The present Pyrenean population of bearded vulture (*Gypaetus barbatus*): Its genetic characteristics

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The Pyrenean population of the endangered bearded vulture (*Gypaetus barbatus*) is the largest natural population in Europe. In this study, its current genetic variability was assessed using 110 animals of the recent population in order to know what the present situation. Sex identification by DNA methodology in the 110 bearded vultures, mitochondrial DNA (mtDNA) and eight microsatellite markers in 87 bearded vultures have been analysed. Our results for sex identification present a number of 49 males and 61 females; no significant differences for number of males and females in this population have been observed. mtDNA studies indicate that nucleotide and haplotype diversities and number of variable sites were low. Tajima's D test and Fu and Li's D* and F* tests suggest that mutations are selectively neutral and the population is expanding. A mean number of alleles per locus and a mean observed heterozygosity have been obtained by microsatellite analysis. F_{IS} is not high, and inbreeding depression could be discarded in the near future. The results suggest that the Pyrenean population of bearded vultures have to be controlled in order to avoid the loss of genetic variability. This data should be taken into account when considering conservation plans for the species.

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1. Introduction

The bearded vulture (*Gypaetus barbatus*) is an endangered large raptor. The species experienced a dramatic population decline in western Europe during the last century (BirdLife 2008). This decline was due to direct and indirect human causes [e.g. shooting, poisoning, electrocution, collision with electric lines or human disturbance (Margalida *et al.* 2008)]. Currently, the Pyrenean Mountains (Spain and France) support most of the European population of bearded vultures with 119 breeding pairs recorded in 2007 (FCBV-Heredia 2008), located mainly in Aragón and Western Catalonia. Other bearded vulture populations occur in other European mountainous areas [namely, 8 breeding pairs in Corsica (Xirouchakis and Nikolakakis 2002; FCBV-Fasce 2008), 6 breeding pairs in Crete (Bretagnolle *et al.* 2004; FCBV-Xirouchakis 2008) and in the Alps (Frey *et al.* 1995); this last population has been recently

Keywords. Bearded vulture Pyrenean population; genetic variability

Abbreviations used: EEP, European Endangered Species Programme; FCQ, Spanish Foundation for the Conservation of the Bearded Vulture; mtDNA, mitochondrial DNA; PIC, polymorphic information content; UPGMA, unweighted pair group method with arithmetic mean

reintroduced], and they are severely endangered due to their reduced population sizes.

Therefore, bearded vultures are a species with conservation concerns in Europe; consequently, interest in achieving a demographic positive trend is increasing. Several conservation plans have been carried out, such as the Breeding Network of the European Endangered Species Programme (EEP) to rear bearded vultures, and the reintroduction programmes. EEP includes breeding centres that help in the reintroduction plans of bearded vultures into places of historical range for the species (FCBV 2008). A reintroduction project has been running in the Alps since 1986 (Frey *et al*. 1995) and successful natural reproduction has already taken place. The first releases of bearded vultures in southern Spain took place in 2006 in Cazorla Mountains (Simón *et al.* 2007) and in Sardinia (Italy), where three individuals were released in 2008 (FCBV-Fasce 2008). The Aragón region now has the greatest population of bearded vultures in the Pyrenees, and since 1994 (Decreto 184/1994 according to the Spanish Law 4/1989 of wildlife conservation), a Conservation plan exists to increase the bearded vulture populations further. A captive breeding centre has been established in this region in order to maintain this species by the incubation and breeding of nestlings that could not normally survive in the wild. In order to increase the efficiency of these conservation plans, genetic studies have been developed for this article.

Other authors such as Negro and Torres (1999) determined genetic differences within the Pyrenean population of the bearded vulture, but these authors used old samples from museums and different collections. Godoy *et al*. (2004) evaluated the genetic diversity of this species by the analysis of the control region (mitochondrial DNA, or mtDNA). Gautschi *et al.* (2003a) used microsatellite markers to assess the genetic variability of old Pyrenean population and a captive population. Gautschi *et al*. (2003b) combined two genetic systems, microsatellites and mtDNA, to evaluate the relatedness of founders in a Swish captive bearded vulture population. All previous studies have been realized in old bearded vulture Pyrenean population, with samples obtained from museums and old collections together with a few present animals.

Our aim was to determine the recent genetic situation in the present Pyrenean bearded vulture. Our study is only with the wild and present animals in the Pyrenean population using sexing DNA methodology and complementary methodologies involved the analysis of part of the non-coding control region of the mtDNA and microsatellite markers in nuclear DNA. It is necessary to know what the sex relation is in the population, because the future of its conservation depends of the number of males and females presented in the current population. The high rate of nucleotide substitution makes the mtDNA control region a suitable marker for studies of population (Avise *et al*. 1987). Microsatellites, however, have a high mutation rate (Li *et al*. 2002) and are widely used for population structure analysis. For this, our work helps to obtain a more robust assessment and these results can contribute to conservation plans in order to avoid genetic drift. Our results concerning to the present Pyrenean population are compared with the previous genetic studies to check the tendency of this species.

2. Materials and methods

A total of 110 different bearded vultures were studied. All samples were collected from the Pyrenean population (figure 1), with the required legal licences, during the years 2006–2011. Collected samples were two to three feather quills, blood samples kept on FTA® cards and tissues from egg shells. When possible, the use of FTA® cards was preferred because only 2 or 3 drops of blood are required. This collection method minimizes the contact and disturbance of the animals and samples are easily kept at room temperature for a long time in genetic banks of the species (Gutierrez-Corchero *et al.* 2002; García and Arruga 2005, 2006). The Wildlife Conservation Centre of La Alfranca (Zaragoza, Spain) supplied 12 samples and the Spanish Foundation for the Conservation of the bearded vulture (FCQ) 98.

DNA from feather quills and tissues was extracted with DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions and DNA from FTA® cards was extracted using Chelex (Jensen *et al*. 2003; García *et al.* 2009).

A fragment of the *CHD* gene was amplified by PCR using the pair of primers P2 and P8 (Griffiths *et al*., 1998) and P2 and NP (Ito *et al*. 2003). PCR amplification and all sexing methodologies were performed following methods indicated by García *et al.* (2009).

For amplification the mitochondrial control region in 87 different bearded vultures, we used primer3 software (Rozen and Skaletsky 1998). The sequence used was the whole gene described by Roques *et al.* (2004), with GenBank accession number AY542900. It was necessary to design primers for a fragment that included haplotypes described previously, within domain I of the control region and contains most of the polymorphic sites (Godoy *et al*. 2004; Arruga 2010).

PCR amplification was performed in a final volume of 25 μL containing 5 μL of DNA, 1 unit of *Termophilus aquatic* polymerase (Ecogen), 400 nM of each primer [QUEBDL-F (5′- TTGTACATTAAACTATATGCCCCATT-3′) and QUEBDL-R (5′-GTAGTAGAGGATCTTCCTGACACC-3′)], 200 μM of each dNTP, 2 mM $MgCl_2$ and PCR buffer with 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH=8) and 0.01% Tween-20. PCR conditions included an initial denaturing step at 94°C for 4 min 30 s followed by 40 cycles at 94°C for 30 s, 57°C for 45 s and 72°C for 45 s with a final extension step at 72°C for 5 min. PCR products were

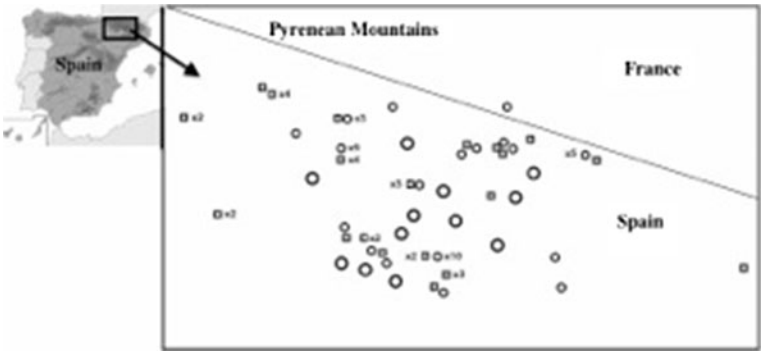

Figure 1. Map of sample collection areas in the Pyrenean area. Names of the villages have been deleted from the map to protect the locations of the nests.

purified and directly sequenced. Sequences were aligned using the program BioEdit (Hall 1999) and corrected manually. Blast program (Zhang *et al*. 2000) was used to search the most similar sequences in GenBank for our resulting sequences. Genetic diversity analysis was conducted using DNASP (Rozas y Rozas 1999) to calculate the number of variable sites, values of haplotype diversity, nucleotide diversity and Tajima's D neutrality test and MEGA version 4 (Tamura *et al*. 2004, 2007) to check pairwise distance calculation and UPGMA phylogenetic tree with 1000 bootstrapping. Haplotypes identifications correspond to the sequences deposited in GenBank with accessions EU934098 (1b), EU934099 (2b), EU934100 (3b), EU934101 (18b) and EU934102 (1/27).

For microsatellite analysis, we selected 8 microsatellites described by Gautschi *et al*. (2000) and we studied in 87 different animals. We used the most variable microsatellite loci: BV5, BV6, BV11, BV12, BV13, BV14 and BV16. For the loci BV11, BV12, BV13 and BV14 we used the newly designed primers by Gautschi *et al*. (2003a) because its shorter size allows for easier amplification from scarce DNA. We also selected BV1 among BV1, BV2 and BV20 (with 3 or 4 alleles per locus) because its expected size allowed multiplexing and its observed heterozygosity was higher than expected heterozygosity (Gautschi *et al.* 2000). Assessment of genetic variability of the bearded vulture samples was conducted using Genetix (Belkhir *et al*. 1996) and Cervus (Marshall *et al*. 1998). We evaluated allele frequencies, mean number of alleles, PIC (polymorphic information content) of the markers, mean observed heterozygosity, mean unbiased expected heterozygosity and Wright's inbreeding coefficient (F_{IS}).

3. Results

From a total of 110 bearded vultures analysed we identified 49 males and 61 females in this recent population. The identification of sex of *G. barbatus* by *Hae*III digestion of *CHD* gene amplification was made following the methods of García *et al.* (2009). Males have one band and females have two bands (figure 2). No significant differences between number of males and females were obtained ($0.30>P>0.20$).

The amplifications of mitochondrial control region obtained in 87 samples (fragment size of 388 bp) were aligned and compared with haplotypes previously described in this species (Godoy *et al*. 2004). The fragments included a part of the variable domain I and a part of the central conserved domain II (including the F box), at positions 184 to 521 of the whole gene described by Roques *et al.* (2004) (AY542900). We confirm that all obtained sequences are monomorphic in the last positions (central domain), being only variable in the part that aligned with the previous haplotypes (domain I, Godoy *et al*. 2004). Sequences of our samples have been deposited in GenBank under accession numbers EU934098 (haplotype 1b), EU934099 (haplotype 2b), EU934100 (haplotype 3b), EU934101 (haplotype 18b) and EU934102 (haplotype 1/27).

The 388 bp segment analysed in the present Pyrenean population included 11 variable sites. DNASP program

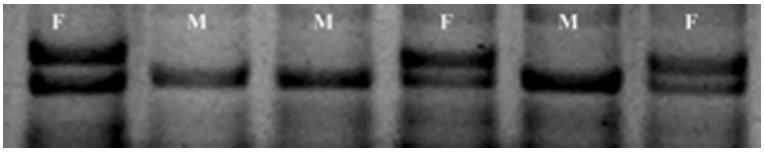

Figure 2. Patterns of amplied DNA after PCR in Bearded vultures. M: males; F: females.

Table 1. Pairwise distances among the five haplotypes identified in mitochondrial control region

	Hap. 1b	Hap. 2b	Hap. 3b	Hap. 18b	Hap. 1/27
Hap. 1b	0.000				
Hap. 2b	0.012 (0.007)	0.000			
Hap. 3b	0.009 (0.006)	0.002 (0.003)	0.000		
Hap. 18 b	0.019 (0.009)	0.021 (0.010)	0.019 (0.009)	0.000	
Hap. 1/27	0.000 (0.000)	0.012 (0.007)	0.009 (0.006)	0.019 (0.009)	0.000

Standard errors (1000 replicates bootstrap) appear in brackets.

(Rozas y Rozas 1999) did not take into account the ambiguous position and identified five haplotypes (1b, 2b, 3b, 18b and 1/27). Haplotype diversity (Hd) was estimated in 0.480 with a variance of 0.00787 and a standard deviation of 0.089. The assessment of nucleotide diversity () resulted in Pi= 0.00828 with a sampling variance of 0.0000031 and a standard deviation of 0.00175. The average number of nucleotide differences is 2.50739.

Tajima's D test of neutrality of the whole samples resulted in D=−0.33987. It was not significant with $P>0.10$. The null hypothesis is neutral evolution (all mutations are selectively neutral). This negative Tajima's D means that there is an excess of low frequency polymorphisms, indicating that there is no selection. Fu and Li's D* test statistic gave −1.53742 (not significant, $P>0.10$) and Fu and Li's F* test statistic gave −1.36778 (not significant, $P>0.10$).

We estimated the evolutionary divergence between the sequences of the haplotypes of our bearded vultures and that of the average evolutionary divergence over all sequence pairs. Analyses were conducted using the Maximum Composite Likelihood method in MEGA version 4 (Tamura *et al*. 2004; Tamura *et al*. 2007). Standard error estimates were obtained by a bootstrap procedure (1000 replicates).

Table 2. Summary of analysis of genetic variability in 87 bearded vultures with eight microsatellite loci

Locus	k	N	H(O)	H(E)	PIC	Null freq
BV1	3	87	0.407	0.408	0.486	−0.0069
BV5	5	87	0.463	0.647	0.448	0.2098
BV6	5	87	0.792	0.728	0.666	−0.0039
BV11	6	87	0.583	0.713	0.647	0.0810
BV12	12	87	0.625	0.831	0.792	0.1043
BV13	6	87	0.667	0.697	0.655	−0.0076
BV14	4	87	0.500	0.516	0.440	0.0780
BV16	5	87	0.625	0.631	0.574	−0.0227

k = mean number of alleles; N = number of samples analysed with each locus; H(O) = observed heterocigosity; H(E) = Hardy–Weinberg expected heterocigosity; PIC = polymorphic information content; Null freq = null allele frequency estimated.

Pairwise distance estimations and standard errors are indicated in table 1. The overall average is 0.012 with a standard error of 0.005. This program did not find any distance between haplotypes 1b and 1/27 although there was an ambiguous position. The minimum distance (0.002) is found between haplotypes 2b and 3b and the maximum distance is found between haplotypes 2b and 18b (0.021) although all distances between 18b and the other haplotypes are higher.

Phylogenetic analysis was conducted in MEGA version 4 (Tamura *et al*. 2004, 2007). The evolutionary history was inferred using the UPGMA method. The sum of the branch length of the optimal tree was 0.02608723. The evolutionary distances were computed using the Maximum Composite Likelihood method.

Microsatellite data were analysed in Genetix (Belkhir *et al*. 1996) and Cervus (Marshall *et al*. 1998), and the results were assessed together (table 2). The mean number of alleles per locus is 5.13. The mean expected Hardy–Weinberg heterozygosity is 0.585, the mean observed heterozygosity is 0.523, the mean PIC is 0.528 and null allele frequency estimate for each locus is as indicated in the same table 2. It is interesting note that all loci are in Hardy–Weinberg equilibrium (HWE) except the locus BV5.

The Wright's inbreeding coefficient (F_{IS}) for these 87 bearded vultures is 0.10911 with an interval confidence of 95% (calculated with 1000 permutations) from −0.03347 to 0.20343. This F_{IS} value is not very high.

4. Discussion

Although previous studies have been developed in Pyrenean populations of bearded vulture (Negro and Torres 1999; Gautschi *et al*. 2003a; Godoy *et al*. 2004), all these authors used old samples from museums, old collections, etc., together with a few present animals. In this study, we used only wild and present animals of the Pyrenean population, in order to know the present situation of this population.

DNA extraction from blood on FTA® cards and tips of feathers have worked well in our studies, and both of them only require a minimal contact with the animals; we strongly recommend one drop of blood on FTA® cards as a collection

method because they are kept at room temperature for a very long time (Gutiérrez-Corchero *et al*. 2002; García and Arruga, 2005, 2006, García *et al*. 2009). The number of males and females identified in this Pyrenean population does not present significant differences. The sexing methodology used in this study has been very useful for the gender identification of bearded vultures in the first days of nestling life, in captive breeding programs or to know the sex ratio in populations in the wild. Finally, this is also very important in conservation management of this endangered species.

We have found many consistent results between the analyses of mtDNA and microsatellite markers (nuclear DNA), but they also offer complementary genetic information. Our results in the analysis of mitochondrial control region indicate that the nucleotide and haplotype diversities as well as number of polymorphic sites are a bit low. Godoy *et al*. (2004) also identified some individuals with haplotype numbers 2b, 3b, 10b and 18b. We identified haplotype numbers 2b, 3b and 18b in our samples of the present Pyrenean population but we could not find any bearded vulture corresponding to haplotype number 10b. Godoy *et al*. (2004) did not show any problems with unclear positions, although Gautschi *et al*. (2003b) and our study are consistent in finding one position. Since five distinct maternal lineages were observed in 87 bearded vultures, the possibility that the sampled birds are descendants of one or a few females is low.

In the Tajima's test of neutrality, our data suggest that mutations in the 388 bp fragment of the mitochondrial control region in the present bearded vultures are selectively neutral. Besides, negative and non-significant results in Fu and Li's D* and F* tests indicate that the studied population is expanding.

All the microsatellite loci, except BV5, are in HWE. Since only few of the several loci analyzed deviate significantly from HWE, there is no strong indication to believe that this observation is reflective of systematic inbreeding or other possible deviations from random mating. This locus is also not known to be associated with any phenotype that may be under the strong influence of natural selection.

Microsatellite analysis show a mean number of alleles per locus of 5.13, which is bigger than 4.3 and 3.7 found by Gautschi *et al*. (2003a) when using 8 or 14 microsatellite markers respectively. The mean observed heterozygosity (0.523) is very similar compared to that found by Gautschi *et al*. (2003a) (0.520) also with 8 microsatellites. With the mean expected Hardy−Weinberg heterozygosity (0.585), our data is higher than that in the previous study (Gautschi *et al*. 2003a). Our results show a higher variability and it suggests that there has not been a bottleneck effect.

The inbreeding coefficient (F_{IS}) for the studied population is 0.10911 and Gautschi *et al*. (2003a) presented F_{IS}=0.037. It has been an increased F_{IS} value, but it is not big enough to cause an inbreeding depression of the studied population at present.

The results we obtained describe the present genetic situation of the Pyrenean bearded vultures. The previous authors used samples from museums and different collections and a few present samples of Pyrenean bearded vulture (Gautschi *et al*. 2003a; Godoy *et al*. 2004). The differences obtained between our results and the previous results can be due to two reasons: the Pyrenean bearded vulture population has changed from the genetic point of view and more number of animals have been analysed in this study. Concerning the present Pyrenean population, our results show the genetic tendency of this species.

Maintenance of genetic diversity has to be a priority goal for genetic management strategies in long-term conservation programmes of the present Pyrenean bearded vulture. The results presented in this article are of great interest and can be included in the proposal of the plans to conserve and preserve this emblematic species in Europe.

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