

# Population genetics after fragmentation: the case of the endangered Spanish imperial eagle (*Aquila adalberti*)

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## Abstract

The highly endangered Spanish imperial eagle, *Aquila adalberti*, has suffered from both population decline and fragmentation during the last century. Here we describe the current genetic status of the population using an extensive sampling of its current distribution range and both mitochondrial control region sequences and nuclear microsatellite markers. Results were evaluated in comparison to those obtained for the Eastern imperial eagle, *Aquila heliaca*, its nearest extant relative. Mitochondrial haplotype diversity was lower in the Spanish than in the Eastern species whereas microsatellite allelic richness and expected heterozygosity did not differ. Both allelic richness and expected heterozygosity were lower in the small Parque Nacional de Doñana breeding nucleus compared to the remaining nuclei. A signal for a recent genetic bottleneck was not detected in the current Spanish imperial eagle population. We obtained low but significant pairwise  $F_{ST}$  values that were congruent with a model of isolation by distance.  $F_{ST}$  and exact tests showed differentiation among the peripheral and small Parque Nacional de Doñana population and the remaining breeding subgroups. The centrally located Montes de Toledo population did not differ from the surrounding Centro, Extremadura and Sierra Morena populations whereas the latter were significantly differentiated. On the other hand, a Bayesian approach identified two groups, Parque Nacional de Doñana and the rest of breeding nuclei. Recent migration rates into and from Parque Nacional de Doñana and the rest of breeding nuclei were detected by assignment methods and estimated as 2.4 and 5.7 individuals per generation, respectively, by a Bayesian approach. We discuss how management strategies should aim at the maintenance of current genetic variability levels and the avoidance of inbreeding depression through the connection of the different nuclei.

**Keywords:** bottleneck, genetic variability, migration rates, population fragmentation, population structure, Spanish imperial eagle

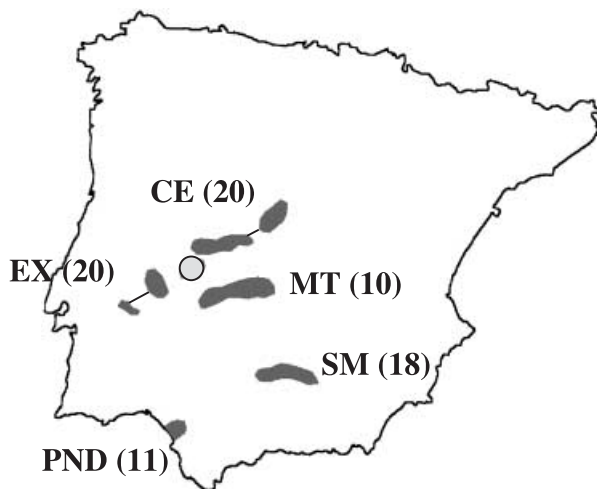
Received 24 February 2004; revision received 25 March 2004; accepted 25 March 2004

## Introduction

Demographic changes induced by anthropogenic pressure can have major genetic consequences in wild populations. A demographic scenario of bottleneck and fragmentation is expected to alter the genetic composition of a population through the effect of drift in two main ways: by reducing genetic diversity and by increasing the intrapopulation levels of inbreeding and the genetic structure among resulting subpopulations. These genetic alterations might in turn compromise the long- and short-term viability of the species,

respectively, potentially precipitating the extinction of the population through what has been called the 'extinction vortex' (Gilpin & Soulé 1986; Brook *et al.* 2002). Consequently, management strategies for endangered species must be designed to prevent the deleterious effects of inbreeding and the further loss of genetic diversity. Depending on the degree of fragmentation, the distribution of population sizes in the fragments, the distances between them, and the species dispersal behaviour, these genetic effects would be more or less severe (Frankham *et al.* 2002). Furthermore, when the two-dimensional spatial arrangement of fragments is taken into consideration, edge populations are expected to have lower population sizes with higher temporal variation with respect to core populations, and thus will

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**Fig. 1** Distribution of the Spanish imperial eagle breeding nuclei (taken from Ferrer 2001). Numbers between brackets represent individuals sampled in each nucleus. The Tiétar valley nucleus, from where no samples were obtained, is shown in grey. Bars connect subgroups ascribed to the same breeding nucleus. CE, Centro; EX, Extremadura; MT, Montes de Toledo; PND, Parque Nacional de Doñana; SM, Sierra Morena.

be subjected to more intense genetic drift (e.g. Cassel & Tammaru 2003; Vucetich & Waite 2003).

Listed in Appendix I of the CITES (Convention on International Trade of Endangered Species of Wild Fauna and Flora) and considered 'Vulnerable' by the IUCN (2003 IUCN Red List of Threatened Species), the Spanish imperial eagle (*Aquila adalberti*) is one of the most threatened birds of prey in the world (Collar & Andrews 1988). In Spain it is classified as 'Endangered' in the National Catalogue of Threatened Species (1990). The species suffered a demographic decline during the last century (González *et al.* 1989), mainly as a result of human pressure and population fragmentation (Collar & Andrews 1988). Moreover, since the 1950s, myxomatosis and viral haemorrhagic disease have decimated rabbit populations in Spain (Villafuerte *et al.* 1995), which are the main food resource for these eagles (Ferrer 2001). In the last decades, electrocution in power lines (Ferrer & Hiraldo 1992) and poisoning (Ferrer 2001) further pushed the species to the brink of extinction. Many traditional breeding areas were lost, resulting in the present patchy distribution of breeding nuclei (see Fig. 1, González *et al.* 1989) confined to the southwestern corner of Spain (Ferrer 2001). In the early 1980s, the population began to recover in response to protective measures (Ferrer 2001). In the 1990s the population stabilized at 130 pairs and in 2000 around 140 breeding pairs were estimated in the wild (Ferrer 2001).

Previous studies on the genetic variability of the Spanish imperial eagle have been undertaken, with varying results. Negro & Hiraldo (1993) showed a complete lack of genetic variability in 22 allozyme loci studied in 42 samples from

the whole distribution range, whereas Padilla *et al.* (2000) reported high levels of genetic variability in a study of 25 individuals of one Spanish imperial eagle population with random amplified polymorphic DNA (RAPD). As a result of the low variability commonly found in allozyme markers (Avice 1994) and the generally recognized limitations of RAPD genotyping (Pérez *et al.* 1998) it is obvious that more adequate markers in conjunction with extensive sampling are needed to evaluate adequately the genetic status of the species.

In this study we have applied nuclear multilocus genotypes and mitochondrial hypervariable sequences to a wide survey covering the current distribution range of the species. The present study had two main goals. First, to evaluate whether current genetic diversity could have been affected by the species decline, we compare current estimates with those found in its sister species, the Eastern imperial eagle, *Aquila heliaca*. While both species have most aspects of breeding and diet in common, the eastern imperial eagle numbers over 5000 individuals over a wide geographical range (Ferrer & Negro 2004). Multilocus genotypes are also used to test the presence of a genetic signature for a recent bottleneck in the current population. Second, we analyse the genetic structure of the species through the use of both classical distance-based and more recent model-based methods. Two models of population structure were tested and the effect of drift relative to migration was compared among nuclei. Recent migration rates were estimated to test whether reduced gene flow could explain genetic differentiation. The consequences of these results for the conservation of the species are discussed.

## Materials and methods

### Samples

Blood samples from 35- to 45-day-old Spanish imperial eagle nestlings were taken from the radial vein in Parque Nacional de Doñana and Sierra Morena during the years 2000, 2001 and 2002. Blood samples from birds in other populations were kindly contributed by different administrations and private collaborators (see Appendix 1). Sixty samples (30 eaglets, 14 adults, six juveniles and 10 birds of unknown age) were used to carry out the mitochondrial DNA study, whereas 79 samples (47 eaglets, 16 adults, three juveniles and 13 of unknown age) were selected for the microsatellite study. Monitoring of ringed birds was used to avoid pseudo-replicates (half- or full-sibs from the same or different cohorts) in most of the cases. Unringed individuals that were captured incidentally or living in captivity were assumed to be unrelated. Samples were grouped into breeding nuclei as defined by González (1991), except for the Tiétar valley from which no sample was obtained, as follows: (i) Centro, with 34 territories in

the Guadarrama and Gredos mountain ranges and the woods of holm-oaks in Madrid; (ii) Montes de Toledo, with 12 territories in the oriental Oretana mountain ranges; (iii) Parque Nacional de Doñana, with 14 territories in the Guadalquivir surroundings; (iv) Sierra Morena, with 12 territories in the mountain ranges of Jaén and Ciudad Real; (v) Extremadura, including the two neighbouring nuclei of Sierra de San Pedro, with 14 territories in the western side of the mountain range that separates Cáceres and Badajoz regions and Tajo mountain ranges, with 11 territories in the mountain ranges situated along the Tajo river edge, as no exact information on the location of origin was given in every case. In addition, to identify the immigrant parent of a mixed ancestry eaglet, six adult feathers were collected under the nest and neighbouring perching sites in the corresponding territory. As for the Eastern imperial eagle, blood samples were taken from three nonsib nestlings in Hungary in the summer of 1997, from 20 in the Naurzum National Park in the Northwest of Kazakhstan during the summer of 2000 and 11 additional samples were obtained from captive adults of unknown origin. (See Appendix I for complete information on the samples.)

#### *DNA extraction and amplification*

Total DNA from blood was extracted using a proteinase K digestion followed by a LiCl protocol (Gemmell & Akiyama 1996). DNA was stored at a final concentration of 100 ng/μL in TE buffer at -20 °C. DNA from moulted feathers was extracted from a recently discovered source that usually yields enough DNA quantity and quality to perform reliable genotyping (Horvath *et al.* in press). A 345-base-pair (bp) fragment of the hypervariable Domain I of the mitochondrial control region was amplified using primers AID1 (5'-AAGGGCCATTATTGCCAAA-3') designed specifically for this study and Fbox (5'-GGGTTGCTGRTTTCACGTGAG-3') designed for the bearded vulture (Godoy *et al.* 2004). Polymerase chain reaction (PCR) amplifications were performed in a final volume of 25 μL containing 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP, 0.5 U *Taq* DNA polymerase (Bioline), 0.25 μM of primers and 31.25 ng 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. Microsatellite amplifications were carried out as in Martínez-Cruz *et al.* (2002). All amplifications were performed in a MJ Research PTC-100 thermocycler using the same conditions for the Spanish and the Eastern imperial eagles.

#### *Sequencing and genotyping*

Spanish and Eastern imperial eagle amplicons were sequenced with primers Fbox and AID1. Sequencing reac-

tions were carried out using the ABI Big Dye TM v2.0 chemistry (Applied Biosystems), the fragments were purified with Sephadex™ G-50 Fine (Amersham Pharmacia Biotech) and analysed in an ABI 310 Genetic Analyser (Applied Biosystems). Chromatograms were generated with SEQUENCE ANALYSIS and sequence alignments were performed with SEQUENCHER™ 4.1 (Gene Codes Corporation). Mitochondrial DNA sequences were deposited in the EMBL nucleotide database under accession numbers AJ567366, AJ567367 and AJ574878–AJ574885. Fluorescently labelled microsatellite amplification products were also analysed in an ABI 310 Genetic Analyser (Applied Biosystems). Alleles were sized and assigned using GENOTYPER 2.5 (Applied Biosystems) with the commercial molecular weight marker TAMRA350 (Applied Biosystems).

#### *Data analysis*

**Mitochondrial DNA.** The haplotype diversity and nucleotide ( $\pi$ ) diversity for both species was calculated using DNASP software (Rozas & Rozas 1999). Levels of haplotype 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, 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 within groups, measured as the number of alleles per locus (*k*), the allelic richness and the observed and expected heterozygosities ( $H_O$  and  $H_E$ ) were calculated with FSTAT (Goudet 1995). Levels of allelic richness and expected heterozygosity between species were compared using a Wilcoxon sign-rank test. Differences in mean allelic richness and expected heterozygosity per locus among nuclei were tested with analysis of variance. 'Nuclei' was tested as a fixed factor and 'locus' was included in the analyses as a random factor (or 'block' factor, see Zar 1998). The inclusion of locus as a random factor allowed us to control for differences among loci in the degree of allelic richness and heterozygosity. To test our hypothesis of lower levels of allele diversity in Parque Nacional de Doñana because of its relative situation and its small size, allelic richness and expected heterozygosity in this nucleus were compared against levels in the four other nuclei through *a posteriori* contrasts. Statistical analyses were performed with JMP 5.1 (SAS Institute Inc.).

A test for heterozygote excess (BOTTLENECK, Cornuet & Luikart 1996) was used to detect recent population bottlenecks in the Spanish imperial eagle population and in the three largest nuclei: Centro, Extremadura and Sierra

Morena. The program BOTTLENECK (Cornuet & Luikart 1996) was run under the two-phase model that is supposed to fit microsatellite evolution better (di Rienzo *et al.* 1994), i.e. with 10% of the infinite allele model and 90% of the stepwise mutation model. The  $M$ -ratio test (Garza & Williamson 2001) was also calculated for the whole population and contrasted with that expected under equilibrium.  $M$ -ratio was also used to test for greater deviations among nuclei, assuming that equilibrium  $4N_e\mu$  was the same across the sampled nuclei prior to habitat fragmentation. Values for the proportion of one-step mutations ( $p_s$ ) and the average size of multistep mutations ( $\Delta_s$ ) parameters were set to 90% and 3.5, respectively, as recommended by the authors. The program assumes a mean mutation rate of  $5 \times 10^{-4}$  for the ensemble of markers. Differences in  $M$  among nuclei were tested for with a nonparametric Kruskal–Wallis analysis of variance, including locus as a block factor.

The computer program BOTTLESIMV2.6 (Kuo & Jansen 2003) was used to simulate a population bottleneck similar to that observed in Parque Nacional de Doñana. The simulation allowed us to determine whether the observed losses of genetic variation in this population were consistent with a simulated scenario of genetic drift. BOTTLESIM is a program specifically designed for simulating the genetic consequences of bottlenecks and post-bottleneck population growth for long-lived species, allowing for an overlapping-generation model. The initial conditions of the simulation were based on the current allele frequencies in the species. Assuming historical panmixia, bottlenecks were modelled of 24 and 12 individuals, which were the upper and mean estimates of the effective population size in the park, with an estimated pre-bottleneck population of 500 individuals (data not shown) (1000 iterations per simulation).

A global  $F_{ST}$  value for the Spanish imperial eagle was obtained and tested for significance by performing 10000 permutations with the program GENETIX 4.03 (Belkhir *et al.* 1996–2001).  $R_{ST}$  indices would underestimate differentiation when loci do not fit an strict stepwise mutation model (Balloux *et al.* 2000) and when populations are not highly structured (Balloux & Goudet 2002), whereas frequency-based estimates have been shown to be more appropriate when comparing closely related populations (Paetkau *et al.* 1997). Exact tests of differentiation between pairs of the five breeding nuclei were performed and tested for significance with FSTAT 2.9.3.2 (Goudet 1995).

Correlation analysis between genetic and geographical distances (Mantel test) was calculated with the ISOLDE program implemented in GENEPOP 3.1 as proposed by Rousset (1997).

The Bayesian clustering method described by Pritchard *et al.* (2000) and implemented in the program STRUCTURE 1.0 was applied to the Spanish imperial eagle data. This approach uses multilocus genotypes to assign individuals

to the distinct groups in the sample,  $K$  (where  $K$  may be unknown). To choose the burn-in length and to estimate the number of iterations necessary, several runs were assayed at each number of groups. A length of  $10^7$  iterations after a burn-in period of  $10^5$  iterations was determined as giving consistent results. Posterior probabilities of the number  $K$  were estimated assuming uniform prior values on  $K$  between 1 and 6 and comparing the Ln of the probability of the data for each one. The highest likelihood value is assumed to indicate the number of groups in our pool of data. In a second step, we used prior population information (POPINFO option switched on), assuming the  $K$  deduced above. When incorporating population information, either inferred as above or known *a priori*, this procedure allows the fractional assignment of individual multilocus genotypes to each cluster, the identification of migrants and the estimation of the probabilities of ancestry in populations other than the one of origin.

Two models of population structure were tested with the program 2MOD (Ciofi *et al.* 1999) to infer the relative likelihood of immigration drift equilibrium versus pure drift among nuclei. The first model assumes that the gene frequencies within nuclei are determined by a balance between genetic drift and immigration. In the pure drift model, one ancestral panmictic population separated into several units diverging independently in complete isolation. Both models assume that the effects of microsatellite mutations are negligible. The program is based on coalescent theory and uses a Markov's Chain Monte Carlo simulation approach with Metropolis–Hastings sampling to explore the alternative models. The program also estimates  $F$ , the probability that two genes share a common ancestor within a population. We carried out two independent runs with 100 000 iterations to check the convergence of the posterior probabilities of the models. The first 10% of points were dropped to avoid independence on initial starting values.  $F$ -values were checked for convergence by comparing the means and time-series standard errors for the two runs.

The contemporary rate of gene flow between Parque Nacional de Doñana and the remaining breeding nuclei as a whole (RBN) was estimated using a recently published Bayesian inference method of recent migration rates among populations using a Markov Chain Monte Carlo approach, BAYESASS 1.1 (Wilson & Rannala 2003). The program also allows for the estimation of inbreeding coefficients, among other parameters of potential interest. This method assumes relatively low levels of migration and the proportion of migrant individuals into a population cannot exceed one-third in each generation. Loci are assumed to be in linkage equilibrium, but deviations from Hardy–Weinberg equilibrium are allowed. To examine the convergence of Markov's Chain Monte Carlo algorithm, the posterior probability density of each allele frequency at each locus in each population was compared for two independent runs differing in

**Table 1** Mitochondrial control region diversity in both Spanish (*Aquila adalberti*) and Eastern (*Aquila heliaca*) imperial eagle species *n* stands for the number of samples used

Species	<i>n</i>	No. of haplotypes	No. of polymorphic sites	Haplotype diversity	Nucleotide diversity	Mean no. of pairwise differences
<i>Aquila adalberti</i>	60	3	2	0.3215 ± 0.0730	0.00098 ± 0.00024	0.338 ± 0.0632
<i>Aquila heliaca</i>	34	7	8	0.7790 ± 0.0420	0.00548 ± 0.00068	1.891 ± 0.2665

Haplotype	Nucleotide positions													Populations					
														A. adalberti			A. heliaca		
	5	6	8	3	4	4	5	2	9	7	9	8	9	CE	EX	MT	PND	SM	Unk
HapA	A	G	C	C	G	A	G	G	C	A	T	A	C	11	11	5	10	11	1
HapB	.	.	.	.	.	.	.	.	.	.	C	.	.	5					1
HapC	G	.	.	.	.	.	.	.	.	.	.	.	.	1	2			1	1
HapD	.	A	.	A	.	G	C	A	T	.	.	.	.						11
HapE	.	A	.	A	.	G	C	A	.	.	.	.	.						11
HapF	.	A	.	A	A	G	C	A	T	.	.	.	.						1
HapG	.	.	T	A	.	G	C	A	.	G	.	.	.						2
HapH	.	.	T	A	.	G	C	A	.	.	C	.	.						5
HapI	.	.	T	A	.	G	C	A	.	.	.	.	.						3
HapJ	.	A	.	A	.	G	C	A	T	.	G	A	.						1

**Fig. 2** Variable sites found in a fragment of 345 bp of the control region in 60 Spanish imperial eagles defining three haplotypes and their distribution in nuclei and 34 Eastern imperial eagles defining seven haplotypes. Haplotype labels are shown on the left and nucleotide positions relative to the beginning of the sequence are indicated by digits on the top. These sequences have been deposited in EMBL nucleotide database under accession numbers AJ567366, AJ567367 and AJ574878–AJ574885. CE, Centro; EX, Extremadura; MT, Montes de Toledo; PND, Parque Nacional de Doñana; SM, Sierra Morena; Unk, unknown origin.

initial random seed number and initial values of migration rate, *m*, and inbreeding coefficient, *F*, using three million iterations and a burn-in period of one million, as recommended by the authors. Those values were chosen as optimal and samples were collected every 2000 iterations to infer posterior probability distribution of the parameters.

## Results

### Genetic diversity

**Mitochondrial DNA.** Observed numbers of haplotypes and estimates of haplotype and nucleotide diversity for each group are shown in Table 1. Only three haplotypes, each differing by a single base, were found in the Spanish imperial eagle population (Fig. 2). The most frequent haplotype (HapA) was present in 82.3% of the individuals sequenced while two less frequent haplotypes were present in 9.7% (HapB) and 8.0% (HapC) of the individuals. HapA was present in all breeding nuclei, being fixed in both Centro and Parque Nacional de Doñana. We also found HapC in Montes de Toledo, while all three haplotypes were present in Extremadura and Sierra Morena breeding nuclei. In the Eastern imperial eagle, seven haplotypes differing in one or two bases occurred in more homogeneous frequencies (HapD 32.4%, HapE 32.4%, HapF 2.9%, HapG 5.9%, HapH 14.7%, HapI 8.8% and HapJ 2.9%, Fig. 2). Both gene and

nucleotide diversity in the Spanish imperial eagle were significantly lower than in the Eastern imperial eagle (Welch's approximate *t*-test, *t'* = 38, *P* < 0.01).

**Microsatellites.** The individual genotypes at 18 microsatellite loci were determined in 79 Spanish imperial eagles covering the whole distribution range and grouped in five subpopulations, corresponding to six out of the seven breeding nuclei described (Fig. 1, González 1991). Thirty-four Eastern imperial eagles were also genotyped for the same set of microsatellite loci. Deviation from Hardy–Weinberg equilibrium was detected in the Spanish imperial eagle population (*P* < 0.01). However, each breeding nucleus considered showed no deviation from equilibrium at any locus, suggesting a Wahlund effect. Loci Aa04, Aa56 and Aa43 showed a heterozygote deficit (*P* < 0.05) after sequential Bonferroni correction for the whole Spanish population. Deviation from equilibrium was also detected in the Eastern imperial eagle (*P* = 0.01).

Significant linkage disequilibrium was detected between four pairs of loci in the current Spanish imperial eagle population: Aa56–Aa53, Aa53–Aa43, Aa41–Aa43 and Aa11–Aa51 (*P* < 0.05 after sequential Bonferroni correction). However, four significant results out of 153 tests are expected by chance alone for a nominal *P*-value of 0.05. As no linkage disequilibrium was detected between those pairs of loci in the Eastern imperial eagle population, we consider it

**Table 2** Microsatellite number of alleles ( $k$ ), allelic richness (AR), expected and observed heterozygosity ( $H_E$  and  $H_O$ , respectively) in the Spanish (*Aquila adalberti*) and Eastern (*Aquila heliaca*) imperial eagle populations

Species	Population	$k$	AR	$H_E$	$H_O$
<i>Aquila adalberti</i>	Centro	66	3.259	0.522	0.491
	Extremadura	68	3.375	0.535	0.533
	Montes de Toledo	63	3.500	0.483	0.483
	Parque Nacional de Doñana	53	2.866	0.483	0.490
	Sierra Morena	65	3.292	0.559	0.559
	All	88	4.881	0.549	0.516
<i>Aquila heliaca</i>		96	5.211	0.627	0.563

unlikely that these pairs of loci are physically linked. For all the analyses below we have considered the full set of 18 markers as unlinked.

Number of alleles, allelic richness, expected and observed heterozygosities for the two species, as well as for the five breeding nuclei considered in the Spanish imperial eagle, are summarized in Table 2. After genotyping 79 individuals with 18 markers a total of 88 alleles were found in the Spanish imperial eagle population and 96 alleles in 34 Eastern imperial eagles. Number of alleles per locus ranged between two and nine for the Spanish imperial eagle population and between two and 12 for the Eastern species. Both allelic richness (El Mousadik & Petit 1996) and expected heterozygosity were similar for both species (Wilcoxon signed rank test,  $T_+ = 68.5$ ,  $n = 18$ ,  $P = 0.50$  and  $T_+ = 55$ ,  $n = 18$ ,  $P = 0.20$ , respectively). Results from the analysis of variance indicated significant differences among nuclei in the mean allelic richness ( $F_{4,68} = 3.095$ ,  $P = 0.02$ ) and the same tendency in expected heterozygosity ( $F_{4,68} = 2.136$ ,  $P = 0.09$ ). The proportion of variance explained by the locus effect was 74.2% for allelic richness and 51.7% for expected heterozygosity. Contrasts showed that allelic richness and expected heterozygosity were significantly smaller in Parque Nacional de Doñana ( $F_{1,68} = 10.50$ ,  $P < 0.01$ , and  $F_{1,68} = 7.16$ ,  $P = 0.01$ , respectively).

**Bottleneck analysis.** The observed proportion of heterozygotes was not significantly different to that expected under equilibrium for the observed number of alleles ( $P = 0.66$ , Wilcoxon test). Heterozygote excess was not significant either in the three largest nuclei: Centro, Extremadura and Sierra Morena ( $P = 0.28$ ,  $P = 0.53$  and  $P = 0.25$ , respectively). Average  $M$  among the 18 microsatellites was 0.84, a value that was not significantly lower than that obtained under simulation for a population of a size estimated to reflect the Imperial eagle's pre-bottleneck size ( $P = 0.14$  for  $\theta = 1.0$ , B. Martínez-Cruz, J.A. Godoy & J.J. Negro, unpublished data). Furthermore,  $M$ -ratios in the five nuclei were not signi-

**Table 3** Differentiation indexes among the five breeding nuclei

	CE	EX	MT	PND	SM
CE		0.0227*	0.0078	0.1100***	0.0274**
EX	0.0025*		-0.0001	0.0790**	0.0263**
MT	0.0644	0.2364		0.0557**	0.0018
PND	0.0001**	0.0001**	0.0031*		0.1015***
SM	0.0008**	0.0021*	0.2441	0.0002**	

CE, Centro; EX, Extremadura; MT, Montes de Toledo; PND, Parque Nacional de Doñana; SM, Sierra Morena.

$F_{ST}$  probability values above the diagonal and for the exact test of differentiation under the diagonal.

Levels of significance are \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

ficantly different from each other ( $\chi^2_{0.054} = 5.20$ ,  $P = 0.27$ , Kruskal-Wallis test).

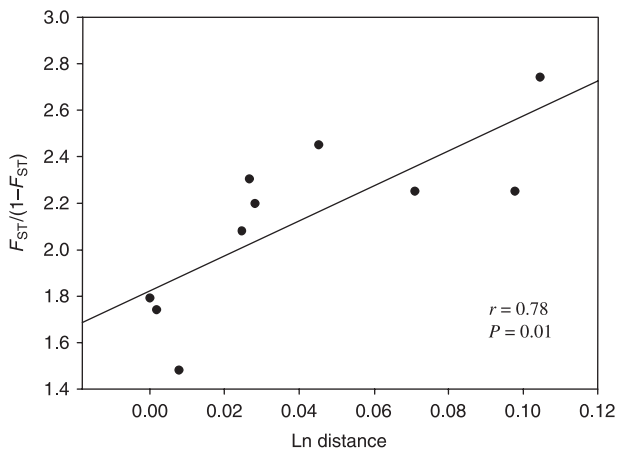
Simulations under realistic scenarios predicted changes in allelic diversity and loss of heterozygosity that were consistent with the observed changes in Parque Nacional de Doñana. The estimate of effective population size for that population was 12 individuals (based on genetic data, B. Martínez-Cruz, J.A. Godoy & J.J. Negro, unpublished data). A simulated bottleneck of 12 individuals for 50 years (three generations, the mean generation time being around 16.4 years in the park, Ferrer & Calderón 1990) produced estimates of allelic diversity and expected heterozygosity similar than observed ( $2.85 \pm 0.24$  simulation,  $2.94 \pm 0.80$  observed;  $T_- = 77.5$ ,  $n = 18$ ,  $P = 0.728$ )  $0.480 \pm 0.03$  simulation,  $0.464 \pm 0.20$  observed;  $T_+ = 84$ ,  $n = 18$ ,  $P = 0.948$  respectively, Wilcoxon signed rank test). Values of both simulated parameters were significantly different from the values observed in the population of departure ( $T_- = 13$ ,  $n = 18$ ,  $P < 0.01$  for allelic diversity and  $T_+ = 0$ ,  $n = 18$ ,  $P < 0.01$  for expected heterozygosity, Wilcoxon signed rank test).

### Structure in the Spanish imperial eagle population

**Distance-based methods.** Global  $F_{ST}$  value (Weir & Cockerham 1984) for the five Spanish imperial eagle breeding nuclei was low but still significant ( $\theta = 0.04054$ ,  $P < 0.01$ ). Values of pairwise  $F_{ST}$  were significantly different from zero in all pairwise comparisons involving Parque Nacional de Doñana ( $P < 0.05$ ). In contrast, Montes de Toledo did not differ significantly from Sierra Morena, Centro and Extremadura, although the latter three were significantly differentiated from each other (all  $P < 0.05$ ). Exact tests of pairwise differentiation yielded coincident results. (Table 3).

A pattern of isolation by distance (IBD) was indicated by a significant correlation of genetic and geographical distances (Mantel test,  $R = 0.778$ ,  $P = 0.01$ ; Fig. 3).

**Bayesian clustering method.** Initially, all the individuals were pooled together and no population information was



**Fig. 3** Mantel test showing the correlation between genetic and geographical distances among the five nuclei ( $r = 0.78$ ,  $P = 0.01$ ).

used for determining the most likely number of clusters. The highest likelihood was found for  $K = 3$ , suggesting that the population was subdivided into three genetically different clusters. However, when we estimated the proportion of membership of each individual ( $q$ ) to each cluster with  $K = 3$ , only a small fraction of the individuals clustered clearly in group I, while the rest of individuals were partially clustered in groups II and III in the same proportion. A strong departure from Hardy–Weinberg equilibrium was detected within this subsample of individuals that could lead to the estimation of the wrong number of groups. Thus we repeated the analysis with only those individuals clustering partially in groups II and III, with priors  $K = 1$  and  $K = 2$ . The maximum likelihood was for  $K = 1$ . We interpreted all these results as an indication that the whole population was divided into two subpopulations.

When prior population information was incorporated, individuals from Parque Nacional de Doñana were classified as group I and individuals from the remaining breeding nuclei (RBN) as group II, with an average proportion of membership of 0.87 and 0.96, respectively. In each subpopulation we detected two admixed individuals (migrants or its offspring). One subadult captured in 1995 and one nestling sampled in 2001 in Parque Nacional de Doñana were assigned to RBN subpopulations with probabilities of 0.99 and 0.96, respectively. In the case of the chick hatched in Parque Nacional de Doñana, its parents' genotypes were subsequently analysed from field-collected feathers; the female parent clustered in RBN with a probability of  $P = 0.86$ , identifying it as a migrant. This female replaced the previous one in this nest in 1999 as a juvenile of the 3rd to 4th year. Being a nonringed individual, it was suspected of being an immigrant, since during 1995–96 almost every nestling in Parque Nacional de Doñana was ringed. On the other hand, one adult captured in Toledo and one nestling sampled in Extremadura in 1996 were assigned to Parque Nacional de

Doñana subpopulation with probabilities of  $P = 1.00$  and  $P = 0.98$ , respectively. As for the nestling, the probability of assignment to Parque Nacional de Doñana was mainly the result of its first-generation ancestors.

**Model of population structure and migration rates.** We tested whether the observed differentiation pattern could be better explained by a pure drift model or a model of equilibrium between drift and gene flow. The likelihood of the gene flow model was more than three times that of the pure drift model ( $P(\text{gene flow}) = 0.77 \pm 0.001$ , Bayes factor = 3.3). The relative interaction between gene flow and drift was inferred with  $F$  (probability that two genes share a common ancestor within a population) and  $M$  (migration rate obtained from  $F$ , Ciofi *et al.* 1999) values. High levels of immigration relative to drift were inferred in Centro [ $F = 0.04$ , 90% highest posterior density (HPD) range: 0.02–0.07;  $M = 11.5$ , 90% HPD range: 6.7–23.3], Extremadura ( $F = 0.03$ , 90% HPD range: 0.01–0.05;  $M = 17.5$ , 90% HPD range: 9.7–49.5), Montes de Toledo ( $F = 0.01$ , 90% HPD range: 0.001–0.03;  $M = 35$ , 90% HPD range: 14.2–499.5) and Sierra Morena ( $F = 0.03$ , 90% HPD range: 0.01–0.05;  $M = 16.5$ , 90% HPD range: 8.8–49.5) nuclei. In contrast, in the small Parque Nacional de Doñana nucleus, drift had a higher effect relative to immigration ( $F = 0.16$ , 90% HPD range: 0.10–0.23;  $M = 2.6$ , 90% HPD range: 1.6–4.5).

The average estimates of recent migration rates from RBN into Parque Nacional de Doñana and vice versa were  $0.11 \pm 0.06$  and  $0.02 \pm 0.01$ , respectively. Considering the estimated breeding census size of both populations (around 22 individuals in Parque Nacional de Doñana and 260 in RBN) this would mean that in each generation, a mean of 2.4 individuals migrate from RBN to Parque Nacional de Doñana and 5.7 migrate in the other direction.

## Discussion

The analysis of both mitochondrial and microsatellite data in conjunction with an extensive sampling of the species allowed us to characterize the genetic composition of the current eagle population in terms of both diversity and structure. The relative support for alternative demographic scenarios giving rise to the observed pattern needs to be discussed, and these in turn will call for different management strategies for the conservation of the species.

### Genetic variability

The fact that differences among haplotypes are of a single base and that one haplotype was common resulted in very low nucleotide and haplotype diversities for the Spanish imperial eagle, lower than diversities found in the Eastern sister species, as well as in other species of the genus *Aquila* (Masuda *et al.* 1998; Väli 2002). However, levels of variability

in microsatellites do not differ between these species and are similar to those in other nonendangered raptors (Nichols *et al.* 2001; Kretzmann *et al.* 2003). Signatures of a genetic bottleneck in the nuclear genome were not detected by several independent tests, indicating that the demographic bottleneck suffered during the twentieth century was neither critical nor lasting enough to have an impact on nuclear genetic variation at the species level. Moreover, Garza & Williamson (2001) suggested that high allelic diversity and high  $M$  ratio, i.e. the pattern obtained here, were indicative of populations that have been small for a long time in contrast with populations that have been recently reduced in size. In contrast, mitochondrial DNA diversity might have been directly affected by the demographic decline, because of its four-fold lower effective population size. Alternatively, a model where the population of the Spanish imperial eagle had suffered moderate size fluctuations strong enough to affect mitochondrial DNA but not microsatellite diversity (e.g. range expansions and contractions during glaciations) could also explain current diversity levels as well as the lack of signal of a recent genetic bottleneck in the nuclear genome. These hypotheses are being directly tested with the analysis of pre-bottleneck museum samples.

#### *Genetic structure of the Spanish imperial eagle*

Genetic structure was detected in the Spanish imperial eagle population by both distance- and model-based methods. Distance-based methods identified Parque Nacional de Doñana as a subpopulation but detected only slight structure among the remaining breeding nuclei (RBN), affecting peripheral nuclei. On the other hand, the model-based method only differentiated Parque Nacional de Doñana from the rest of breeding nuclei.

To understand the implications of the present pattern of structure in the population we need to investigate whether this is an equilibrium situation or not. If that is the case, then the structure must have been there historically, when the species had a widespread distribution in the Iberian Peninsula. Although structure has always been interpreted in the light of external factors (e.g. selection, barriers to dispersal), recent studies have shown that even in the absence of external influences, structure may appear between the extremes of a continuous distribution range if dispersal distances and population sizes are sufficiently low (Hoelzer 2001; Irwin 2002). Young imperial eagles always abandon their natal areas and settle temporarily in one or several locations from where they periodically return to their parents' territories (Ferrer 2001). The mean dispersal distance between natal and temporary settlement locations has been estimated as 138.61 km, being in some occasions up to 400 km (Ferrer 2001). Data on effective dispersal distances are scarce except for Parque Nacional de

Doñana. However, these include proof of long-distance effective migration to Doñana. One individual banded as a nestling in Cáceres (Extremadura), successfully mated and reproduced in Doñana, 300 km away, for several years (Monitoring Group of Natural Processes of the Doñana Biological Station databases). Moreover, our study shows genetic evidence for successful migration both to and from Doñana, between breeding nuclei over 300 km distant, and relatively high estimates of migration. These observations seem incompatible with an equilibrium pattern of genetic structure in the Spanish Imperial eagle.

Evidence for the occurrence of migration-drift equilibrium might be obtained from the analysis of a plot of genetic distance against geographical distance. In a population at equilibrium, where the dispersal capability of the organisms is constrained by distance (i) genetic distances would increase in a monotonic and positive way with the geographical distances, (ii) the scatter of the pairwise points would increase outward from being narrow at the origin to wider at further geographical distances, and (iii) the scatter plot would originate near the origin (Hutchinson & Templeton 1999). While the first criterion is satisfied for the Spanish imperial eagle, indicating the influence of distance-limited dispersal in the observed pattern, the other two are not, indicating that this is not an equilibrium situation. Nonetheless this may reflect insufficient statistical power.

In a different scenario, the current structure could have been a consequence of the recent population fragmentation (five to seven generations since in the 20th century, Negro & Hiraldo 1993) and the disappearance of intermediate breeding areas. This would require an intense drift driven by very small effective population size and/or very low migration rates. A pure drift model was not supported by the data and effective migration has been detected to and from Parque Nacional de Doñana (asymmetrical migration) both from field (González 1989) and genetic data (the present study). Nevertheless, migration seems insufficient to counteract the effects of drift in Parque Nacional de Doñana as seen in a significant genetic differentiation and reduced genetic diversity. Both a relatively low level of immigration in Parque Nacional de Doñana together with a low effective population size ( $N_e$ ) are most likely the factors responsible for the current situation. The carrying capacity of the population has been estimated to be only 15–16 pairs (Ferrer & Donázar 1996). Moreover, the population has fluctuated from 6 to 16 pairs in the last 40 years (Ferrer & Donázar 1996; Ferrer *et al.* 2003). In addition, a strong breeding success bias in favour of the good quality territories, situated in the edge of the marshes (Ferrer & Donázar 1996), certainly diminish  $N_e$  to a handful of individuals. Besides that, data from the last four decades show a high variation in productivity (Ferrer 2001), decreasing in the last 10 years because of the anomalously high

recruitment of juveniles to the breeding population (Ferrer *et al.* 2003). This situation of very low  $N_e$  driving drift may have been the direct result of the disappearance of the Huelva and Cádiz populations, that occurred by the first half of the twentieth century (González 1991). These populations connected Doñana with the others, presumably promoting gene flow and increasing  $N_e$  (M Ferrer, personal communication). Actually, simulations indicate that a bottleneck of 12 individuals starting at this time would explain the decrease in genetic diversity observed in the population of Doñana. Moreover, as our simulations did not take immigration into account, the effective population size might be even lower.

Even if a conclusive answer will only be given by the analysis of old specimens predating 1900, our results indicate that Parque Nacional de Doñana population differentiation might be the result of evolution under drift despite continued gene flow following fragmentation.

### Conservation implications

As the observed genetic differentiation is interpreted to be the consequence of the demographic decline and thus has no long-term evolutionary significance, and the risks of genetic erosion in peripheral populations are considered to be of overriding concern, we recommend the management of the whole population as a single unit, both *in situ* and *ex situ*.

Genetic factors, including loss of diversity and inbreeding depression, will increase the extinction risks by reducing adaptive potential and decreasing average fitness, respectively (Brook *et al.* 2002). Although levels of variability in the mitochondrial genome are low, we do not find evidence of genome-wide genetic erosion in the Spanish imperial eagle, as nuclear diversity shows normal levels, similar to those of its more widely distributed and more abundant sister species. However, Parque Nacional de Doñana shows strong genetic differentiation and lower levels of diversity than the remaining breeding nuclei as a whole, suggesting that this population is suffering the consequences of an extremely low  $N_e$  and reduced migration rates. Similar but less intense tendencies are suggested for the other peripheral nuclei. Management strategies should aim to preserve the extant diversity and to minimize inbreeding in peripheral populations, with special attention being given to the Parque Nacional de Doñana subpopulation. This would require an increase of population sizes and the interconnection of the different nuclei. For the Parque Nacional de Doñana population, both objectives could be achieved in the long term through the reintroduction of the species in surrounding populations in Huelva and Cádiz, that might act as a stepping stone between Parque Nacional de Doñana and the rest of the distribution range (M. Ferrer, personal communication). In the meanwhile, the translocation of

individuals from other nuclei into Parque Nacional de Doñana might be considered.

### Acknowledgements

We are very grateful to all the institutions and private persons that kindly contributed samples to this study: the administrations from Andalucía, Castilla la Mancha, Castilla-León, Extremadura and Madrid, the Spanish Ministry of Environment, M. Ferrer, J. M. Blanco, T. Katzner, L. Haraszthy, MME Birdlife Hungary and J. A. Padilla. B.M.C. benefited from a grant from the Government of La Rioja for the training of researchers (F.P.I.). This project is funded by the Spanish Ministry of Science and Technology (Ref.: REN2001-2310).

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Begoña Martínez-Cruz is developing her PhD thesis on the population genetics of the Spanish imperial eagle and is especially interested in the conservation genetics and molecular ecology of endangered species. 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 loss, inbreeding and hybridization.

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# Appendix 1

Summary list of samples used in this study

Sample ID*	Sampl code	Species	Locality/area	Date	Tissue†	Haplotype	Nucleus‡	Sampler
OW	Aad001	<i>A. adalberti</i>	Villa del Pardo (Madrid)	—	B	HapA	CE	M. Ferrer
12	Aad002	<i>A. adalberti</i>	El Pardo (Madrid)	—	B	—	CE	M. Ferrer
14	Aad003	<i>A. adalberti</i>	S <sup>a</sup> Toreros (Cáceres)	—	B	—	EX	M. Ferrer
28	Aad006	<i>A. adalberti</i>	Extremadura	—	B	HapA	EX	M. Ferrer
4P	Aad007	<i>A. adalberti</i>	Madrid	—	B	HapA	CE	M. Ferrer
JJ	Aad009	<i>A. adalberti</i>	Coto del Rey (Parque Nacional de Doñana)	1999	B	HapA	PND	Authors
LM	Aad011	<i>A. adalberti</i>	Jaén	1996	B	HapA	SM	Authors
G373	Aad014	<i>A. adalberti</i>	Madrid	1991	B	HapA	CE	J. Oria
LW	Aad015	<i>A. adalberti</i>	Peguerinos (Segovia)	2000	B	HapA	CE	Authors
T21	Aad016	<i>A. adalberti</i>	Segovia	2000	B	HapA	CE	J. Oria
LU	Aad017	<i>A. adalberti</i>	Andújar (Jaén)	2000	B	—	SM	Authors
L5	Aad018	<i>A. adalberti</i>	Pajarera (Parque Nacional de Doñana)	2000	B	HapA	PND	Authors
5X	Aad020	<i>A. adalberti</i>	Punta del Caño (Parque Nacional de Doñana)	2000	B	HapA	PND	Authors
AIIOO3*	Aad023	<i>A. adalberti</i>	Unknown	—	B	HapB	—	—
133/OO	Aad024	<i>A. adalberti</i>	El Pardo (Madrid)	—	B	HapA	CE	—
134/00	Aad025	<i>A. adalberti</i>	El Pardo (Madrid)	—	B	HapA	CE	—
LC	Aad026	<i>A. adalberti</i>	La Carolina (Jaén)	2000	B	HapA	SM	Authors
T25	Aad029	<i>A. adalberti</i>	Madrid	2000	B	HapA	CE	J. Oria
LV	Aad030	<i>A. adalberti</i>	Sierra Norte (Seville)	2000	B	HapA	SM	Authors
LX*	Aad031	<i>A. adalberti</i>	Vetalarrena (Parque Nacional de Doñana)	2000	B	HapA	PND	Authors
T-11	Aad032	<i>A. adalberti</i>	Madrid	2000	B	—	CE	J. Oria
G377	Aad033	<i>A. adalberti</i>	Madrid	1991	B	—	CE	J. Oria
P99ZJ (43)*	Aad035	<i>A. adalberti</i>	Sierra de S Pedro (Cáceres)	—	B	HapB	EX	Authors
B6*	Aad038	<i>A. adalberti</i>	El Acebuche (Huelva)	—	B	HapA	PND	C. Sánchez
24	Aad039	<i>A. adalberti</i>	Parque Hornachuelos (Badajoz)	—	B	HapA	SM	C. Sánchez
26	Aad040	<i>A. adalberti</i>	Toledo	—	B	HapC	MT	—
16	Aad041	<i>A. adalberti</i>	Herreruela (Cáceres)	—	B	HapA	EX	J.M. Blanco
4-PA	Aad042	<i>A. adalberti</i>	Aliseda (Cáceres)	1996	B	HapB	EX	—
1-31/11/98	Aad045	<i>A. adalberti</i>	Extremadura	1996	B	HapA	EX	—
2-31/11/98	Aad046	<i>A. adalberti</i>	Extremadura	1996	B	HapA	EX	—
CO-8231	Aad047	<i>A. adalberti</i>	Monfragüe (Cáceres)	2000	B	HapA	EX	J. Caldera
N116/01	Aad050	<i>A. adalberti</i>	Segovia	—	M	—	CE	—
A	Aad051	<i>A. adalberti</i>	Huerto Zorros (Parque Nacional de Doñana)	1995	B	—	PND	C. Sánchez
5A	Aad052	<i>A. adalberti</i>	Corral de la Liebre (Parque Nacional de Doñana)	1995	B	HapA	PND	C. Sánchez
C	Aad064	<i>A. adalberti</i>	Extremadura	1998	B	HapA	EX	C. Sánchez
D*	Aad065	<i>A. adalberti</i>	Unknown	1995	B	HapA	—	C. Sánchez
5T	Aad076	<i>A. adalberti</i>	P. Porquera (Parque Nacional de Doñana)	1997	B	HapA	PND	C. Sánchez
E	Aad078	<i>A. adalberti</i>	Huévar (Seville)	1995	B	HapA	PND	C. Sánchez
M11 M	Aad079	<i>A. adalberti</i>	Yébenes (Toledo)	—	B	HapA	MT	J.M. Blanco
M11 H	Aad080	<i>A. adalberti</i>	Cabañeros (Ciudad Real)	—	B	HapA	MT	J.M. Blanco
M13 M	Aad082	<i>A. adalberti</i>	Sierra del Castañar (Toledo)	—	B	HapC	MT	J.M. Blanco
M15 M	Aad084	<i>A. adalberti</i>	Yébenes (Toledo)	—	B	HapA	MT	J.M. Blanco
M16 M	Aad085	<i>A. adalberti</i>	Toledo	—	B	HapA	MT	J.M. Blanco
26 SEV*	Aad087	<i>A. adalberti</i>	Unknown	—	B	HapC	—	J.M. Blanco
4L	Aad090	<i>A. adalberti</i>	Aliseda (Cáceres)	—	B	HapA	EX	J.M. Blanco
4T	Aad091	<i>A. adalberti</i>	Ávila	—	B	HapA	CE	J.M. Blanco
M2	Aad111	<i>A. adalberti</i>	Vetalarrena (Parque Nacional de Doñana)	2001	B	—	PND	Authors

## Appendix 1 Continued

Sample ID*	Sampl code	Species	Locality/area	Date	Tissue†	Haplotype	Nucleus‡	Sampler
M5	Aad114	<i>A. adalberti</i>	Navazo Adelfas (Parque Nacional de Doñana)	2001	B	—	PND	Authors
NC	Aad115	<i>A. adalberti</i>	Cazalla (Seville)	2001	B	—	SM	Authors
NA	Aad118	<i>A. adalberti</i>	Vilches (Jaén)	2001	B	HapA	SM	Authors
N5	Aad121	<i>A. adalberti</i>	Jaén	2001	B	HapA	SM	Authors
P98 SSPedro	Aad125	<i>A. adalberti</i>	Sierra de S Pedro (Cáceres)	1998	B	—	EX	J. Caldera
H277	Aad126	<i>A. adalberti</i>	Monfragüe (Cáceres)	2001	M	HapA	EX	J. Caldera
Diana	Aad127	<i>A. adalberti</i>	Coria (Cáceres)	—	B	—	EX	J. Caldera
MC	Aad128	<i>A. adalberti</i>	Soto Chico (Parque Nacional de Doñana)	2001	B	HapA	PND	Authors
2837	Aad133	<i>A. adalberti</i>	Abenojar (Ciudad Real)	1996	DNA	HapA	SM	J.M. Blanco
8110	Aad137	<i>A. adalberti</i>	Cerrajeros (Ciudad Real)	2000	DNA	HapA	SM	J.M. Blanco
2840	Aad138	<i>A. adalberti</i>	Toledo	1996	DNA	HapA	CE	J.M. Blanco
2842	Aad139	<i>A. adalberti</i>	La Ribera (Ciudad Real)	1996	DNA	HapA	SM	J.M. Blanco
2843	Aad140	<i>A. adalberti</i>	El Molinillo (Toledo)	2000	DNA	HapA	MT	J.M. Blanco
3825	Aad141	<i>A. adalberti</i>	Pulgar (Toledo)	1997	DNA	—	MT	J.M. Blanco
3831	Aad144	<i>A. adalberti</i>	Fuencaliente (Ciudad Real)	1997	DNA	HapA	SM	J.M. Blanco
9122	Aad146	<i>A. adalberti</i>	Almadén (Ciudad Real)	—	DNA	HapC	SM	J.M. Blanco
AI	Aad147	<i>A. adalberti</i>	Navalagamella (Madrid)	1999	B	—	CE	P. Lanzarot
Cebreros	Aad148	<i>A. adalberti</i>	Cebreros (Madrid)	2000	Br	HapA	CE	M. Díez
Pollo2-JF	Aad151	<i>A. adalberti</i>	Sierra Norte (Seville)	2002	B	—	SM	A. Llopis
T1	Aad153	<i>A. adalberti</i>	Andujar (Jaén)	2002	B	—	SM	Authors
T4	Aad156	<i>A. adalberti</i>	Santisteban (Jaén)	2002	B	—	SM	Authors
T5	Aad157	<i>A. adalberti</i>	Santisteban (Jaén)	2002	B	—	SM	Authors
MJ	Aad161	<i>A. adalberti</i>	Cañuelas (Parque Nacional de Doñana)	2002	B	HapA	PND	Authors
Metal 41	Aad164	<i>A. adalberti</i>	Sierra Norte (Seville)	2002	B	HapA	SM	Authors
9	Aad171	<i>A. adalberti</i>	Aliseda (Cáceres)	—	B	HapB	EX	—
10	Aad172	<i>A. adalberti</i>	Monroy (Cáceres)	—	B	—	EX	—
15	Aad177	<i>A. adalberti</i>	Aliseda (Cáceres)	—	B	HapC	EX	—
24-feb	Aad185	<i>A. adalberti</i>	Membrío (Cáceres)	—	B	HapB	EX	—
25	Aad186	<i>A. adalberti</i>	Puerto del Clavín (Cáceres)	—	B	HapB	EX	—
27	Aad188	<i>A. adalberti</i>	Aliseda (Cáceres)	—	B	HapA	EX	—
28	Aad189	<i>A. adalberti</i>	Cáceres	—	B	HapA	EX	—
29	Aad190	<i>A. adalberti</i>	Cáceres	—	B	HapA	EX	—
T23	Aad191	<i>A. adalberti</i>	Madrid	—	B	—	CE	J. Oria
T14	Aad192	<i>A. adalberti</i>	Madrid	—	B	—	CE	J. Oria
T30	Aad193	<i>A. adalberti</i>	Madrid	2002	B	—	CE	J. Oria
Ávila 2002	Aad194	<i>A. adalberti</i>	Valle de Hiruelas (Ávila)	2002	B	—	CE	J. Oria
Sev.02-02	Aad196	<i>A. adalberti</i>	Los Navalmorales (Toledo)	2002	B	—	MT	J.M. Blanco
Sev.03-02	Aad197	<i>A. adalberti</i>	Los Navalmorales (Toledo)	2002	B	—	MT	J.M. Blanco
IE.22	Ahe001	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.24	Ahe002	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.25	Ahe003	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.27	Ahe004	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapF	—	T. Katzner
IE.28	Ahe005	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.29	Ahe006	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.30	Ahe007	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.33	Ahe008	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.35	Ahe009	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapG	—	T. Katzner
IE.36	Ahe010	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.38	Ahe011	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.40	Ahe012	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.01	Ahe013	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.02	Ahe014	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.03	Ahe015	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner

Appendix 1 Continued

Sample ID*	Sampl code	Species	Locality/area	Date	Tissue†	Haplotype	Nucleus‡	Sampler
IE.05	Ahe016	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.06	Ahe017	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
IE.08	Ahe018	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.10	Ahe019	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapE	—	T. Katzner
IE.23	Ahe020	<i>A. heliaca</i>	Naurzum Park (Kahzakstan)	2000	DNA	HapD	—	T. Katzner
SLOVA	Ahe021	<i>A. heliaca</i>	Hungary	1997	DNA	HapD	—	Authors
77	Ahe022	<i>A. heliaca</i>	Hungary	1997	DNA	HapG	—	Authors
79	Ahe023	<i>A. heliaca</i>	Hungary	1997	DNA	HapE	—	Authors
H1	Ahe024	<i>A. heliaca</i>	Unknown	1998	DNA	HapE	—	M. Ferrer
H10	Ahe025	<i>A. heliaca</i>	Unknown	1998	DNA	HapH	—	M. Ferrer
H11	Ahe026	<i>A. heliaca</i>	Unknown	1998	DNA	Hapl	—	M. Ferrer
H2	Ahe027	<i>A. heliaca</i>	Unknown	1998	DNA	HapH	—	M. Ferrer
H3	Ahe028	<i>A. heliaca</i>	Unknown	1998	DNA	HapE	—	M. Ferrer
H4	Ahe029	<i>A. heliaca</i>	Unknown	1998	DNA	HapH	—	M. Ferrer
H5	Ahe030	<i>A. heliaca</i>	Unknown	1998	DNA	Hapl	—	M. Ferrer
H6	Ahe031	<i>A. heliaca</i>	Unknown	1998	DNA	HapH	—	M. Ferrer
H7	Ahe032	<i>A. heliaca</i>	Unknown	1998	DNA	Hapl	—	M. Ferrer
H8	Ahe033	<i>A. heliaca</i>	Unknown	1998	DNA	HapH	—	M. Ferrer
H9	Ahe034	<i>A. heliaca</i>	Unknown	1999	DNA	HapJ	—	M. Ferrer

\*indicates samples not used in the microsatellite study.

†The type of tissue sampled is indicated as B, blood; Br, brain; M, muscle; DNA, samples provided as extracted DNA.

‡CE, Centro; EX, Extremadura; MT, Montes de Toledo; PND, Parque Nacional de Doñana; SM, Sierra Morena.