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Population fragmentation leads to spatial and temporal genetic structure in the endangered Spanish imperial eagle

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Abstract

The fragmentation of a population may have important consequences for population genetic diversity and structure due to the effects of genetic drift and reduced gene flow. We studied the genetic consequences of the fragmentation of the Spanish imperial eagle (*Aquila adalberti*) population into small patches through a temporal analysis. Thirty-four museum individuals representing the population predating the fragmentation were analysed for a 345-bp segment of the mitochondrial control region and a set of 10 nuclear microsatellite loci. Data from a previous study on the current population (*N* = 79) were re-analysed for this subset of 10 microsatellite markers and results compared to those obtained from the historical sample. Three shared mitochondrial haplotypes were found in both populations, although fluctuations in haplotype frequencies and the occurrence of a fourth haplotype in the historical population resulted in lower current levels of haplotype and nucleotide diversity. However, microsatellite markers revealed undiminished levels of nuclear diversity. No evidence for genetic structure was observed for the historical Spanish imperial eagle population, suggesting that the current pattern of structure is the direct consequence of population fragmentation. Temporal fluctuations in mitochondrial and microsatellite allelic frequencies were found between the historical and the current population as well as for each pairwise comparison between historical and current Centro and historical and current Parque Nacional de Doñana nuclei. Our results indicate an ancestral panmictic situation for the species that management policies should aim to restore. A historical analysis like the one taken here provides the baseline upon which the relative role of recent drift in shaping current genetic patterns in endangered species can be evaluated and this knowledge is used to guide conservation actions.

Keywords: ancient DNA, genetic diversity, genetic drift, genetic structure, population fragmentation, Spanish imperial eagle

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Introduction

One fundamental concern in biodiversity conservation is that population fragmentation might seriously compromise the long-term survival of many wild species. Concomitant genetic effects of population decline due to drift can act synergistically with environmental factors to precipitate population extinction (Gilpin & Soulé 1986; Brook *et al*. 2002). Population genetics theory predicts that, following fragmentation, a panmictic population will become genetically structured, due to reduced effective population sizes (N_e) in, and reduced gene flow among, the resulting fragments, potentially leading to reduction of genetic diversity and accumulation of inbreeding. The intensity of these processes will vary among the resulting fragments depending on their effective population sizes and their immigration rate from other fragments.

Many studies have tried to elucidate the effects of fragmentation on genetic diversity, but only a fraction of them have also investigated its effect in the genetic structure. For this latter purpose, three different approaches have been used: (i) model-based methods (e.g. O'Ryan *et al*. 1998; Ciofi *et al*. 1999); (ii) comparisons of the degree of

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differentiation in fragmented vs. continuous populations — spatial approach (e.g. Mossman & Waser 2001; Caizergues *et al*. 2003; Keller & Largiadèr 2003; Vandergast *et al*. 2004); and (iii) comparison of the population before and after the fragmentation event — temporal approach (e.g. Dayanandan *et al*. 1999; Pertoldi *et al*. 2001). These kinds of temporal studies are scarce, as they rely on the availability of genetic material pre-dating fragmentation or decline (e.g. museum specimens, subfossil and permafrost samples). Moreover, being based on old specimens collections, sampling cannot be optimally designed, is limited in size, and critical information like location and date of sampling might be missing.

Nonetheless, only the analysis of the population pre-dating the perturbation would unambiguously reveal the baseline against which to evaluate the current genetic status of the species. Not only must the remnant genetic diversity be carefully investigated, but also how it is geographically distributed with respect to the recent past in order to evaluate the relative contribution of the evolutionary forces of genetic drift and selection in shaping the present genetic structure of the population. Otherwise, the current pattern of genetic structure in populations that are immersed in a process of decline could be erroneously assigned unwarranted evolutionary significance.

With only 185 breeding pairs counted in the 2003 survey (Spanish imperial eagle working group), the Spanish imperial eagle, *Aquila adalberti*, is listed under the Appendix of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES) and considered 'Endangered' by the IUCN (BirdLife International 2004). Although there is no precise estimation of the size of the historical population by the end of the 19th century, the species was considered abundant in the Iberian Peninsula (González *et al*. 1989 and references therein). During the 20th century, for six to seven generations (Negro & Hiraldo 1993), and mainly due to anthropic pressures, many breeding areas were lost and the population was fragmented into several breeding nuclei distributed in the southwestern quadrant of the Iberian peninsula (González 1991). Furthermore, since the 1950s, myxomatosis and viral haemorrhagic disease have decimated rabbit populations (Villafuerte *et al*. 1995), which is considered Spanish imperial eagle's main prey (Ferrer 2001). Electrocution in power lines and poisoning further pushed the species to the brink of extinction in the last decades (Ferrer & Hiraldo 1992; Ferrer 2001). Considered philopatric (Calderón *et al*. 1988; González 1991; Ferrer 1993), the Spanish imperial eagle juveniles settle temporarily in one or several locations from where they periodically return to their natal place (138.61 km mean dispersal distance) (Ferrer 2001). However, long-effective migrations up to 300 km have been reported from field observations (Monitoring Group of Natural Processes of the Doñana Biological Station databases) and genetic data reflect this process (Martínez-Cruz *et al*. 2004). The population has been shown to be genetically structured, with the most geographically isolated nucleus of Parque Nacional de Doñana differentiated from, and with lower levels of genetic diversity than, others (Martínez-Cruz *et al*. 2004). In comparison with its sister species, the Eastern imperial eagle (*Aquila heliaca*), the Spanish imperial eagle shows lower levels of diversity in the mitochondrial genome, although similar diversity levels for 18 microsatellites studied (Martínez-Cruz *et al*. 2004). Based upon indirect evidences on the recent demography of the species, we hypothesized that the current level of structure could have been the direct consequence of fragmentation of the population (Martínez-Cruz *et al*. 2004).

Here we investigate, through a temporal approach, the genetic consequences of population fragmentation for the Spanish imperial eagle. We applied mitochondrial and microsatellite markers to an almost exhaustive sampling of museum specimens pre-dating the decline, and covering the most distant nuclei of the current distribution. Our aim was to test two main hypotheses: first, whether the genetic diversity of the species has been reduced following the decline; second, whether the current pattern of structure is the consequence of the recent fragmentation or rather a reflection of a historical equilibrium situation. Despite the reduced sample sizes imposed by the limited availability and incomplete identification of museum specimens, and the low quality of DNA obtained, our data suggest the emergence of structure following fragmentation and decline and illustrate the potential of museum collections in conservation genetics.

Materials and methods

Samples

Fifty-one museum skins from individuals captured prior to 1904 (Aad1 population) were sampled either from footpad, feather tip plus a little piece of surrounding skin, or the clot from the superior umbilicus (Horvàth *et al.* 2005). These individuals represent almost all the naturalized individuals from the last half of the 19th century currently available in museum collections all over the world. Only 34 samples yielded enough DNA for the mitochondrial DNA study, whereas 32 samples could be used in the microsatellite study (see below) (13 from Doñana National Park (PND1), 8 from Centro (CE1), and no precise location could be assigned to the remaining ones) (see Appendix for a complete description of the samples). These historical samples were collected over a time span of 40 years (around three generations). In pooling these samples, we are assuming a temporal stability of the genetic composition of the species in this time span, a period in which the species was considered abundant in the peninsula (González *et al.* 1989).

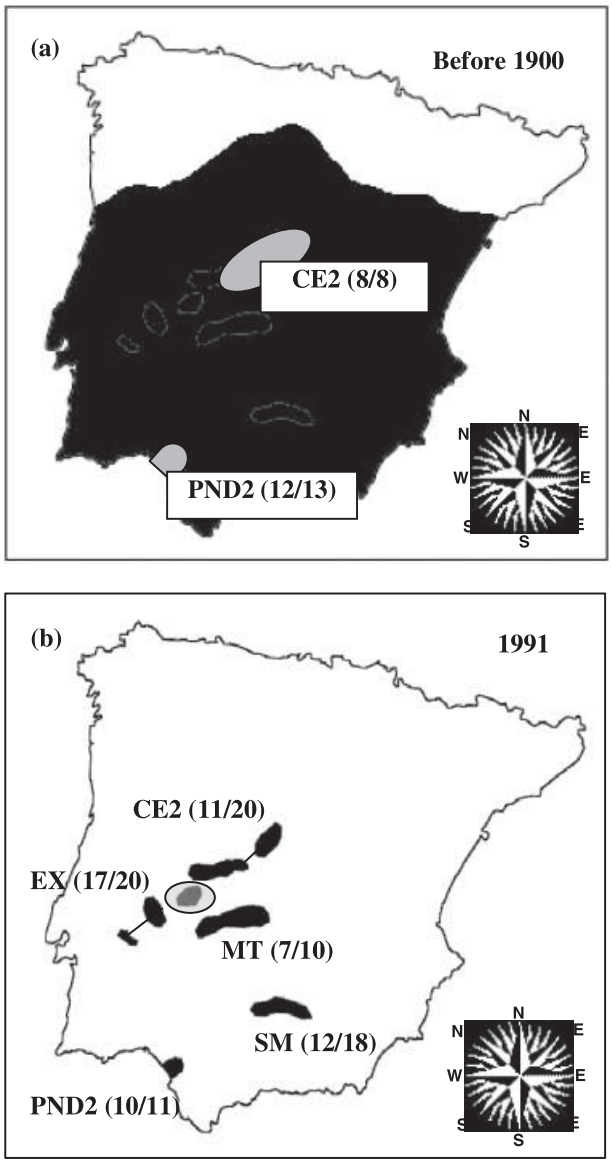

Fig. 1 Historical (after González *et al*. 1989) (a) and 1990s (b) distributions of the Spanish imperial eagle samples in the Iberian peninsula (redrawn from Ferrer 2001). Numbers between brackets represent individuals sampled in each nucleus. The first figure indicates the number of samples included in the mtDNA study and the second one the number included in the microsatellite study. In grey is the Tiétar valley nucleus, from where no samples were obtained. Bars connect subgroups ascribed to the same breeding *nucleus*. CE1, historical Centro; PND1, historical Parque Nacional de Doñana; CE2, current Centro; EX, Extremadura; MT, Montes de Toledo; PND2, current Parque Nacional de Doñana; SM, Sierra Morena.

Data from a previous study, comprising an extensive sampling of the current population, subdivided in five breeding nuclei (Martínez-Cruz *et al*. 2004; see Fig. 1), were re-analysed for the subset of 10 microsatellite loci used in this study. Historical PND1 and CE1 populations are geographically coincident with current PND2 and CE2 populations, respectively, as described in Martinez-Cruz and collaborators (2004) (see Fig. 1).

DNA extraction and amplification

Feather/skin samples were first washed extensively in excess NTE (0.05 M Tris-HCl, 0.01 M NaCl, 0.02 M EDTA, pH 9.0; Goetz *et al*. 1985) and GTE (0.1 M glycine, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA) (Shedlock *et al*. 1997) to remove enzyme inhibitors (Hall *et al*. 1997). Total DNA was extracted using the DNA Tissue Kit (QIAGEN) following the manufacturer's instructions. DNA was resuspended in a final volume of 120 μL of elution buffer and stored at –20 °C.

Extractions were carried out in a separate room specially conditioned for working with ancient and noninvasive material, which was UV irradiated when not in use. During all the extractions, the researcher wore gloves, mask and blouse to avoid contamination. All the manipulations were made in a laminar-flow hood that remained UV irradiated when not in use. We performed a negative extraction control and a negative polymerase chain reaction (PCR) control for every extraction and amplification to monitor for contamination. DNA concentration was measured fluorimetrically with a Hoefer-Dyna Quant 200 Fluorimeter, using Hoetsch-33258 dye.

A total 345-bp segment of the hypervariable Domain I of the mitochondrial control region was amplified in two partially overlapping fragments as follows: (i) a 210-bp fragment using primers AID1 (Martínez-Cruz *et al*. 2004) and AIR (Horvàth *et al*. 2005) (ii) a 230-bp fragment using primers AIF (5′-ATACGGGCATGCATCTCTTT-3′) and Fbox (Godoy *et al*. 2004). PCR amplifications were performed in a final volume of 25 μL containing 16 mM $(NH_4)SO_4$, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.16 μg/μL BSA, 0.5 U *Taq* DNA polymerase (Bioline), 0.25 μM of primers and 3 μL DNA, under the following conditions: an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of 2 min at 94 °C, 30 s at 64°, and 1 min at 72°, and a final extension step of 72 °C for 5 min. Ten microsatellite *loci* specifically developed for the Spanish imperial eagle (GenBank Accession nos AF469499, AF469501–AF469505, AF469507, AF469509, AF469510 and AF469512) were amplified under the same conditions as in Martínez-Cruz & Collaborators (2002). All amplifications were carried out in an MJ Research PTC-100 thermocycler.

Sequencing, genotyping and data analysis

Sequencing reactions were carried out using the ABI PRISM BigDye TM v2.0 chemistry (Applied Biosystems); the reaction products were purified with Sephadex G-50 Fine (Amersham Pharmacia Biotech) and analysed in an ABI PRISM 310 Genetic Analyser (Applied Biosystems).

Chromatograms were generated with SEQUENCE ANALYSIS and SEQUENCE EDITION and alignments were performed with SEQUENCHER 4.1 (Gene Codes Corporation). One of the haplotypes was found only once and it was quite divergent from the other haplotypes (HapO, GeneBank Accession no. DQ834904). This haplotype was consistently recovered in three independent extractions and showed no ambiguities in the chromatograms, indicating that it was not an artefact or a nuclear insertion.

For the 19 out of 51 samples for which DNA concentration was in the order of nanograms, we performed a unique genotyping for the set of 10 microsatellites. For the remaining 32 samples, preliminary amplifications were carried out in order to test its reliability for genotyping as follows. Three PCRs per sample were performed for each of three selected microsatellite loci (Aa36, Aa39 and Aa49). Thirteen samples out of those 32 rendered good amplifications, and were further genotyped following the multitube approach proposed by Taberlet & Waits (1996), and the 19 that failed were then eliminated from the analysis. We ended up with a total of 32 historical samples analysed. In this multitube experiment, two allelic dropouts were observed out of 636 genotypes (two different amplifications of the same locus in the same individual). Fluorescently labelled microsatellite amplification products were analysed in an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Alleles were assigned using GENOTYPER 2.5 (Applied Biosystems).

Genetic diversity mtDNA. The observed number of haplotypes (N_H), haplotype diversity (H_D) and nucleotide diversity (π) were calculated using DNASP software (Rozas & Rozas 1999). Levels of genetic diversity were compared using the Welch approximate *t*-test to account for the possible difference in variance among samples (Hoelzel 1999).

Microsatellites. Deviations from Hardy–Weinberg equilibrium (HWE), heterozygote deficits and linkage equilibrium were tested with GENEPOP 3.1 [(update of version 2.1 described in Raymond & Rousset (1995)] using a Markov chain method to estimate without bias the exact *P* value in these tests (Guo & Thompson 1992).

Genetic diversity, measured as the number of alleles per locus (*k*), allelic richness (A_R) (El Mousadik & Petit 1996) were calculated with FSTAT (Goudet 1995) and the observed and expected heterozygosities (H_O and H_E) with GENETIX (Belkhir *et al*. 1996–2001). Differences in A_R and H_E between groups were tested with Wilcoxon signed-ranked test and paired *t*-tests, respectively, including locus as a block factor.

Genetic structure

F_{ST} and exact tests of differentiation for the set of 79 current samples as well as between historical Centro and Parque Nacional de Doñana were performed and tested for significance under 1000 permutations with the program ARLEQUIN (Schneider *et al*. 2000) for mtDNA data and with the programs GENETIX (F_{ST}) (Belkhir *et al*. 1996–2001) and FSTAT (exact test) (Goudet 1995) for microsatellite data. In the latter case, 95% confidence intervals were estimated by bootstrapping over loci with FSTAT. In order to detect a possible type II error due to small sample sizes, we randomly drew with replacement a number of microsatellite genotypes from each of the two current Doñana (PND2) and Centro (CE2) populations ($F_{ST} = 0.12$, $P < 0.001$) equal to the number of sampled museum individuals. The procedure was repeated 5000 times and we drew the frequency distribution of pairwise F_{ST} values obtained for the randomized datasets using GENETIX. F_{ST} value between historical Centro and Parque Nacional de Doñana was then contrasted against this distribution. The level of differentiation between CE1 and PND1 was further compared with the one observed between CE2 and PND2 by testing the significance of the difference in F_{ST} values with a Wilcoxon signed-ranked test.

Median-joining networks for historical and current populations were estimated for mtDNA using the software NETWORK version 3.1.1.1 (http://www.fluxus-eingeneering.com/sharenet.htm) assigning equal weights to all variable sites (Bandelt *et al*. 1999).

Temporal fluctuations in allelic frequencies

Fluctuations in haplotype frequencies for mtDNA among the historical and the present population were performed and tested for significance under 1000 permutations with the program ARLEQUIN (Scheider *et al*. 2000). Fluctuations in allelic frequencies were evaluated through the estimation of F_{ST} (Weir & Cockerham 1984), testing for significance under 10 000 permutations, and exact tests of differentiation (Raymond & Rousset 1995) with the programs GENETIX (Belkhir *et al*. 1996–2001) and FSTAT, respectively. Temporal comparisons for Doñana and Centro, the two nuclei for which historical genetic population data was available were also performed (PND1 vs. PND2; CE1 vs. CE2).

Results

Genetic diversity

Mitochondrial DNA. Haplotype and nucleotide diversity in the historical population were 0.322 (± 0.073) and 0.001 (± 0.0002). Four different haplotypes were found in the historical population, and three of them were those found in the current population (Martínez-Cruz *et al*. 2004) (Fig. 2). Their proportions in the historical sample were: HapA, 61.8%; HapB, 32.4%; HapC, 2.9%. The fourth haplotype, HapO, was present in only one individual representing the 2.9% of the sample and differed in five or six sites from the other three haplotypes. The results obtained evidenced

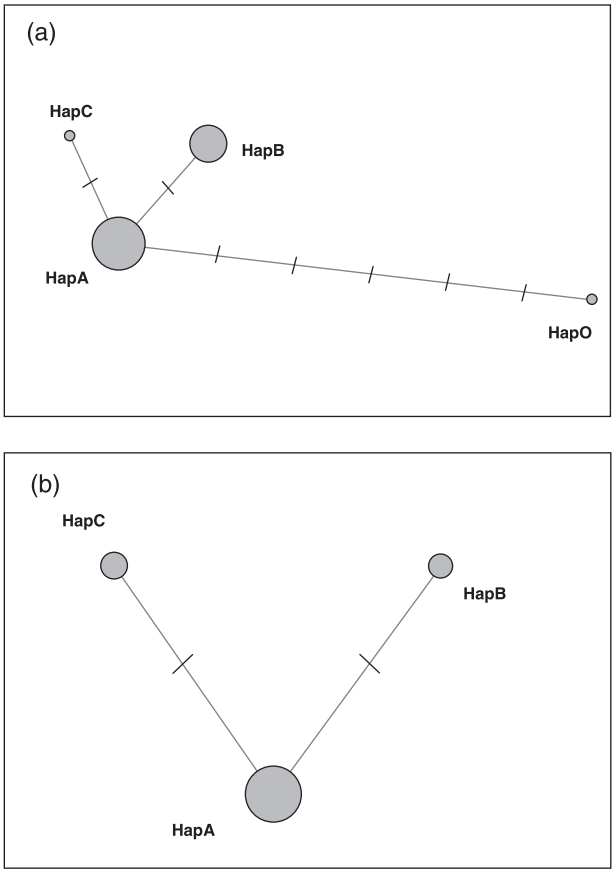

Fig. 2 Median-joining networks of historical (a) and contemporary (b) Spanish imperial eagle haplotypes. The size of the node indicates the relative frequency of the corresponding haplotype in each data set. Bars indicate nucleotide differences.

a change in the frequencies of shared haplotypes with respect to the current situation (82.3%, 9.7% and 8.0% for HapA, HapB and HapC, respectively, Martínez-Cruz *et al*. 2004). Due to a more even distribution of the frequencies of these three haplotypes and the occurrence of the fourth, both H_D and π were significantly higher in the historical population (Aad1) than in the current population (Aad2) (Welch's approximate *t*-test, $t' = 14$, $P < 0.001$; $t' = 9$, $P < 0.001$, respectively). When the individual with HapO was omitted, both H_D and π were still higher in the historical population (Welch's approximate *t*-test, $t' = 12$, $P < 0.01$; $t' = 10$, $P < 0.01$, respectively).

Microsatellites. No global deviation from HWE was found at any locus in the historical sample ($P > 0.05$). Significant linkage disequilibrium was detected between Aa36 and Aa53 loci in the historical sample. Since no linkage disequilibrium for this pair of loci was detected in the present Spanish imperial eagle population (Martínez-Cruz *et al*. 2004), we consider unlikely that this pair of loci is physically linked. For all the analyses below, we have considered the full set of 10 markers as unlinked. A_R and H_E and H_O values for historical and current populations are summarized in Table 1. Neither global A_R nor H_E were significantly different in historical and current populations (Aad1 vs. Aad2; $\chi^2 = 0.14$, d.f. = 1, $P = 0.71$ Wilcoxon signed-ranked test, and $t = 1.12$, d.f. = 18, $P = 0.14$ paired *t*-test, respectively), for historical and current Parque Nacional de Doñana nuclei (PND1 vs. PND2; $\chi^2 = 0.82$, d.f. = 1, $P = 0.36$ Wilcoxon signed rank test, and $t = 1.55$, d.f. = 18, $P = 0.14$ paired *t*-test, respectively), and for historical and current Centro nuclei (CE1 vs. CE2; $\chi^2 = 0.12$, d.f. = 1, $P = 0.73$, and $\chi^2 = 0.69$, d.f. = 1, $P = 0.41$, Wilcoxon signed-ranked test, respectively). No significant differences were found either between historical Centro and Parque Nacional de Doñana nuclei (CE1 vs. PND1 $\chi^2 = 0.97$, d.f. = 1, $P = 0.33$ Wilcoxon signed-ranked test, and $t = -0.89$, d.f. = 18, $P = 0.39$ paired *t*-test, respectively).

Table 1 Microsatellite diversity in historic and current Spanish imperial eagle (Aad1 and Aad2, respectively). H_E and H_O expected and observed heterozygosities, respectively

	Allelic richness		Number of alleles		H_E		H_O	
Locus	Aad1	Aad2	Aad1	Aad2	Aad1	Aad2	Aad1	Aad2
Aa36	3.5	4.2	4	5	0.674	0.695	0.656	0.658
Aa39	8.2	7.3	10	10	0.806	0.755	0.714	0.740
Aa50	3.0	3.8	3	4	0.584	0.617	0.483	0.595
Aa53	3.5	3.9	4	4	0.656	0.648	0.700	0.608
Aa15	2.9	2.6	3	3	0.545	0.364	0.379	0.354
Aa35	6.0	5.5	6	9	0.694	0.485	0.438	0.456
Aa26	4.7	5.1	6	7	0.567	0.637	0.483	0.544
Aa27	3.3	2.8	4	3	0.476	0.500	0.469	0.519
Aa41	3.0	3.4	3	4	0.662	0.618	0.656	0.582
Aa49	4.8	4.7	6	6	0.610	0.711	0.656	0.646
Overall	4.3	4.3	49	55	0.627	0.603	0.563	0.570

Re-analysis of structure in the current population

Microsatellites. The re-analysis of the current population for the subset of 10 microsatellite markers used in this study showed the same overall pattern observed with the whole marker set (Martínez-Cruz *et al*. 2004). Only in the case of SM and CE2 the exact test became nonsignificant when the reduced set was used, which can be due to the loss of power due to the decrease in the number of markers used (data not shown).

Structure in the historical population

We evaluated the level of historical differentiation using the 21 historical samples that could be unambiguously

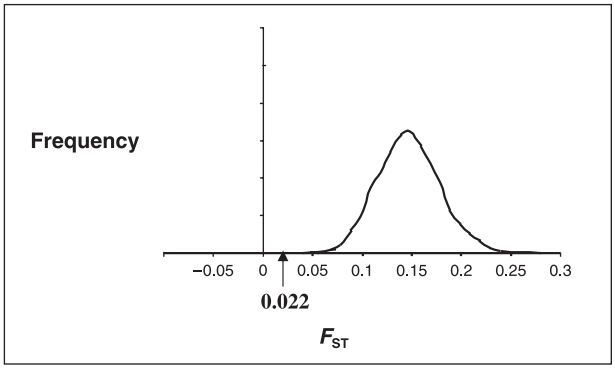

Fig. 3 F_{ST} frequency distribution resulting from randomizations of the contemporary samples (CE2, PND2) to the size of the historical (CE1, PND1) under the hypothesis of genetic substructuring. The F_{ST} value observed between CE1 and PND1 (F_{ST} = 0.022, P = 0.19) is indicated with an arrow.

assigned to Centro (n = 8) or to Parque Nacional de Doñana (n = 13). Our power to detect genetic structure with mtDNA data was hampered by its low polymorphism, more so in the contemporary population. Thus, although F_{ST} value estimated for the comparison of historical CE1 and PND1 was low and not significant (F_{ST} = −0.07; P = 0.80), these same populations now share a single haplotype rendering the comparison meaningless. For microsatellite data, F_{ST} and exact test of differentiation indicated no genetic differentiation between these two groups (CE1 vs. PND1; F_{ST} = 0.022, 95% CI = −0.018–0.068, P = 0.19; exact test P = 0.13), whereas the corresponding contemporary comparison showed higher and significant values (CE2 vs. PND2; F_{ST} = 0.12, 95% CI = 0.051–0.190, P < 0.001; exact test P < 0001). Furthermore, the observed historical F_{ST} value fell outside the distribution of F_{ST} obtained by resampling the current populations to the sizes of the historical samples (mean F_{ST} = 0.15, 95% CI = 0.09–0.21; Fig. 3). Furthermore, F_{ST} values for the historical Centro and Doñana populations were significantly lower than current values consistently across loci (χ^2 = 8.69, d.f. = 1, P = 0.003 Wilcoxon signed-ranked test). These results indicate that the current level of structure is not a historical pattern and that the observed difference in structure between the two periods is not solely due to lack of power because of smaller sample sizes in the historical populations.

Temporal fluctuations in allelic frequencies

The intensity of drift between the two periods was assessed by quantifying the fluctuations in allelic or haplotypic frequencies with F_{ST} statistics. F_{ST} between Aad1 and Aad2 populations was low but still significant for mitochondrial DNA (F_{ST} = 0.079, P = 0.02) and nuclear microsatellite markers (F_{ST} = 0.032, P < 0.001; exact test P < 0.001). For mtDNA, this differentiation was mainly due to the changes in the haplotypic frequencies of the three coincident haplotypes, since the F_{ST} remained similar (F_{ST} = 0.077, P = 0.02) when the individual carrying the rare haplotype HpO was excluded from the analysis.

Current and historical Centro were significantly differentiated for microsatellites (CE2 vs. CE1; F_{ST} = 0.042, P < 0.01, exact test P = 0.02), but not for mitochondrial DNA (F_{ST} = 0.20, P = 0.14), whereas current and historical Parque Nacional de Doñana were significantly differentiated for both microsatellite (PND2 vs. PND1; F_{ST} = 0.107, P < 0.01, exact test P = 0.01) and mitochondrial DNA (F_{ST} = 0.20, P = 0.03) markers.

Discussion

The use of museum samples allowed us to directly assess the changes in genetic compositions suffered by the Spanish imperial eagle during a period of decline and fragmentation. The power of the analysis might have been hampered to some extent by small sample sizes and the necessary pooling of historical samples across generations, hard to overcome limitations given our seemingly exhaustive sampling of collections. Nevertheless, results showed robust to the sample size problem and important allelic frequency fluctuations across generations are unlikely in the historical period analysed when the species was abundant and widely distributed.

Mitochondrial diversity in the Spanish imperial eagle has been reduced by the recent decline, due mostly to the fluctuation of haplotype frequencies resulting in a more skewed distribution and perhaps to the disappearance of haplotypes occurring historically at low frequencies. Nevertheless, it was already low by the end of the 19th century when compared to its sister species, the Eastern imperial eagle (Martínez-Cruz *et al*. 2004). Meanwhile, diversity at the nuclear genome has remained constant during this period at levels similar to its sister species. Such disparity between mitochondrial and nuclear levels of diversity may be caused by the four times lower N_e of the mitochondrial genome that makes it more susceptible to genetic erosion or the consequence of ancestral evolutionary forces (i.e. selective sweeps, founder effects, etc.) having acted preferentially on the mitochondrial genome. Thus, the observed pattern suggests an ancestral event affecting only the mitochondrial genome.

Six to seven generations of population fragmentation and demographic decline (Negro & Hiraldo 1993) have resulted in fluctuations in allelic frequencies and the emergence of spatial genetic structure from an apparently less structured historical pattern. This increase in genetic structure has occurred with no concomitant loss of genetic diversity, except in the extreme case of PND — situated 300 km apart from any other nucleus, with 14 breeding territories (Ferrer 2001), and having suffered intense demographic fluctuations in the last decades (Ferrer *et al*. 2003) — where both processes

have already occurred (Martínez-Cruz *et al*. 2004). Our results go in the same direction as those of Keyghobadi & collaborators (2005) and suggest that fluctuations in allelic frequencies may be taken as an early warning signal of genetic endangerment. The continued effect of drift and isolation could eventually lead to loss of genetic variability and the accumulation of inbreeding and to a consequent increase of the probabilities of extinction of local populations (Gilpin & Soulé 1986).

Considering that conservation genetic studies focus especially on species involved in a declining process, a contemporary pattern of genetic structure present in those populations may be the direct consequence of anthropogenic perturbation and thus have no evolutionary significance. Distinguishing a recently derived genetic structure from one of equilibrium is crucial to design an effective management strategy, which should in the former case aim to revert the population to its historical panmictic condition through habitat restoration, translocation or other appropriate methods. In the case of the Spanish imperial eagle, the study of the historical population has revealed recent drift as the main process shaping current genetic structure. This highlights the necessity of increasing the connection among nuclei — particularly in the case of PND — and the recognition of the whole population as a single evolutionary unit and its consequent management as a whole. The present study illustrates the potential of a temporal approach and the value of museum collections in conservation genetics.

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References

Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37–48.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (1996–2001) GENETIX *4.02: Logiciel sous Windows TM pour la génétique des populations*. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier, France.

BirdLife International (2004) *Threatened Birds of the World 2004*. CD-ROM. BirdLife International, Cambridge, UK.

Brook BW, Tonkyn DW, O'Grady JJ, Frankham R (2002) Contribution of inbreeding to extinction risk in threatened species. *Conservation Ecology*, **6**, 16.

Caizergues A, Rätti O, Helle P, Rotelli L, Ellison L, Rasplus J-Y (2003) Population genetic structure of male black grouse (*Tetrao tetrix* L.) in fragmented versus continuous landscapes. *Molecular Ecology*, **12**, 2297–2305. (DOI: 10.1046/j.1365-294X.2003.01903.x).

Calderón J, Castroviejo J, García L, Ferrer M (1988) El águila imperial (*Aquila adalberti*): dispersión de los jóvenes, estructura de edades y mortalidad. *Doñana Acta Vertebrata*, **15**, 79–98.

Ciofi C, Beaumont MA, Swingland IR, Bruford MW (1999) Genetic divergence and units for conservation in the Komodo dragon Varanus komodoensis. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **266**, 2269–2274. (DOI: 10.1098/rspb 1999.0918).

Dayanandan S, Dole J, Bawa K, Kesseli (1999) Population structure delineated with microsatellite markers in fragmented populations of a tropical tree, *Carapa guianensis* (Meliaceae). *Molecular Ecology*, **8**, 1585–1592. (DOI: 10.1046/j.1365-294X.1999.00735.x).

El Mousadik A, Petit RJ (1996) High level of genetic differentiation for allelic richness among populations of the argan tree *Argania spinosa* (L.) Skeels endemic to Morocco. *Theoretical Applications in Genetics*, **92**, 832–839.

Ferrer M (1993) Juvenile dispersal behaviour and natal philopatry of a long-lived raptor, the Spanish imperial eagle *Aquila adalberti*. *Ibis*, **135**, 132–138.

Ferrer M (2001) *The Spanish Imperial Eagle*. Lynx Editions, Barcelona.

Ferrer M, Hiraldo F (1992) Man-induced sex-biased mortality in the Spanish imperial eagle. *Biological Conservation*, **60**, 57–70.

Ferrer M, Penteriani V, Balbontín J, Pandolfi M (2003) The proportion of immature breeders as a reliable early warning signal of population decline: evidence from the Spanish imperial eagle in *Doñana*. *Biological Conservation*, **114**, 463–466 (DOI: 10.1016/S0006-3207(03)00085-5).

Gilpin ME, Soulé ME (1986) Minimum viable populations: processes of species extinction. In: *Conservation Biology: The Science of Scarcity and Diversity* (ed. Soulé ME), pp. 19–34. Sinauer, Sunderland, Massachusetts.

Godoy JA, Negro JJ, Hiraldo F, Donazar JA (2004) Phylogeography, genetic structure and diversity in the bearded vulture (*Gypaetus barbatus*, L.), as revealed by mitochondrial DNA. *Molecular Ecology*, **13**, 371–390.

Goetz SE, Hamilton SR, Vogelstein B (1985) Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochemical and Biophysical Research Communications*, **130**, 118–126.

González LM (1991) *Historia Natural del Aguila Imperial Ibérica (Aquila adalberti Brehm, 1861): Taxonomía, Población, Análisis de la Distribución Geográfica, Alimentación, Reeproducción y Conservación*. Instituto Nacional para la Conservación de la Naturaleza, Madrid.

González LM, Hiraldo F, Delibes M, Calderón J (1989) Reduction in the range of the Spanish Imperial Eagle (*Aquila adalberti* Brehm, 1861) since AD 1850. *Journal of Biogeography*, **16**, 305–315.

Goudet J (1995) FSTAT version 1.2: a computer program to calculate *F*-statistics. *Journal of Heredity*, **86**, 485–486.

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics*, **48**, 359.

Hall LM, Wilcox MS, Jones DS (1997) Association of enzyme inhibition with methods of museum skin press. *BioTechniques*, **22**, 928–930.

Hoelzel AR (1999) Impact of population bottlenecks on genetic variation and the importance of life-history, a case study of the northern elephant seal. *Biological Journal of the Linnean Society*, **68**, 23–39.

Horvàth M, Martínez-Cruz B, Negro JJ, Kálmar L, Godoy JA (2005) An overlooked DNA for non-invasive genetic analysis in birds. *Journal of Avian Biology*, **36**, 84–88. (DOI: 10.1111/j.0908-8857.2005.03370.x).

Keller I, Largiadèr C (2003) Recent habitat fragmentation caused by major roads leads to reduction of gene flow and loss of genetic variability in ground beetles. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **270**, 417–423. (DOI: 10.1098/rspb.2002.2247).

Keyghobadi N, Roland J, Matter SJ, Strobeck C (2005) Among- and within-patch components of genetic diversity respond at different rates to habitat fragmentation: an empirical demonstration. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **272**, 553–560. (DOI: 10.1098/rspb.2004.2976).

Martínez-Cruz B, David VA, Godoy JA *et al.* (2002) Eighteen polymorphic microsatellite markers for the highly endangered Spanish imperial eagle (*Aquila adalberti*) and related species. *Molecular Ecology Notes*, **2**, 323–326.

Martínez-Cruz B, Godoy JA, Negro JJ (2004) Population genetics after fragmentation: the case of the endangered Spanish imperial eagle (*Aquila adalberti*). *Molecular Ecology*, **13**, 2243–2255. (DOI: 10.1111/j.1365-294X.2004.02220.x).

Mossman CA, Waser PM (2001) Effects of habitat fragmentation on population genetic structure in the white-footed mouse (*Peromyscus leucopus*). *Canadian Journal of Zoology*, **79**, 285–295. (DOI: 10.1139-cjz-79-2-285).

Negro JJ, Hiraldo F (1993) Lack of allozyme variation in the Spanish imperial eagle *Aquila adalberti*. *Ibis*, **136**, 87–90.

O'Ryan C, Harley EH, Bruford MW, Beaumont M, Wayne RK, Cherry MI (1998) Microsatellite analysis of genetic diversity in fragmented South African buffalo populations. *Animal Conservation*, **1**, 85–94.

Pertoldi C, Moller Hansen M, Loeschcke V, Bo Madsen A, Jacobsen L, Baagoe H (2001) Genetic consequences of population decline in the European otter (*Lutra lutra*): an assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **268**, 1775–1781. (DOI: 10.1098/rspb.2001.1762).

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozas J, Rozas R (1999) DNASP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN version 2000: A software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Shedlock AM, Haygood GM, Pietsch TW, Bentzen P (1997) Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens. *BioTechniques*, **22**, 394–400.

Taberlet P, Waits LP (1996) Non-invasive genetic sampling. *Trends in Ecology & Evolution*, **13**.

Vandergast AG, Gillespie RG, Roderick GK (2004) Influence of volcanic activity on the population genetic structure of Hawaiian Tetragnatha spiders: fragmentation, rapid population growth and the potential for accelerated evolution. *Molecular Ecology*, **13**, 1729–1743. (DOI: 10.1111/j.1365-294X.2004.02179.x).

Villafuerte R, Calvete C, Blanco JC, Lucientes J (1995) Incidence of viral hemorrhagic disease in wild rabbit populations in Spain. *Mammalia*, **59**, 651–659.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Begoña Martinez-Cruz has developed her PhD thesis on the conservation genetics of the Spanish imperial eagle. Currently she is developing her research on the population genetics of Central Asian human populations. She is interested in the conservation genetics of endangered species and in the study of the evolutionary forces shaping the genetic diversity of populations. José A. Godoy is a geneticist involved in various studies of conservation genetics and molecular ecology of raptors, carnivores and plants. Juan J. Negro is interested in the genetics of small populations of birds, including variability lose, inbreeding and hybridization.

Appendix

Description of the 51 samples collected in several museums over the world

Sample code*	Sample ID	Contributor†	Locality/Area	Date	Nucleus‡	Tissue	Haplotype§	Sampler
P049*	17137	MHNN (Nantes)	Coto de Doñana	1880	PND1	Footpad	—	M.L. Guerin
P051	17139	MHNN (Nantes)	Guadalquivir marshes (Huelva)	1880	PND1	Footpad	B	M.L. Guerin
P052*	17140	MHNN (Nantes)	Coto de Doñana	1880	PND1	Footpad	—	M.L. Guerin
P057*	17145	MHNN (Nantes)	Guadalquivir marshes (Huelva)	1880	PND1	Footpad	B	M.L. Guerin
P060	17148	MHNN (Nantes)	Guadalquivir marshes (Huelva)	1876	PND1	Footpad	B	M.L. Guerin
P061	4516	SMNS (Stuttgart)	Spain	1860	—	Feather/Skin	A	F. Woog
P066*	44278	NMW (Wien)	Spain	1880	—	Feather/Skin	B	A. Gamauf
P067	1871.8.15.2	NHM (Tring)	Sevilla	1870	—	Footpad	B	M. Adams
P068*	1871.8.15.3	NHM (Tring)	Sevilla	1870	—	Footpad	A	M. Adams
P069*	1875.11.22.50	NHM (Tring)	Madre del Rocío (Huelva)	1869	PND1	Footpad	A	M. Adams
P070*	1879.4.5.316	NHM (Tring)	Madrid	1868	CE1	Footpad	B	M. Adams
P071*	1887.11.1.287	NHM (Tring)	Sevilla	1876	—	Footpad	A	M. Adams
P072*	1887.11.1.288	NHM (Tring)	Sevilla	1876	—	Footpad	A	M. Adams
P073*	1895.5.1.5	NHM (Tring)	Coto de Doñana	1879	PND1	Footpad	A	M. Adams
P074*	1905.6.28.1274	NHM (Tring)	Coto de Doñana	1872	PND1	Footpad	A	M. Adams
P075*	1934.1.1.1098	NHM (Tring)	Coto de Doñana	1898	PND1	Footpad	A	M. Adams
P077	1934.1.1.1100	NHM (Tring)	Coto de Doñana	1898	PND1	Footpad	A	M. Adams
P078*	1955.6.N.20.252	NHM (Tring)	Sevilla	1874	—	Footpad	B	M. Adams
P079	1955.6.N.20.253	NHM (Tring)	Sevilla	1869	—	Footpad	B	M. Adams
P081*	535161	AMNH (New York)	Madrid	1861	CE1	Footpad	A	P. Sweet
P082*	535162	AMNH (New York)	Madrid	1904	CE1	Footpad	B	P. Sweet
P083*	535163	AMNH (New York)	Madrid	1904	CE1	Footpad	A	P. Sweet
P084*	535165	AMNH (New York)	Málaga	1904	—	Footpad	A	P. Sweet
P085*	535166	AMNH (New York)	Sevilla	1904	—	Footpad	B	P. Sweet
P086*	535167	AMNH (New York)	Coria del Río (Sevilla)	1903	—	Footpad	A	P. Sweet
P087*	535169	AMNH (New York)	Madrid	1861	CE1	Footpad	A	P. Sweet
P088*	535159	AMNH (New York)	Spain	1860	—	Footpad	A	P. Sweet
P089	164,D	FCTUC (Coimbra)	Spain	1881	—	Feather	A	I. Carreira
P102	1956.3	NMS (Edinburgh)	Sevilla	1880	—	Feather/Skin	—	A. Kitchener
P104*	1956.3 (2182)	NMS (Edinburgh)	Coto de Doñana	1873	PND1	Feather/Skin	A	A. Kitchener
P105	1956.32175	NMS (Edinburgh)	Madrid	1865	CE1	Feather/Skin	A	A. Kitchener
P107*	1956.32178	NMS (Edinburgh)	Coto de Doñana	1872	PND1	Feather/Skin	A	A. Kitchener
P108	1956.3	NMS (Edinburgh)	Madrid	1865	CE1	Feather/Skin	A	A. Kitchener
P109	1956.3 (2176)	NMS (Edinburgh)	Andalousia	1874	—	Feather/Skin	—	A. Kitchener
P111	1956.32177	NMS (Edinburgh)	Andalousia	1874	—	Feather/Skin	—	A. Kitchener
P115*	1956.32185	NMS (Edinburgh)	Spain	—	—	Feather/Skin	—	A. Kitchener
P116	1956.32183	NMS (Edinburgh)	Madrid	1865	CE1	Feather/Skin	A	A. Kitchener
P117	1956.32184	NMS (Edinburgh)	Spain	1862	—	Feather/Skin	—	A. Kitchener
P118	1956.32181	NMS (Edinburgh)	Madrid	1862	CE1	Feather/Skin	—	A. Kitchener
P119*	1896.133.1543	NMS (Edinburgh)	Sevilla	1875	—	Feather/Skin	B	A. Kitchener
P120*	1885.4.2	NMS (Edinburgh)	Coto de Doñana	1876	PND1	Feather/Skin	C	A. Kitchener
P112	1956.3	NMS (Edinburgh)	Coto del Rey, RBD	1872	PND1	Footpad	O	A. Kitchener
P145*	2000.H1411	Hancock Museum (New Castle)	Coto de Doñana	1891	PND1	Footpad	—	L. Jessop
P147*	2000.H1031	Hancock Museum (New Castle)	Coto de Doñana	1891	PND1	Footpad	—	L. Jessop
P148*	2000.H1410	Hancock Museum (New Castle)	Coto de Doñana	—	PND1	Footpad	— —	L. Jessop
P149	2000.H1030	Hancock Museum (New Castle)	Coto de Doñana	1883	PND1	Footpad	—	L. Jessop

Appendix *Continued*

Sample code*	Sample ID	Contributor†	Locality/Area	Date	Nucleus‡	Tissue	Haplotype§	Sampler
P150	ZMB21125	Museum für Naturkunde (Berlin)	Spain	1855 –1891	—	Footpad	—	F. Steinheimer
P151	ZMB538	Museum für Naturkunde (Berlin)	Spain	Before 1854	—	Footpad	—	F. Steinheimer
P152*	7646	MNCN (Madrid)	Madrid	1853	CE1	Feather clot	—	Authors
P153*	81	MNCN (Madrid)	Madrid	1891	CE1	Feather clot	—	Authors
P154*	82	MNCN (Madrid)	El Pardo (Madrid)	1888	CE1	Feather clot	—	Authors

*Indicates samples used in the microsatellite study; †Collaborating institutions abbreviations; MHNN: Muséum d'Histoire Naturelle de Nantes, France; SMNS: Staatliches Museum für Naturkunde, Stuttgart, Germany; NMW: Naturhistorische Museum Wien, Austria NHM: Natural History Museum, Tring, England; AMNH: American Museum of Natural History, New York, USA; FCTUC: Museu Zoológico da Universidade de Coimbra, Portugal; NMS: National Museums of Scotland, Edinburgh, Scotland; MNCN: Museo Nacional de Ciencias Naturales, Madrid, Spain; ‡CE1, historical Centro; PND1, historical Parque Nacional de Doñana; — unknown locality of origin; §mtDNA study was conducted with the samples for which the haplotype is indicated.