. 1995). Whenever possible samples were assigned to groups according to their geographical origin and these groups were regarded as populations for population-genetic analysis. In many instances localities of collection were not registered or only broad geographical localizations were available (i.e. former Soviet Union, Russia). With these latter samples, a group of Eastern samples with no precise location was made (EAS). The Pyrenean samples were subdivided into contemporary (NSC) and historic museum samples dated before 1960 (NSH), to evaluate any temporal changes in genetic diversity or gene frequencies. For the other extant

populations, museum and contemporary samples were pooled when both available.

DNA isolation, polymerase chain reaction (PCR) and sequencing

DNA from blood and tissue samples was extracted with a salting-out (Gemmell & Akiyama 1996) and a standard phenol–chloroform method, respectively. DNA from museum samples was extracted with DNeasy Tissue Kits (Qiagen) following the manufacturer's instructions, but previous washes in excess NTE (0.05 M Tris-HCl, 0.01 M NaCl, 0.02 M EDTA, pH 9.0) were included to remove possible protease or PCR inhibitors (Hall *et al*. 1997). Contamination with modern DNA or PCR products was monitored by including two extraction blanks in every extraction round and prevented by performing all museum samples extractions in a dedicated 'clean' laboratory, kept free of good-quality DNA and PCR products.

A previous sequence characterization of the whole mitochondrial control region in bearded vulture in a few individuals of diverse origin (Roques *et al*. 2004) was used to define the target sequence and to design primers for this study. A contiguous segment, centrally located within domain I of the control region, was found to include most of the polymorphic sites observed in a preliminary analysis of a few individuals. Amplification of this approximately 500 base pairs (bp) fragment of the control region was performed using primers tThrF (5′-TTGGTCTTGTAAACCAAARANTGAAG-3′) and Fbox-R (5′-GGGTTGCTGRTTTCACGTGAG-3′). Primers QHD1-2F (5′-TGCCCCATTATAATGCACTATTCT-3′) and Fbox2-R (5′-GTAGGTTCGACAGGAAATGGC-3′), internal to this segment were designed additionally to amplify a shorter 273 bp in museum samples. Maternal mode of inheritance of the sequenced fragment was confirmed with two independent captive families, indicating that they were of mitochondrial origin and not nuclear insertions of mitochondrial sequences.

DNA amplification reactions contained 67 mM Tris-HCl pH 8.0, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM dNTPs, 1 μM of each primer, 0.5 U of *Taq* polymerase and either 50–100 ng of total DNA or 5 μL of museum DNA extracts as template. Bovine serum albumin was included at a concentration of 0.1 μg/μL for amplification of blood DNA and at 0.8 μg/μL for museum DNA. Amplification reactions were performed in an MJ Research thermocycler, Model PTC-100, programmed for an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 62–64 °C (depending on primers) for 30 s and extension at 72 °C for 30 s. All reactions were finished with a final extension at 72 °C for 5 min. To control the performance of the process and monitor for contamination, positive (diluted blood DNA) and negative (water) DNA controls, respectively, were included with each set of PCR reactions. Additionally, negative extraction controls (mock extractions with no starting material) were included in all amplifications of museum extracts. Amplification products were separated by electrophoresis in 1.5% agarose gels in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) in the presence of 0.5 mg/L EtBr. Gels were visualized under UV and photographed with a digital image system (Eastman Kodak Company). PCR products were cleaned by ultrafiltration through Microcon-YM100 or Microcon-PCR (Millipore Corp.) and sequenced on an automated DNA sequencer (ABI-310, Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit version 2.0 (Applied Biosystems, Inc.) following the manufacturer's instructions, and with the same primers used for the amplification. Sequences were edited, assembled and aligned using the program SEQUENCHER™ version 4.1 (Gene Codes Corporation).

Phylogenetic analysis

The model of DNA substitution that best fitted the data was selected with the program MODELTEST, version 3.06 (Posada & Crandall 1998). The models K80 + I + G (I = 0.798; alpha = 0.525) and TrN + I + G (I = 0.775, alpha = 0.341) were selected by the hierarchical likelihood ratio test and the Akaike Information Index criterion, respectively. Phylogenetic relationships among haplotypes were analysed by distance and maximum likelihood approaches in PAUP 4.0b10 (Swofford 2002) assuming the model of evolution selected by MODELTEST. In addition, a Bayesian inference approach using a variant of Markov Chain Monte Carlo was also applied (MRBAYES version 3.064; Huelsenbeck & Ronquist 2001). In this case the tree shown is a consensus of 7000 trees sampled from the posterior distribution with mean branch lengths, once the first 3000 trees were excluded as the 'burn-in'. Statistical support for nodes was estimated by their Bayesian posterior probability (BPP) and by bootstrapping (BS) distance-based trees. Intraspecific genealogies are typically multifurcating and descendant genes coexist with their ancestor. In this situation, as well as in others (recombination, horizontal gene transfer), relationships among DNA sequences are better represented in the form of a network, which also allows the visualization of reverse and parallel homoplasious mutations as cycles or loops (Posada & Crandall 2001). Median-joining networks were estimated using the software NETWORK version 3.1.1.1 (http://www.fluxus-engineering.com) assigning equal weights to all variable sites and with default values for the epsilon parameter (epsilon = 0). The same final network was obtained when the median-joining algorithm (Bandelt *et al*. 1999) was run on the reduced data set generated by the reduced median algorithm from a binary transformed data matrix (Bandelt *et al*. 1995).

Population sizes and demographic trends

Inferences about population sizes and demographic tendencies can be obtained from genealogies through a number of different approaches (reviewed in Emerson *et al*. 2001). The parameter theta and the growth rate under an exponential model were estimated simultaneously by maximum likelihood taking into account genealogical information with the program FLUCTUATE 1.3 (Kuhner *et al*. 1995; Kuhner *et al*. 1998). Phylogenetic and nonphylogenetic estimates may be considered to reflect historical vs. current demography and their comparison can indicate recent changes in demographic tendencies (Crandall *et al*. 1999; Vilà *et al*. 1999). Finally, past demography was investigated through the analysis of the distribution of coalescence events in the genealogy. The shape of the number of lineages through time plots can be interpreted in terms of demographic tendencies, a concave-up plot indicating a stable or declining population and a concave-down plot indicating population growth (Nee *et al*. 1996). On the other hand, the more recently developed generalized skyline plots provide a nonparametric estimate of effective population size through time obtained from the inferred genealogy (Strimmer & Pybus 2001). Both LTT and Skyline plots were obtained with the software GENIE 3.0 (http://evolve.zoo.ox.ac.uk/software/Genie/).

Population structure and gene flow

Evidence for population genetic structure was assessed using an analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) and exact permutation tests (Raymond & Rousset 1995), as implemented in ARLEQUIN 2000 (Schneider *et al*. 2000). Φ_{ST} values between pairs of population were obtained with and without the consideration of the molecular distance among haplotypes. In the latter analysis the influences of the genealogical relationships are neglected and the focus is made on historic dispersal connectivity among populations. A nonparametric permutation test (random permutations of sequences among populations) was used to test whether these statistics are significantly different from zero. Estimates of the absolute number of female migrants ($M_f = N_f m_f$) exchanged per generation by each population pair were obtained from the pairwise Φ_{ST} values assuming migration-drift equilibrium, applying the formula: $M_f = (1 - \Phi_{ST})/2\Phi_{ST}$. For the computation of geographical distances between populations we first obtained coordinates for reference cities located within the population (SSH: Jaen, NSH: Jaca, ALP: Brig, SAR: Oristano, GRE: Thessaly, CRE: Iraklion, CAS: Naryn, ETH: Adis Ababa) from the TGN website (http://www.getty.edu/research/tools/vocabulary/tgn/) and then calculated pairwise distances in kilometres with the module 'Geographic distances' in the R software package (Casgrain & Legendre 1998). The correlation between the matrix of linearized pairwise differentiation indexes [$\Phi_{ST}/(1 - \Phi_{ST})$] and the geographical distance (ln km) was evaluated with Mantel tests using the R software package.

Genetic diversity

Nucleotide diversity, haplotype diversity and the mean number of differences among sequences and their standard deviations were estimated for the entire sample and different subgroups using the ARLEQUIN 2000 software package (Schneider *et al*. 2000). Differences between historic and contemporary haplotypic diversity in Pyrenean populations were tested for statistical significance with a Welch's test to account for the possible difference in variance between the populations compared (Hoelzel 1999).

Results

Phylogeography

The 228 bp segment analysed in 172 samples of bearded vultures includes 28 variable sites, of which three are indels; only one shows three variants and 21 are parsimony informative (Table 1). Nucleotide diversity for the whole data set is estimated in 0.029 (SD = 0.015), and the mean number of nucleotide differences is 6.65 (SD = 3.16). These variable sites define a total of 50 haplotypes, resulting in a global haplotype diversity of 0.932 (SD = 0.012) and a nucleotide diversity of 0.0292 (SD = 0.0153).

The resolution of the phylogenetic analysis was limited by the large number of haplotypes found and a relatively small number of variable sites, a few of which are highly variable. Nevertheless, a few major phylogenetic associations clearly and consistently showed up as well supported nodes in all analyses performed. Haplotypes are grouped consistently into two major divergent clades, A and B, that differ by a mean of 9.26 substitutions (100% BPP, 67% BS) (Fig. 1). A few other internal nodes within clade A are statistically supported, defining at least three sublineages. Much less structure is apparent within clade B, with most groupings having little statistical support. An exception to this is the high Bayesian support obtained for a subclade grouping haplotypes 44 and 46 (100% BPP, 61% BS) and moderate Bayesian support for a subclade grouping haplotypes 6, 7 and 50 (92% BPP, 40% BS) and for the basal positioning of haplotypes 29 and 30 (90% BPP, 35% BS) within clade B. The distinction of the two mayor lineages is also evident in the network analysis (Fig. 2). Homoplasy is reflected in the high number of loops in the network indicating alternative routes for the generation of many of the haplotypes. Within each major clade, internal and terminal nodes can be interpreted as old vs. recently derived haplotypes (Posada, Crandall 2001). The basal

Table 1 Variable sites found in a fragment of 228 bp of the control region in 172 bearded vultures defining 50 haplotypes and their distribution in populations. Haplotype numbers are shown on the left and nucleotide positions relative to the beginning of the sequence are indicated by digits on the top. Gaps are indicated by -. These sequences have been deposited in GenBank under Accession nos AJ566850–AJ566899.

	Nucleotide positions	Populations																	
	11111111111111122	Iberian Peninsula													Africa				
	33355666899134444555577888812																		
Haplotype	03467358839600689278916035 70	NSC	NSH	CSH	SSH	IBH	SAR	ALP	GRE	CRE	CAU	CAS	EAS	YEM	NAF	ETH	SAF	AFR	Total
Gba_H01	GATACTGCAACTCCACAGACTTCACGTA	26	7	1			1	1											36
Gba_H02	A...T..TG.T.....T.C.	5	1		1														7
Gba_H03	....T..T..T.....T.C.	1	1																2
Gba_H04	...T.CA..G..T.....C..T...											1	1						2
Gba_H05	...T.CAT.G..T.....C..T.C.												1						1
Gba_H06	...C.CA..G..T.....T.C..T...												1						1
Gba_H07	...T.C...G..T.....T.C..T...												1						1
Gba_H08	...C.CATGG.CT.....C..T...												1						1
Gba_H09	...C.CAT.G..T.G.....C..T.C.									1									1
Gba_H10	...T..A..G..T.....C..T...	1											3						4
Gba_H11	...T.CAT.G..T.....C....C.												2						2
Gba_H12	...T.CAT.G..T.....C..T...											1	1						2
Gba_H13	...T..A..G..T.....C..T.C.											3	2						5
Gba_H14	...T.CAT.G.....C..T.CG												1						1
Gba_H15	...T.CA.....T.....C..T...												1						1
Gba_H16	...C.CAT.G..T.....C..T...												2						2
Gba_H17	...T.CA..G..T.....C..T.C.												1						1
Gba_H18	...C.CAT.G..T.G.....T...	1																	1
Gba_H19	...T.C.T.G..T.....C..TA..												1						1
Gba_H20	...C.CAT.G..T.....C..T.CG								1										1
Gba_H21	.....A..												1						1
Gba_H22	...T.CAT.G..T.....C..T..G												1						1
Gba_H23	...C..A..G..T.....C..T.C.											1							1
Gba_H24	A...T..TG.T.....T.CG				5			1											6
Gba_H25	....T.AT.....G.....CG			1					1										2
Gba_H26	...C.CAT.G..T.GT.....C..T...									2									2
Gba_H27	.....C.				4	1		5			1				1				12
Gba_H28	...C.CAT.G..T..T.....C..T...									1									1
Gba_H29	.....CA..G..T.....C..T.C.															1			1
Gba_H30	.....CAT.G..T.....C..T.C.														1	5	2	1	9
Gba_H31	...C.CAT.G..T.G.....C..T...						1	12						1					14
Gba_H32	...C.CAT.G..T.G.....C.CT...							2											2
Gba_H33	....T.ATG.T.....T.CG						10	2				1							13
Gba_H34	....T.AT.....G.....C.		1		1			1			1								4
Gba_H35	....T..TG.T.....T.CG		2				1	1											4
Gba_H36	....T..TG.T.....T.C.		1				4	1											6
Gba_H37	....T.AT.....G.G.....CG									1									1
Gba_H38	...T.CAT.G..TT.....C..T.C.											1							1
Gba_H39	...C.CAT.G..T.....C..T.C.											2							2
Gba_H40	.....ATG.....G..-...CT...C.							1											1

Table 1 *Continued*

Haplotype	Nucleotide positions 1111111111111122 3335566689913444455557788812 0346735883960068927891603570	Populations Iberian Peninsula NSC	NSH	CSH	SSH	IBH	SAR	ALP	GRE	CRE	CAU	CAS	EAS	YEM	Africa NAF	ETH	SAF	AFR	Total
Gba_H41	....T.ATG.T.....T.C.						2												2
Gba_H42	....T.ATG.T...G.....T.CG						1												1
Gba_H43	.....ATG.....-...C....C.							1							1				2
Gba_H44	.-–T.CAT....T.....CC..T.C.								1										1
Gba_H45	...C.CAT.G..T.G.....C.....								2										2
Gba_H46	.-–T.CAT....T.....CC..T...								3										3
Gba_H47	.-–T.CAT.G..T.....C..T.C.										1								1
Gba_H48	...T.CA.....T.....C..T.C.											1							1
Gba_H49	...T.CAT.G..T.....C..T.CG											1							1
Gba_H50	...T.CA..G..T.....T.C..T...										1								1
Total		34	13	2	11	1	20	28	8	5	4	12	21	1	3	6	2	1	172

NSC, Northern Spain Contemporary; NSH, Northern Spain Historic; CSH, Central Spain Historic; SSH, Southern Spain Historic; IBH, Iberian Peninsula Historic; SAR, Sardinia; ALP, Alps; GRE, Continental Greece; CRE, Crete; CAU; Caucasus; CAS; Central Asia; EAS, Eastern samples with no precise location; YEM, Yemen; NAF, North Africa; ETH, Ethiopia; SAF, South Africa; AFR, Africa.

position of haplotypes 29 and 30 in trees is also reflected in their internal positioning within the network.

The rooting of the genealogy was hindered by the lack of suitable outgroups. The bearded vulture's sister species, the Egyptian vulture, shows a highly divergent control region sequence, and alignment is complicated by the presence of tandem repeats (Roques *et al*. 2003). Control region sequences of more distantly related species, such as those of the Spanish imperial eagle, *A. adalberti* (GB | AJ567366, Martínez-Cruz *et al*. unpublished) and crested serpent-eagle, *Spilornis cheela* (GB | AY140546.1; Horng *et al*. unpublished), could be aligned and were used as outgroups in different phylogenetic analysis. Overall, the position of the root between clades A and B was supported by more approaches than any other alternative, being the position inferred by midpoint rooting of maximum-likelihood and distance trees, and outgroup rooting of distance and maximum-parsimony trees.

A clear geographical pattern can be superimposed onto the tree and network, with Eastern and African samples classifying preferentially in group B and Western samples in group A. Strict monophyly is not observed, however: a few haplotypes found in western birds do belong to clade B and in two cases (H_21 and H_33) group A haplotypes were found on eastern birds. Haplotype H_21 was detected in sample GbC059 corresponding to individual GB199 from the Breeding Network, recorded as originating from the Former Soviet Union, and haplotype H_33 was found in sample GbS098 from Kazakhstan. Among the several occurrences of clade B haplotypes in western birds, we found H_10 in GB122, an offspring of BG010, recorded as Pyrenean. Because individual GB010 also classified among Asian birds according to its nuclear genotype (Gautschi *et al*. 2003), this might be a case of sample misidentification or a recent immigrant from Asia in the Pyrenees. Interestingly, clade A haplotypes in eastern populations and clade B haplotypes in western populations tend to be placed as terminal or external nodes in the network, suggesting a recently derived origin, with the only exception of the probably misidentified H_10 haplotype (Fig. 2).

In Africa, two closely related haplotypes classifying in basal positions in clade B (H_30 an H_29) were found in all Ethiopian ($n = 7$) and South African ($n = 2$) samples, as well as in an African sample of unknown origin. On the other hand, one of the three samples analysed from North Africa presented the major African haplotype (H_30), whereas the remaining two presented clade A haplotypes shared with Western European populations (H_27 and H_43).

The geographical pattern of haplotype distribution is shown in Fig. 3, indicating a sharp East–West clinal distribution of clade proportions in populations ranging from Iberian Peninsula to Central Asia. More than 95% of birds from Iberia or Sardinia harboured clade A haplotypes, while

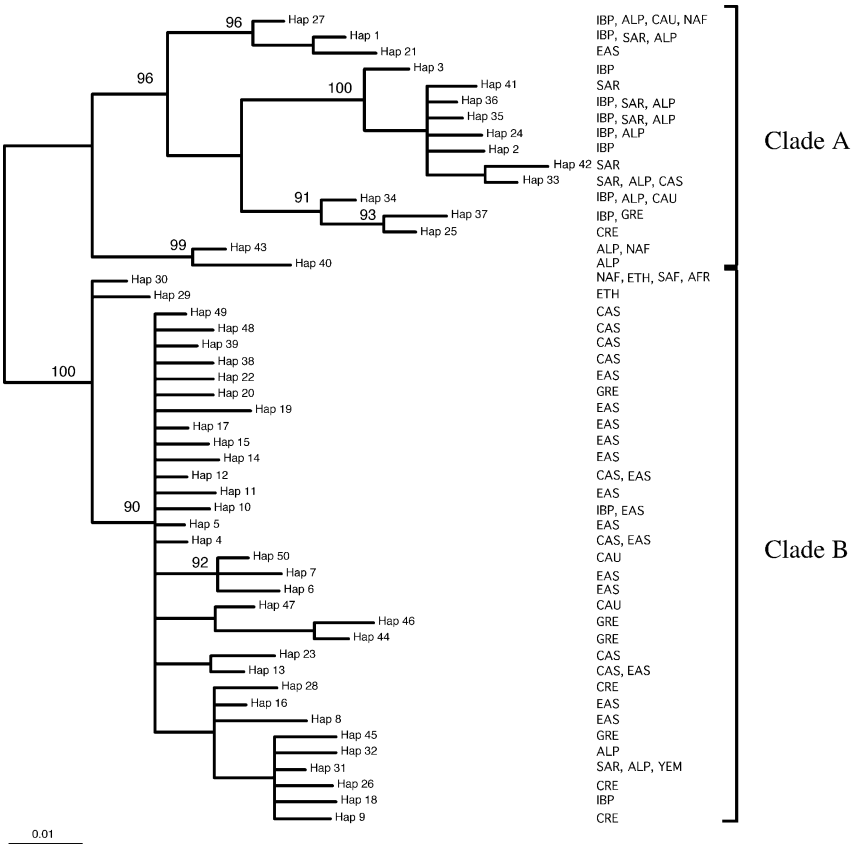

Fig. 1 Phylogenetic relationships among bearded vulture mitochondrial haplotypes. The tree shown is the consensus of 7000 trees sampled from the Bayesian posterior distribution. Numbers at nodes indicate the proportion of times in percentage that a node is found among these trees, as a measure of support. Populations where each haplotype was found are indicated to the right.

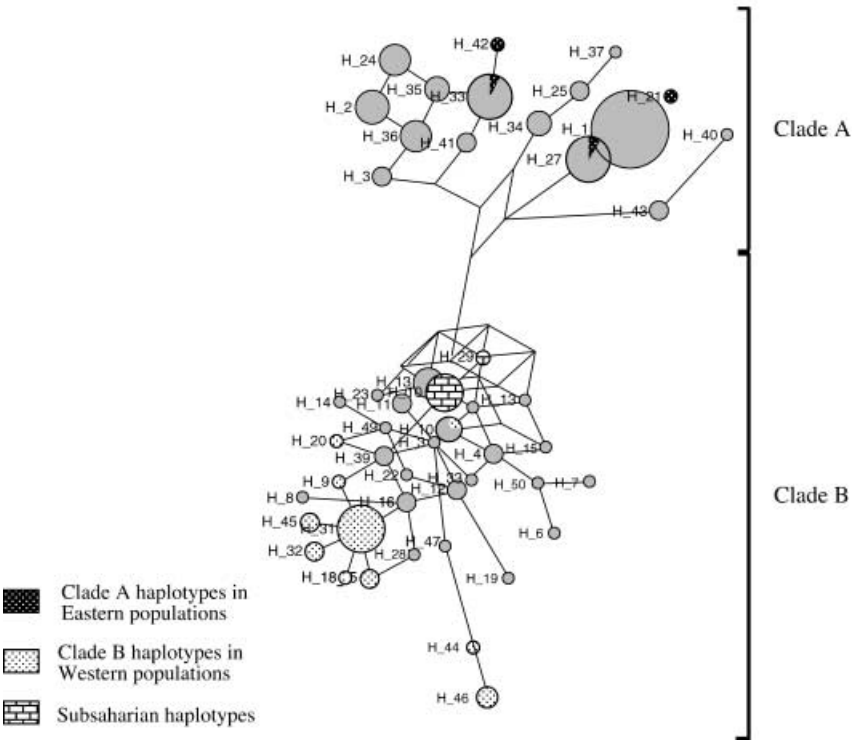

Fig. 2 Median joining network of bearded vulture haplotypes. The size of the node indicates the relative frequency of the corresponding haplotype in the whole data set. The position of 'misplaced' and African haplotypes is indicated to show their relative external and internal position, respectively.

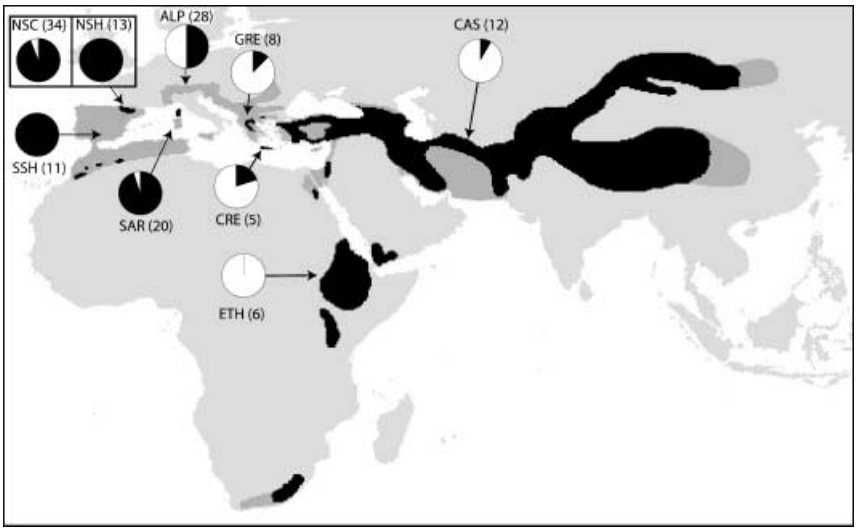

Fig. 3 Map showing the past (grey) and current (black) distribution of the species, based on Mundy *et al*. (1992) and Heredia & Heredia (1991). Pie diagrams depict the proportions of clade A (black) and clade B (white) haplotypes in different bearded vulture populations. Numbers in parenthesis refer to sample size.

the opposite was true for birds from central Asia or Africa. Mixing of the two clades was maximal in the Alps, where roughly equal proportions from each clade were found.

The observed pattern might have originated by divergence of the two clades in allopatry and secondary mixing after range expansion, as discussed below. Dating of the population separation event requires a reliable estimation of the evolutionary rate and the assumption of rate constancy and homogeneity. In the absence of an internal calibration, a general uncorrected rate for the hypervariable domain I of the control region in birds is estimated at 20% per million years (Quinn 1992; Avise & Walker 1998; Marshall & Baker 1998). According to this rate, a separation date of 124 250 years is estimated from the mean net number of substitutions per site, corrected for within-clade diversity, of 0.02485. The 95% confidence interval estimated for the Jukes–Cantor corrected mean is 117 950–140 150 years (Steel *et al*. 1996).

Demographic analysis

The shape of the mismatch distribution function for the whole data set is bimodal, reflecting the deep divergence between the two clades. 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). Estimates of theta taking into account genealogical information are 0.0228 (SD = 0.0028) for clade A and 0.125 (SD = 0.0098) for clade B. Both estimates are higher than those derived from summary statistics (i.e. nucleotide diversity, π) that might be interpreted to reflect more recent demography ($\pi_A = 0.0189 \pm 0.0105$ and $\pi_B = 0.0147 \pm 0.0085$). On the other hand, the maximum-likelihood estimates for the growth parameter under an exponential demographic model, expressed as $1/\mu$, is negative and low for clade A and positive and relatively high for clade B ($g = -16.32$ and $g = 253.05$, respectively). Finally, the shapes of the number of lineages through time plots and skyline plots indicate a stationary population for clade A sequences and a growing population for clade B (Fig. 4). Taking all the information into account, a historic demographic expansion is thus well supported by the sequence data for clade B sequences, while a historically stationary or decreasing population is inferred from clade A sequences.

Population structure and gene flow

With a subset of the samples for which reliable and specific information on location was available, 10 populations with more than five samples could be defined (Table 1). A strong genetic differentiation among populations was indicated by a global Φ_{ST} value of 0.553 and 0.260, for distance or frequency-based analysis, respectively, both being significantly different from zero ($P < 0.00001$). When the AMOVA analysis was based on a matrix of genetic distances under the most likely model of sequence evolution (K2P + G, $\alpha = 0.014$), significant differentiation was recorded between NSH, SSH and SAR and between each of them and all other populations, while more eastern populations, including ALP, were not differentiated among them nor with Ethiopia (data not shown). These results reflect mainly the differential distribution of clades A and B haplotypes and the low divergence among clade B haplotypes. On the contrary, when the analysis was based only on haplotype frequencies, significant genetic differentiation was also found between ALP, GRE, CAS, and ETH (Table 2). CRE did not show Φ_{ST} values significantly different from zero against any of the other populations, but the exact test excluded the null hypothesis of no differentiation between CRE and SSH, SAR or ALP; in the remaining cases, small sample size might have compromised

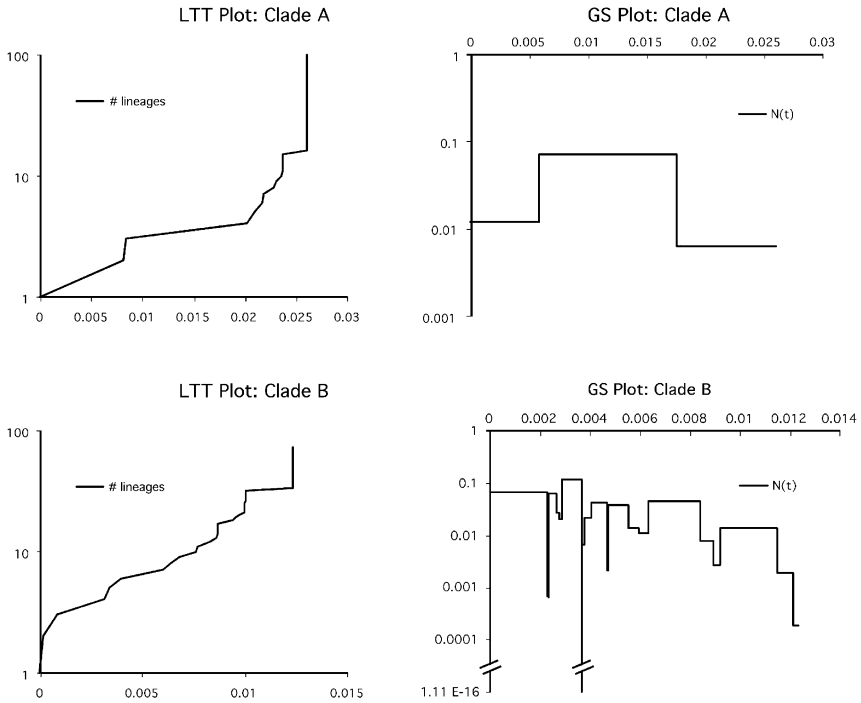

Fig. 4 Demographic history of the two bearded vulture lineages as revealed by lineage-through-time (LTT) and generalized skyline (GS) plots. Horizontal axis represents time since present in substitutions per site and the vertical axis corresponds to the number of lineages in LTT plots and estimated effective population size × substitution rate in GS plots.

Table 2 Genetic differentiation between bearded vulture populations. Pairwise Φ_{ST} values based on haplotype frequencies (above the diagonal) and the corresponding number of female migrants per generation (below the diagonal), assuming mutation-drift equilibrium and an island population model. Significance is evaluated by permuting haplotypes among samples (10 000 permutations) and adjusted for multiple tests by the sequential Bonferroni procedure (*$P < 0.05$, **$P < 0.01$). Significant differentiation in exact differentiation test is shown in bold type in the upper-right half-matrix

	NSH	SSH	SAR	ALP	GRE	CRE	CAS	ETH
NSH	—	**0.2762****	**0.23953****	**0.21611****	**0.21922***	0.210	**0.17277****	**0.4299****
SSH	1.31	—	**0.28128****	0.17351*	**0.22186***	**0.213**	**0.17405****	**0.44261****
SAR	1.59	1.28	—	**0.18311****	**0.21886***	**0.209**	**0.13833***	**0.40917****
ALP	1.81	2.38	2.23	—	**0.18036***	**0.167**	**0.13557***	**0.35969****
GRE	1.78	1.75	1.78	2.27	—	0.124	0.0992*	**0.38082****
CRE	1.88	1.85	1.89	2.49	3.54	—	0.077	0.402
CAS	2.39	2.37	3.11	3.19	4.54	5.97	—	0.3099**
ETH	0.66	0.63	0.72	0.89	0.81	0.74	1.11	—

the power of the tests. The group of eastern samples with no precise location (EAS) did not appear differentiated from CAS in any of the analyses performed, suggesting that these samples might come from the same broad Central Asian population defined in this study.

The combined analysis of pairwise Φ_{ST} values did not show a significant correlation with geographical distances (Mantel test, $P = 0.239$; Fig. 5). The relationship between these two variables might be obscured by the widely divergent haplotypic diversity found in different populations. For example, pairwise F_{ST} values between the Central Asian population and the rest of the populations were relatively low, even though it shared no haplotypes with western populations. As pointed out by Hedrick (1999), this might be due to high gene diversity ($H = 0.94$ in CAS) imposing a low upper limit on F_{ST} values. The opposing effect might bias F_{ST} values for ETH where haplotypic diversity was lowest ($H = 0.33$). The significance of the correlation approached significance after the exclusion of CAS (Mantel test, $P = 0.062$), but the exclusion of both CAS and ETH — leaving only European populations — resulted in a nonsignificant correlation (Mantel test, $P = 0.552$).

Genetic diversity

Genetic diversity estimates varied among populations but were especially low for ETH, where six of seven samples shared one haplotype (the major African haplotype H_30),

Table 3 Estimates of genetic diversity in several bearded vulture populations (for population codes see the legend to Table 1)

Population	No. of samples	No. of haplotypes	No. of polymorphic sites	Gene diversity (SD)	Nucleotidic diversity × 100 (SD)	Mean no pairwise differences (SD)
NSC	34	5	14	0.40 (0.10)	1.21 (0.73)	2.76 (1.50)
NSH	13	6	10	0.72 (0.13)	1.69 (1.02)	3.85 (2.07)
SSH	11	4	9	0.71 (0.10)	1.80 (1.10)	4.11 (2.22)
SAR	20	7	14	0.73 (0.09)	1.14 (0.71)	2.59 (1.45)
ALP	28	11	19	0.79 (0.07)	2.92 (1.58)	6.65 (3.24)
GRE	8	5	14	0.86 (0.11)	2.68 (1.62)	6.11 (3.25)
CRE	5	4	13	0.90 (0.16)	2.54 (1.71)	5.80 (3.34)
EAS	21	16	14	0.97 (0.02)	1.55 (0.91)	3.52 (1.87)
CAS	12	9	12	0.94 (0.06)	1.54 (0.95)	3.52 (1.92)
ETH	6	2	1	0.33 (0.22)	0.15 (0.19)	0.33 (0.38)

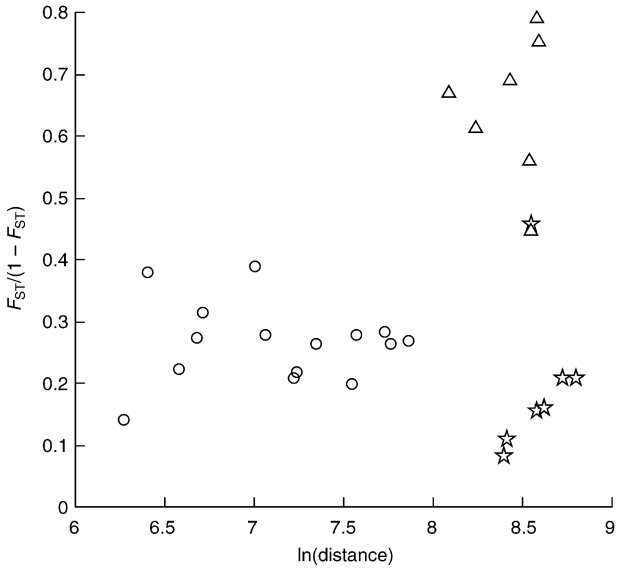

Fig. 5 Genetic differentiation with geographical distance among pairs of bearded vulture populations. Stars and triangles correspond to comparisons involving CAS and ETH populations, respectively.

while the other haplotype differed in one single position from this ($H = 0.33 \pm 0.22$; $P = 0.15 \pm 0.19$) (Table 3). At the other extreme, the Central Asian population showed the highest haplotypic diversity ($H = 0.94$, SD = 0.06), due perhaps in part to the wider geographical area sampled. Haplotypic diversity of European populations ranged from 0.71 (SD = 0.10) in SSH to 0.90 (SD = 0.16) in CRE. We found no evidence for lower diversity in islands (Sardinia or Crete), as has been described for other species, including the sister taxon Egyptian vulture in Fuerteventura (Kretzmann *et al*. 2003; Godoy, Negro, Donazar and Hiraldo, unpublished). On the other hand, nucleotide diversity and mean number of pairwise differences are highest for ALP, GRE and CRE, due to the concurrence of highly divergent clade A and clade B haplotypes in similar proportions.

The direct comparison of current vs. historic diversity in the Pyrenean population allows us to test whether the demographic contraction in the last decades has significantly affected the genetic composition of this population. Historic (NSH) and contemporary (NSC) haplotypic diversity in Pyrenees are significantly different ($t' = 8.03$, $P < 0.001$), while the haplotype distributions are only slightly different, as reflected in nonsignificant Φ_{ST} values ($\Phi_{ST} = 0.043$, $P = 0.108$) but a significant exact test of differentiation ($P = 0.039$).

Discussion

Recent evolutionary history of the species

Patterns of mitochondrial DNA variation in bearded vulture have been shaped by a combination of historic evolutionary and contemporary ecological factors. At the evolutionary level, the phylogenetic analysis of control region sequences reveals the existence of two major evolutionary lineages. Even though a striking geographical pattern is observed in the global distribution of these lineages, a strict geographical separation is not observed. Both lineages coexist in several of the populations analysed reaching almost equal frequencies in the Alps, resulting in polyphyletic groupings. Theory predicts that two populations that become isolated will go progressively from polyphyly to paraphyly and finally to reciprocal monophyly (Avise 2000). Instances in which monophyly is observed allows the inference of a period of evolution in allopatry. However, two alternative historic scenarios might result in polyphyly: (i) a recent separation of the two populations leading to incomplete lineage sorting, or (ii) a secondary admixture following range expansion (Avise 2000). Several lines of evidence indicate that the latter might better explain the pattern observed for the bearded vulture. First, the clinal distribution of clade proportion is not necessarily expected under the lineage-retention hypothesis but a necessary consequence of secondary admixture.

Second, as illustrated by Avise (2000), the coalescence times of retained lineages in the two populations will predate the vicariant event separating the two populations, while recent immigrants under the secondary contact scenario will present haplotypes identical or closely related to haplotypes in the donor population; the bearded vulture data accommodate to this latter pattern, thus supporting the admixture hypothesis. Third, most of clade B haplotypes in western populations and clade A haplotypes in eastern populations are placed as terminal or otherwise external nodes in the network, suggesting a recent origin, as would be expected for haplotypes in the limits of a expanding range (Posada, Crandall 2001).

Under this interpretation, the two lineages have evolved in allopatry and have been admixed after range expansion. This pattern is common for many other species in Europe (Taberlet *et al*. 1998; Hewitt 1999) and North America (e.g. Quinn 1992) and have been usually interpreted in relation to the glacial cycles. The Southern European peninsulas have been shown to have acted as glacial refugia for many of the contemporary flora and fauna species and this may also have been the case for bearded vultures. On the other hand, the location of the other refugia in which the second lineage evolved is less clear, although Southern Asia and Africa could be both considered. In support for Africa as the origin of the B lineages is the basal position of the current African haplotypes within this clade, although the statistical support for this is not definitive. In addition, African haplotypes occupy internal positions within the haplotype network, again suggesting that they were ancestors to the other B haplotypes. However, the low haplotype diversity found currently in Africa is far lower than what would be expected for a refugium. A secondary, more recent, bottleneck or a historically low effective population size might account for the current lack of variation in Africa, although independent evidence for this is lacking. If so, the causes for these can only be guessed, but the extreme climatic fluctuations occurring in the continent after the last glacial maximum (Coetzee 1993) and the scarcity of suitable habitat might have seriously impacted the species demography in Africa.

Climatic and geographical factors should be invoked to explain the observed phylogeographical pattern: on one hand, a past geographical isolation that led to the divergence of the two lineages, and on the other hand, a more recent range expansion from Asia towards the West, and also from Eastern and Southern Africa towards the North, that originated the Central Europe and North Africa suture zones, respectively. First, the Sahara desert has probably acted as a permanent barrier for the species. Bearded vultures may have never inhabited the scanty and relatively low-altitude Saharan mountains due to the limitation of nesting sites and lack of food (Mundy *et al*. 1992). During the last glacial maximum temperatures dropped by 4–9.5 °C in Africa, causing glaciations on high mountains (including the Atlas, Hoggar, and East African mountains), and a substantial increase in aridity occurred that caused a shift of more that 300 km southward in the southern boundary of the Sahara desert (Coetzee 1993). Under these conditions bearded vultures were most probably also absent from Northeast Africa so that Eastern or Southern Africa and Mediterranean populations were isolated effectively by a continuous band of thousands of kilometres of unsuitable habitat extending from coast to coast. When the temperature rose after 14 000 BP, the Atlantic monsoon was re-established which led to more humid conditions, under which the potential habitat in Northeastern Africa became available for bearded vultures. Thus, this key population might have acted as an intermittent stepping-stone that allowed the eventual connection of the two glacial refugia and the formation of the secondary suture zone in North Africa.

The deeper phylogenetic structure and longer branches within clade A when compared to clade B suggests older coalescence times and/or higher historical effective population sizes for western populations. A similar pattern has been observed for other species (reviewed in Taberlet *et al*. 1998; Hewitt 1999) and indicates that the Western Europe glacial refugia might have served as such in repeated glacial maxima and/or that they have harboured larger populations. However, this observation contrasts with the smaller distribution range of clade A haplotypes and suggests limitations in the recolonization ability from Mediterranean peninsulas. A global pattern of stasis in the demographic history of the western clade is probably reflecting this limitation. On the contrary, clade B occupies a large geographical area and signs of vigorous population growth can be detected in the patterns of mitochondrial sequence variation. For other terrestrial species, range expansion limitations from Mediterranean peninsulas has been attributed to the Pyrenees and the Alps acting as effective barriers (Hewitt 1999; Taberlet *et al*. 1998). This should not be the case for a bird inhabiting mountains, so that other factors must have affected the differential ability of the two lineages for range expansion. Differences in the geographical continuity of suitable habitat (mountain chains) and/or the availability of food (mainly ungulate carcasses) might be two of such factors.

The pattern of intraspecific genetic variation described in this study does not support the currently accepted distinction of *G. b. barbatus* and *G. b. meridionalis* subspecies, with the former covering all Eurasia and North Africa and the latter South and East Africa (Hiraldo *et al*. 1984). Morphological characters upon which this classification was made (plumage characteristics and body size) might have been fixed by chance in African bearded vultures, and the low haplotypic diversity found suggests a strong founder or bottleneck effect that might have affected both morphological and molecular diversity. Alternatively,

the morphological characters involved might be subjected to natural selection in Africa. The distinction of subspecies under the complex historic scenario described by the mitochondrial DNA does not seem appropriate; differentiation between the two clades might be viewed as an early stage of allopatric speciation that was aborted by the secondary contact in Central Europe and Northern Africa.

Gene flow among bearded vulture populations

Significant structuring of genetic diversity was found among most bearded vulture populations, even for population pairs separated by only several hundreds of kilometres, indicating low level of gene flow. Although our results from mitochondrial DNA refer only to female dispersal, similar results obtained with nuclear microsatellite markers suggest this is not a sex-specific pattern (Gautschi 2001). These results contrast with a general pattern of low levels of genetic differentiation among populations of birds (Crochet 2000) and with results obtained for its sister species, the Egyptian vulture (Kretzmann *et al*. 2003). Such a high level of population differentiation is specially striking for a bird that usually flies long distances in a single day in search for food (Brown 1988) and that can eventually move hundreds of kilometres, as deduced from occasional sightings of birds far from their origin or from any bearded vulture population. A highly phylopatric dispersal behaviour needs to be invoked to explain this apparent paradox and this hypothesis is supported by field observations (R. Antor, unpublished). Phylopatry also explains the existence of a clear phylogeographical pattern: recurrent dispersal has not been sufficient to obscure the primary historic pattern (Hewitt 1996). Despite low estimated rates, significant interchange of individuals among populations occurred in the past and hence might be expected to occur in the future between the reintroduced population in the Alps and the other remnant European populations. The lack of correlation between geographical and genetic distances might in part be an artefact due to the variance in genetic diversity among populations as discussed above, but could also be taken as evidence for the absence of equilibrium between migration and drift over the analysed scale (Hutchinson & Templeton 1999).

Implications for conservation

The extinction of genetically differentiated bearded vulture populations has resulted in the loss of a significant proportion of the species genetic diversity and this process of genetic erosion continues as the small populations of Crete and Corsica approach extinction. Remnant European populations, and especially the largest Pyrenean population, should be given maximum priority for conservation because, due to its genetic divergence from the Eastern and African populations, they make a major contribution to the total species' genetic diversity (Petit *et al*. 1998). The Pyrenean population has already lost a significant proportion of its diversity in the last decades through drift and this process is expected to continue as long as its effective population size remains low and new genetic diversity is not introduced. Immigration from natural remnant populations seems highly unlikely, in view of the estimated low level of historic gene flow and the critical situation of nearby populations. However, migrants from the reintroduced population in the Alps might arrive in the near future if the current trends continue and this population becomes fully established; this would compensate for the loss of genetic diversity through drift and would help to prevent the occurrence of inbreeding depression. While not expected (see below), the occurrence of outbreeding depression should also be monitored.

On the other hand, the identification of conservation units have to take into account both the complex evolutionary history depicted in the mitochondrial DNA and ecological issues. While, according to our interpretation, two evolutionary lineages started diverging some time in the past, this differentiation process was aborted when they came naturally into contact in central Europe, forming a now-extinct hybrid zone. The decline of the species during the last century in Europe has left an apparently large and genetically healthy population in Central Asia and the Pyrenean population, with census sizes around 100 pairs as the largest and most viable European population. These two populations show divergent haplotypes, reflecting historic isolation, but reciprocal monophyly is not observed and thus they cannot be considered as Evolutionary Significant Units as defined by Moritz (1994). They do show different frequencies of mitochondrial haplotype (this study) and also nuclear alleles (Gautschi 2001; Gautschi *et al*. 2003), indicating low levels of historic gene flow, and thus could be considered Management Units (Moritz 1994). It must be noted, however, that the two populations must have been indirectly connected in the past by gene flow through the now extinct Central European populations. Crandall *et al*. (2000) argued against this categorization of conservation units based exclusively in genetic distinctiveness; they proposed a broader categorization based on exchangeability — rather than distinctiveness — that also takes into account ecological factors. In this context, the bearded vulture situation might be described as one of recent genetic exchangeability but absence of past genetic exchangeability. On the other hand, there seems to be no evidence to reject ecological exchangeability either past or recent, as no significant differences in life history traits, morphology, habitat or behaviour have been detected between these two populations (Brown 1988; Heredia & Heredia 1991; Mundy *et al*. 1992; Cramp & Perrins 1994). In this situation, the best strategy to preserve adaptive

diversity and evolutionary processes in bearded vulture would be to treat the species as a single population (Crandall *et al*. 2000). This interpretation validates the mixed population approach undertaken by the European captive breeding programme and justifies the release of mixed ancestry individuals undertaken in the Alps and future reintroductions in Southern Spain. 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 this work.

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This study is one result of the recent incorporation of molecular genetic techniques in our institute through the creation of the Laboratory of Molecular Ecology, which promotes the collaborative interaction between field- and laboratory-based scientists for the study of the ecology and evolutionary biology of wild species of plants and animals. J.A. Godoy acts as coordinator of the laboratory; he is a geneticist whose current interest is in the application of molecular population genetics to the conservation of carnivores and raptors and the study of seed dispersal by frugivorous birds. J.J. Negro, although initially trained as a behavioural ecologist, is also interested in genetic variability issues, hybridization and genetic erosion in small populations. F. Hiraldo is a population ecologist interested in the study and conservation of endangered raptors. J.A. Donázar has conducted research on population dynamics and limiting factors for vultures, both in the New and the Old World.

Appendix 1

Summary list of samples used in this study

Sample ID	Sample code	Locality/ area	Country	Date	Tissue[1]	Data[2]	Haplotype	Population	Contributor[3]	Sampler
Gb-1101048	GbC001	Pyrenees	Spain	1/6/96	B	+	H_01	NSC	FCQ	R. Antor
Gb-1101050	GbC002	Pyrenees	Spain	3/5/94	B	+	H_01	NSC	FCQ	R. Antor
Gb-12002	GbC003	Pyrenees	Spain	12/1/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12003	GbC004	Pyrenees	Spain	9/2/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12004	GbC005	Pyrenees	Spain	30/5/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12005	GbC006	Pyrenees	Spain	5/6/96	B	+	H_01	NSC	FCQ	R. Antor
Gb-12013	GbC007	Pyrenees	Spain	20/5/99	B	+	H_02	NSC	FCQ	R. Antor
Gb-12021	GbC008	Pyrenees	Spain	16/12/94	B	+	H_01	NSC	FCQ	R. Antor
Gb-12022	GbC009	Pyrenees	Spain	12/1/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12023	GbC010	Pyrenees	Spain	10/2/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12024	GbC011	Pyrenees	Spain	30/5/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12031	GbC012	Pyrenees	Spain	3/6/99	B	+	H_01	NSC	FCQ	R. Antor
Gb-12032	GbC013	Pyrenees	Spain	4/5/99	B	+	H_01	NSC	FCQ	R. Antor
Gb-12033	GbC014	Pyrenees	Spain	21/5/99	B	+	H_01	NSC	FCQ	R. Antor
Gb-12041	GbC015	Pyrenees	Spain	2/1/95	B	+	H_02	NSC	FCQ	R. Antor
Gb-12042	GbC016	Pyrenees	Spain	3/2/95	B	+	H_02	NSC	FCQ	R. Antor
Gb-12043	GbC017	Pyrenees	Spain	11/2/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12044	GbC018	Pyrenees	Spain	17/5/95	B	+	H_02	NSC	FCQ	R. Antor
Gb-12049	GbC019	Pyrenees	Spain	15/6/99	B	+	H_03	NSC	FCQ	R. Antor
Gb-12051	GbC020	Pyrenees	Spain	27/5/99	B	+	H_01	NSC	FCQ	R. Antor
Gb-12052	GbC021	Pyrenees	Spain	5/5/99	B	+	H_01	NSC	FCQ	R. Antor
Gb-12061	GbC022	Pyrenees	Spain	6/1/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12062	GbC023	Pyrenees	Spain	3/2/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12063	GbC024	Pyrenees	Spain	11/2/95	B	+	H_01	NSC	FCQ	R. Antor
Gb-12064	GbC025	Pyrenees	Spain	8/7/95	B	+	H_01	NSC	FCQ	R. Antor
Qh.1ZARAGOZA	GbC026	Pyrenees	Spain	6/10/93	M	+	H_01	NSC	FCQ	R. Antor
Qh.4ZARAGOZA	GbC027	Pyrenees	Spain	19/1/98	M	+	H_01	NSC	FCQ	R. Antor
BG-005	GbC028	Kopetdag	Turkmenistan	6/5/98	B	+	H_04	CAS	BVBN	—
BG-009	GbC029	—	Rusia	Contemporary	B	+	H_05	EAS	BVBN	—
BG-016	GbC030	—	Eurasia	6/5/98	B	+	H_06	EAS	BVBN	—
BG-017	GbC031	—	Eurasia	1999	B	+	H_07	EAS	BVBN	—
BG-040	GbC032	—	Eurasia	Contemporary	B	+	H_08	EAS	BVBN	—
BG-044	GbC033	—	Mideast-Asia	Contemporary	B	+	H_04	EAS	BVBN	—
BG-065	GbC034	—	Crete	6/5/98	B	+	H_09	CRE	BVBN	—
BG-070	GbC035	—	Asia	Contemporary	B	+	H_10	EAS	BVBN	—
BG-104	GbC036	—	Russia	Contemporary	B	+	H_10	EAS	BVBN	—
BG-105	GbC037	—	Former USSR	Contemporary	B	+	H_11	EAS	BVBN	—
BG-118	GbC038	—	Former USSR	Contemporary	B	+	H_12	EAS	BVBN	—
BG-128	GbC039	—	Eurasia	6/5/98	B	+	H_13	EAS	BVBN	—

Appendix 1 *Continued*

Sample ID	Sample code	Locality/ area	Country	Date	Tissue[1]	Data[2]	Haplotype	Population	Contributor[3]	Sampler
BG-122	GbC040	Pyrenees	Spain	Contemporary	B	+	H_10	NSC	BVBN	—
BG-124	GbC041	—	Eurasia	23/10/98	B	+	H_14	EAS	CCG	A. Llopis
BG-130	GbC042	—	Former USSR	Contemporary	B	+	H_15	EAS	BVBN	—
BG-131	GbC043	—	Eurasia	Contemporary	B	+	H_16	EAS	CCG	A. Llopis
BG-133	GbC044	—	Former USSR	Contemporary	B	+	H_17	EAS	BVBN	—
BG-175	GbC045	—	Former USSR	Contemporary	B	+	H_10	EAS	BVBN	—
BG-201	GbC046	—	Tajikistan	1999	B	+	H_12	CAS	BVBN	—
BG-232	GbC047	Pyrenees	Spain	17/6/98	B	+	H_18	NSC	CCG	A. Llopis
BG-278	GbC048	—	Eurasia	25/6/98	B	+	H_13	EAS	CCG	A. Llopis
BG-286	GbC049	Pyrenees	Spain	17/6/98	B	+	H_01	NSC	CCG	A. Llopis
BG-014	GbC050	—	Former USSR	Contemporary	B	+	H_16	EAS	BVBN	—
BG-022	GbC051	—	Russia	Contemporary	B	+	H_19	EAS	BVBN	—
BG-031	GbC052	—	Eurasia	Contemporary	B	+	H_11	EAS	BVBN	—
BG-178	GbC053	—	Greece	Contemporary	B	+	H_20	GRE	BVBN	—
BG-199	GbC054	—	Former USSR	Contemporary	B	+	H_21	EAS	BVBN	—
BG-204	GbC055	—	Russia	Contemporary	B	+	H_22	EAS	BVBN	—
—	GbC056	Zoo Alma-ata	Kazakhstan	June 1999	B	+	H_23	CAS	Zoo Alma-ata	J.A. Donázar
—	GbC057	Zoo Alma-ata	Kazakhstan	June 1999	B	+	H_13	CAS	Zoo Alma-ata	J.A. Donázar
—	GbC065	Pyrenees	Spain	4/6/01	F	+	H_01	NSC	—	M. Razin
ACC 1996–13	GbS001	—	—			1878	S/F	—	NHMT	A. Margalida
ACC 1996–14	GbS002	—	Mongolia	1887	S/F	+	H_38	CAS	NHMT	A. Margalida
ACC 1996–15	GbS003	Baïgorri (Pyr.)	France	1872	S/F	+	H_34	NSH	NHMT	A. Margalida
ACC 1996–16	GbS004	—	—	1871	S/F	–			NHMT	A. Margalida
ACC 1996–17	GbS005	—	—	1860	S/F	–			NHMT	A. Margalida
ACC 1996–18	GbS006	—	—	1877	S/F	–			NHMT	A. Margalida
ACC 1996–19	GbS007	—	—	1872	S/F	–			NHMT	A. Margalida
ACC 1996–212	GbS008	—	—	1880	S/F	–			NHMT	A. Margalida
—	GbS009	Pyrenees	Spain	1966	S/F	+	H_01	NSH	EBD	J. Muñoz
—	GbS010	Pyrenees	Spain	1966	S/F	+	H_01	NSH	EBD	J. Muñoz
—	GbS011	Pyrenees	Spain	Pre-1950	S/F	+	H_03	NSH	—	J.A. Donázar
—	GbS012	Pyrenees	Spain	1932	S/F	–			—	J.A. Donázar
—	GbS013	Pyrenees	Spain	1940—1950	S/F	+	H_01	NSH	—	D. Campión
—	GbS014	Thian Shan	Kazakhstan	1999	S/F	+	H_13	CAS	—	J.A. Donázar
—	GbS015	Thian Shan	Kazakhstan	1999	S/F	+	H_39	CAS	—	J.A. Donázar
—	GbS016	Thian Shan	Kazakhstan	1999	S/F	–			—	J.A. Donázar
—	GbS017	Thian Shan	Kazakhstan	1999	S/F	–			—	J.A. Donázar
—	GbS018	Pyrenees	Spain	1986	S/F	+	H_02	NSC	MNCNM	J.J. Negro
Ad.21735	GbS019	Jaca (Pyr.)	Spain	Pre-1950	S/F	+	H_01	NSH	MNCNM	J.J. Negro
Ad.21736	GbS020	Unarre (Pyr.)	Spain	1979	S/F	+	H_01	NSC	MNCNM	J.J. Negro
7661 Ad.Benedito = 19	GbS021	—	Spain	Pre-1950	S/F	+	H_24	SSH	MNCNM	J.J. Negro
17243	GbS022	Madrid	Spain	Late s.XIX	S/F	+	H_25	CSH	MNCNM	J.J. Negro

Appendix 1 *Continued*

Sample ID	Sample code	Locality/ area	Country	Date	Tissue[1]	Data[2]	Haplotype	Population	Contributor[3]	Sampler
97–0169.DARP6827	GbS023	Ribera de Cordos (Pyr.)	Spain	1994	S/F	+	H_01	NSC	MZB	E. Garcia
92–0184	GbS024	Toledo	Spain	1943	S/F	+	H_01	CSH	MZB	E. Garcia
—	GbS025	Pyrenees	Spain	1896	S/F	–			MHNG	A. Fayard
—	GbS026	Sardinia	Italy	1913	S/F	–			MHNG	A. Fayard
—	GbS027	Sardinia	Italy	1935	S/F	+	H_33	SAR	MHNG	A. Fayard
—	GbS028	Sardinia	Italy	1907	S/F	+	H_01	SAR	MHNG	A. Fayard
—	GbS029	Sardinia	Italy	1912	S/F	–			MHNG	A. Fayard
—	GbS030	Sardinia	Italy	1906	S/F	+	H_36	SAR	MHNG	A. Fayard
—	GbS031	Presles	France	—	S/F	+	H_40	ALP	MHNG	A. Fayard
—	GbS032	Sardinia	Italy	—	S/F	+	H_35	SAR	MHNG	A. Fayard
—	GbS033	Crete	Greece	1998	S/F	+	H_26	CRE	—	S. Xirouchakis
—	GbS034	Jaén (Andalusia)	Spain	Pre-1950	S/F	+	H_27	SSH	M. López, Private	J. Muñoz
—	GbS035	Jaén (Andalusia)	Spain	1930–1932	S/F	+	H_24	SSH	M. López, Private	J. Muñoz
—	GbS036	Jaén (Andalusia)	Spain	1910–1920	S/F	+	H_24	SSH	Dña. Eloisa, Private	J. Muñoz
—	GbS037	Granada (Andalusia)	Spain	1874	S/F	+	H_27	SSH	MCG	J. Muñoz
—	GbS038	Murcia	Spain	1950	S/F	+	H_27	SSH	—	J.J. Negro
—	GbS039	Navarra	Spain	Pre-1950	S/F	–			Gov. of Navarra	A. Senosiain
—	GbS040	Navarra	Spain	40 s	S/F	–			Gov. of Navarra	A. Senosiain
—	GbS041	Crete	Greece	1997	S/F	–	—			S. Xirouchakis
—	GbS042	Crete	Greece	1980	S/F	+	H_28	CRE	—	S. Xirouchakis
—	GbS043	Crete	Greece	—	S/F	–	—			S. Xirouchakis
—	GbS046	—	Ethiopia	2000	S/F	+	H_29	ETH	—	J. Juste
—	GbS047	Crete	Greece	2000	S/F	+	H_26	CRE	—	S. Xirouchakis
0407	GbS048	Sardinia	Italy	—	S/F	+	H_33	SAR	SWNS	B. Gautschi*
2164	GbS049	Malans	Switzerland	—	S/F	+	H_31	ALP	SWNS	B. Gautschi*
2270	GbS050	Andeer	Switzerland	1850	S/F	+	H_32	ALP	SWNS	B. Gautschi*
2243	GbS051	Wattensburg	Switzerland	1852	S/F	+	H_31	ALP	SWNS	B. Gautschi*
0406a	GbS052	—	Switzerland	1850–1851	S/F	+	H_31	ALP	SWNS	B. Gautschi*
7206	GbS053	Sardinia	Italy	—	S/F	+	H_41	SAR	MZS	B. Gautschi*
7207	GbS054	Conoraza	Italy	1885	S/F	+	H_31	SAR	MZS	B. Gautschi*
7208	GbS055	Sardinia	Italy	1892	S/F	+	H_33	SAR	MZS	B. Gautschi*
7209	GbS056	Sardinia	Italy	1901	S/F	+	H_36	SAR	MZS	B. Gautschi*
7210	GbS057	Sardinia	Italy	1906	S/F	+	H_33	SAR	MZS	B. Gautschi*
7211	GbS058	Sardinia	Italy	1907	S/F	+	H_33	SAR	MZS	B. Gautschi*
7212	GbS059	Sardinia	Italy	1907	S/F	+	H_33	SAR	MZS	B. Gautschi*
7213	GbS060	Sardinia	Italy	1908	S/F	+	H_42	SAR	MZS	B. Gautschi*
7214	GbS061	—	Ethiopia	1887	S/F	+	H_30	ETH	MZS	B. Gautschi*
Nr.109	GbS062	Glarus	Switzerland	1830	S/F	+	H_31	ALP	NMB	B. Gautschi*
1036716	GbS063	—	North Africa	—	S/F	+	H_30	NAF	NMBB	B. Gautschi*

Appendix 1 *Continued*

Sample ID	Sample code	Locality / area	Country	Date	Tissue[1]	Data[2]	Haplotype	Population	Contributor[3]	Sampler
1025773	GbS064	Grindelwald	Switzerland	1826	S/F	–			NMBB	B. Gautschi*
1025775	GbS065	Graubünden	Switzerland	1847	S/F	–			NMBB	B. Gautschi*
1025774	GbS066	Brienzersee	Switzerland	1823	S/F	+	H_31	ALP	NMBB	B. Gautschi*
1036711	GbS067	Sardinia	Italy	1888	S/F	+	H_36	SAR	NMBB	B. Gautschi*
1029615	GbS068	Zuoz	Switzerland	1854	S/F	+	H_27	ALP	NMBB	B. Gautschi*
1029619	GbS069	Zweilütschinen	Switzerland	1854	S/F	+	H_32	ALP	NMBB	B. Gautschi*
1040559	GbS070	—	Ethiopia	1959	S/F	+	H_30	ETH	NMBB	B. Gautschi*
1040558	GbS071	—	Ethiopia	1959	S/F	+	H_30	ETH	NMBB	B. Gautschi*
1040557	GbS072	Asco (Corsica)	France	1957	S/F	–			NMBB	B. Gautschi*
MHNG 0007.36	GbS073	Graubünden	Switzerland	1820	S/F	+	H_33	ALP	NMG	B. Gautschi*
MHNG 0799.97	GbS074	Martigny	Switzerland	1861	S/F	+	H_27	ALP	NMG	B. Gautschi*
MHNG 0799.62	GbS075	—	Switzerland	—	S/F	–			NMG	B. Gautschi*
92.3184 A	GbS076	Andeer	Switzerland	—	S/F	+	H_27	ALP	MHN	B. Gautschi*
92.3134 B	GbS077	Oberland	Switzerland	1850	S/F	+	H_31	ALP	MHN	B. Gautschi*
92.3184 C	GbS078	Brig (Wallis)	Switzerland	1833	S/F	+	H_31	ALP	MHN	B. Gautschi*
73771	GbS079	Himalaya	India	1839	S/F	+	H_39	CAS	NMW	B. Gautschi*
73772	GbS080	—	Switzerland	1878	S/F	–			NMW	B. Gautschi*
73774	GbS081	Granada (Andalusia)	Spain	1879	S/F	+	H_34	SSH	NMW	B. Gautschi*
37761	GbS082	Steiermark	Austria	1809	S/F	–			NMW	B. Gautschi*
84422	GbS083	—	Austria	—	S/F	+	H_43	ALP	NMW	B. Gautschi*
4591	GbS084	—	Kaukasus	1904	S/F	+	H_27	CAU	NMW	B. Gautschi*
14711	GbS085	—	Switzerland	1818	S/F	+	H_31	ALP	NSF	B. Gautschi*
10700	GbS086	—	Ethiopia	1900	S/F	+	H_30	ETH	NSF	B. Gautschi*
10690	GbS087	—	Greece	1899	S/F	+	H_44	GRE	NSF	B. Gautschi*
10691	GbS088	—	Greece	1899	S/F	+	H_45	GRE	NSF	B. Gautschi*
14710	GbS089	—	Greece	1905	S/F	+	H_45	GRE	NSF	B. Gautschi*
10692	GbS090	Granada (Andalusia)	Spain	1899	S/F	+	H_27	SSH	NSF	B. Gautschi*
ORN 1129	GbS091	Zermatt (Wallis)	Switzerland	1839	S/F	+	H_31	ALP	MZL	B. Gautschi*
ORN 429	GbS092	Wallis	Switzerland	1886	S/F	+	H_27	ALP	MZL	B. Gautschi*
ORN 40a	GbS093	Sardinia	Italy	1915	S/F	+	H_33	SAR	MZL	B. Gautschi*
1104	GbS094	Maggiatal	Switzerland	1869	S/F	–			NSStGM	B. Gautschi*
1106	GbS095	Nauders (Tirol)	Italia	1871	S/F	+	H_31	ALP	NSStGM	B. Gautschi*
1109	GbS096	Parnassus	Greece	1885	S/F	+	H_46	GRE	NSStGM	B. Gautschi*
Nr.23	GbS097	Naryn (Turkestan)	Kazakhstan	1913	S/F	+	H_13	CAS	HNS	B. Gautschi*
Nr.235	GbS098	Naryn (Turkestan)	Kazakhstan	1910	S/F	+	H_33	CAS	HNS	B. Gautschi*
Nr.199	GbS099	Kaukasus	Kaukasus	1912	S/F	+	H_34	CAU	HNS	B. Gautschi*
0535	GbS100	Sardinia	Italy	1985	S/F	+	H_33	SAR	MFSNU	B. Gautschi*

Appendix 1 *Continued*

Sample ID	Sample code	Locality / area	Country	Date	Tissue[1]	Data[2]	Haplotype	Population	Contributor[3]	Sampler
1955.6.N.20.198	GbS101	Bagnères de Bigorre (Pyr.)	France	1955	S/F	+	H_02	NSH	BMNHZMT	B. Gautschi*
1955.6N20.169	GbS102	Valley of Magna, near Nice	France	1955	S/F	+	H_34	ALP	BMNHZMT	B. Gautschi*
1874.4.9.1	GbS103	Jaén (Andalusia)	Spain	1872	S/F	–			BMNHZMT	B. Gautschi*
1891.4.11.19	GbS104	—	Spain	1891	S/F	+	H_27	IBP	BMNHZMT	B. Gautschi*
1873.1.2.1	GbS105	Málaga (Andalusia)	Spain	1869	S/F	+	H_02	SSH	BMNHZMT	B. Gautschi*
—	GbS106	Kaukasus	Kaukasus	1900	S/F	+	H_47	CAU	MNB	B. Gautschi*
106 × 250	GbS107	—	Greece	1908	S/F	+	H_46	GRE	MNB	B. Gautschi*
B 635	GbS108	—	Greece	1908	S/F	+	H_25	GRE	MNB	B. Gautschi*
—	GbS109	Pyrenees	Spain	1904	S/F	+	H_35	NSH	MNB	B. Gautschi*
171	GbS110	—	Switzerland	1891	S/F	+	H_33	ALP	MCHN	B. Gautschi*
173	GbS111	—	Switzerland	1881	S/F	+	H_31	ALP	MCHN	B. Gautschi*
174	GbS112	—	Switzerland	1891	S/F	+	H_35	ALP	MCHN	B. Gautschi*
172	GbS113	—	Switzerland	1891	S/F	+	H_36	ALP	MCHN	B. Gautschi*
25	GbS114	Geneva	Switzerland	1809	S/F	+	H_31	ALP	MRSNT	B. Gautschi*
11	GbS115	Sardinia	Italy	1906	S/F	+	H_33	SAR	MAKB	B. Gautschi*
13	GbS116	Sardinia	Italy	1906	S/F	+	H_36	SAR	MAKB	B. Gautschi*
15	GbS117	Lamentite	Albania	1924	S/F	+	H_46	GRE	MAKB	B. Gautschi*
7	GbS118	Thian Shan	Kazakhstan	1904	S/F	+	H_48	CAS	MAKB	B. Gautschi*
10	GbS119	Sardinia	Italy	1905	S/F	–			MAKB	B. Gautschi*
22	GbS120	Sardinia	Italy	1915	S/F	+	H_33	SAR	MAKB	B. Gautschi*
—	GbS121	Málaga (Andalusia)	Spain	1900	S/F	+	H_24	SSH	MAKB	B. Gautschi*
25	GbS122	Petrowsk (Dagestan)	Russia	1900	S/F	–			MAKB	B. Gautschi*
9	GbS123	Khalatase Ladakh (Kashmir)	India	1933	S/F	+	H_49	CAS	MAKB	B. Gautschi*
38	GbS124	Ladaka (Kashmir)	India	1934	S/F	–			MAKB	B. Gautschi*
—	GbS125	Nislam	Kaukasus	1904	S/F	+	H_50	CAU	MAKB	B. Gautschi*
—	GbS126	—	Tunisia	1887	S/F	+	H_43	NAF	MAKB	B. Gautschi*
24	GbS127	Kisslowodosk	Kaukasus	1890	S/F	–			MAKB	B. Gautschi*
12	GbS128	Sardinia	Italy	1905	S/F	+	H_41	SAR	MAKB	B. Gautschi*
BNM 3647	GbS129	Ftan, Val Tasua	Switzerland	1859	S/F	+	H_27	ALP	BNMC	B. Gautschi*
NMO 30157	GbS130	—	Ethiopia	1886	S/F	+	H_30	ETH	NMO	B. Gautschi*
7040U	GbS131	—	Africa	1882	S/F	+	H_30	AFR	MS	B. Gautschi*
—	GbS132	Crete	Greece	August 2000	S/F	+	H_37	CRE	—	S.Xirouchakis
—	GbS136	—	Yemen's Republic	1997	F	+	H_31	YEM	—	J.A. Donázar
1955.6N20.211	GbS137	—	South Africa	1955	F	+	H_30	SAF	BMNHZMT	B. Martínez

Appendix 1 *Continued*

Sample ID	Sample code	Locality/ area	Country	Date	Tissue[1]	Data[2]	Haplotype	Population	Contributor[3]	Sampler
—	GbS138	Córdoba (Andalusia)	Spain	20 s	F	+	H_24	SSH	—	J.J. Negro
17330	GbS139	—	Switzerland		Fp	+	H_01	ALP	NHMN	B. Martínez
17329	GbS140	Pyrenees	Spain	1872	Fp	–			NHMN	B. Martínez
17327	GbS141	—	Algeria	1856	Fp	+	H_27	NAF	NHMN	B. Martínez
17326	GbS142	Pyrenees	Spain	1872	Fp	+	H_01	NSH	NHMN	B. Martínez
17321	GbS143	Pyrenees	Spain		Fp	+	H_35	NSH	NHMN	B. Martínez
17328	GbS144	Bayona	France	1852	Fp	–			NHMN	B. Martínez
17322	GbS145	—	Switzerland		Fp	+	H_24	ALP	NHMN	B. Martínez
17323	GbS146	Pyrenees	Spain		Fp	+	H_01	NSH	NHMN	B. Martínez
17324	GbS147	Pyrenees	Spain	1868	Fp	+	H_01	NSH	NHMN	B. Martínez
17325	GbS148	St Jean Pied de Port (Pyr.)	France	1872	Fp	–			NHMN	B. Martínez
31753	GbS149	Pyrenees	Spain	1920–30	Fp	+	H_36	NSH	MBC	B. Martínez
31763	GbS150	Pyrenees	Spain	1920–30	Fp	–			MBC	B. Martínez
SMF 1837	GbS151	—	South Africa	1837	Fp	+	H_30	SAF	NSF	—

[1]The type of tissue sampled is indicated as B, blood; S/F, skin/feather; F, feather; M, muscle; Fp, footpad.

[2]Data indicate whether sequence data was finally obtained (+) or not (–); in this latter case it was always due to unsuccessful PCR amplifications.

[3]Institutions or individuals contributing the samples are listed. FCQ: Fundación para la Conservación del Quebrantahuesos; BVBN: Bearded Vulture Breeding Network; CCG: Centro de Cría 'Guadalentín'; NHMT: Natural History Museum of Toulousse; EBD: Estación Biológica de Doñana, Seville; MNCNM: Museo Nacional de Ciencias Naturales de Madrid; MZB: Museu de Zoologia de Barcelona; MHNG: Muséum d'histoire NaturelLe de Grenoble; MCG: Museo de Ciencias de Granada; SWNS: Stadt Wintherthur Naturwissenschafltiche Sammlungen; MZS: Museo Zoologico de 'La Specola' de Firenze; NMB: Naturhistorisches Museum, Basel; NMBB: Naturhistorisches Museum der Burgergemeinde, Bern; NHMG: Natural History Museum of Geneva; MHNN: Museum d'Histoire Naturelle de Neuchâtel; NMW: Naturhistorisches Museum, Wien; NSF: Naturmuseum Senckenberg, Frankfurt; MZL: Musée de Zoologie de Lausane; NSStGM: Naturmuseum Stiftung St Gallen Museen; HNS: Haus der Natur, Salzburg; MFSNU: Museo Friulano di Stori Naturlale, Udine; BMNHZMT: British Museum Natural History Zoological Museum, Tring; MNB: Museum für Naturkunde, Berlin; MCHNN: Musée Cantonal d'histoire Naturelle de Nord; MRSNT: Museo Regionale di Scienze Naturali, Torino; MAKB: Museum Alexander König, Bonn; BNMC: Bündner Natur-Museum, Chur; NMO: Naturmuseum Olten; MS: Museum Solothurn; NHMN: Natural History Museum of Nantes; MBC: Marchant Baréges Collections.

*Samples provided by B. Gautschi as DNA extracts.

