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RESEARCH ARTICLE

Genetic diversity of the great bustard in Iberia and Morocco: risks from current population fragmentation

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Abstract We studied the genetic diversity of great bustards (*Otis tarda*) in Iberia and Morocco, the main stronghold of this globally endangered species. Samples were collected from 327 individuals covering most of the distribution range within the study area. Sequence variation in a 657 bp fragment of the mtDNA control region revealed 20 variable sites defining 22 haplotypes, two of them exclusive to Morocco. Genetic diversity showed marked regional differences (π = 0–0.53, h = 0–0.89). Multidimensional scaling analysis based on F_{ST} values showed a clear division between Morocco and the Iberian Peninsula, with no evidence of current gene flow between them. Our results suggest that Morocco, where few matrilines have persisted to present, was colonized from Iberia thousands of years ago. Last century reports suggest dispersal through Gibraltar, when the species was more abundant at both sides of the Strait but later population declines and the Strait's barrier effect have favoured current genetic isolation. Within Iberia, only the most peripheral populations (Navarra, Aragón and Andalusia) differed significantly from the main ones in central Spain. The first two showed extremely low genetic diversity and are probably threatened by inbreeding depression. Diversity was higher in Andalusia, where three exclusive haplotypes were found, suggesting some degree of isolation from other populations. Andalusia and Morocco could be regarded as separate management units which hold a significant proportion of the current genetic diversity and thus deserve urgent conservation measures.

Keywords Genetic variation · Great bustard · Iberian Peninsula · mtDNA · Morocco · *Otis tarda*

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Introduction

The great bustard (*Otis tarda*) is a globally endangered species whose numbers and distribution range have decreased markedly throughout the last two centuries due mainly to hunting and agricultural transformations (Collar et al. 1994; BirdLife International 2000, 2001, 2007). Its Palaearctic distribution extends from Iberia and Morocco in the West to north-eastern China in the East, but most populations in central Europe and Asia have been decimated (Glutz et al. 1973; Del Hoyo et al. 1996). Today the species' stronghold is found in the Iberian Peninsula, where numbers are estimated around 25,000 birds, >50% the world population (Alonso et al. 2003). However, the distribution is discontinuous in Iberia, with several breeding groups living in marginal areas subject to various human-induced threats, mainly habitat fragmentation and agricultural intensification (Alonso et al. 2003, 2005b). As for the Moroccan population, it is the only one surviving in the African continent (Urban et al. 1986; Del Hoyo et al.

1996), and one of the most endangered worldwide, with ca. 100 estimated birds surviving in the North of the country (Hellmich and Idaghdour 2002; Alonso et al. 2005a).

It is essential to know the genetic structure of the species throughout the whole distribution range, in order to develop wide-scale conservation strategies and plan management actions according to current genetic differentiation (Avise et al. 1987; Moritz 1994; Haig 1998). Genetic analyses have become an important tool in many studies of threatened or endangered species (Moritz 1994; Haig 1998), and by using genetic markers the evolutionary history of a group can be investigated to determine whether smaller management units may exist below the species level (Moritz 1994; Knapen et al. 2003). Furthermore, the amount and geographical patterning of genetic variation within species is an evolutionary consequence of several historical and contemporary processes including vicariance, range expansion, gene flow and fragmentation (Slatkin 1985; Riddle 1996; Taberlet et al. 1998; Hewitt 2001; Sork et al. 2001). Quantifying this variation and in turn the extent to which these processes have acted in shaping genetic structure is a main concern in the field of evolutionary biology.

A previous survey of the mitochondrial and nuclear genetic variation of great bustards across Europe showed that the Iberian population is genetically separated from the central-eastern European population (Pitra et al. 2000). The current main stronghold of this species in Spain is also fragmented in several breeding areas separated by unsuitable habitat patches (Suárez-Seoane et al. 2002; Alonso et al. 2003). Although adult birds, and particularly females, are strongly philopatric to their traditional breeding sites, the different groups are connected genetically through natal dispersal, at least at a regional scale. Natal dispersal is strongly male-biased in this species (Alonso et al. 1998; Martín et al. 2002, 2008), with most juvenile males (ca. 75%) establishing as breeding adults at sites different from their natal ones, whereas 80% of juvenile females are philopatric. However, females are also capable of establishing at far distances from their natal sites (our recorded maximum natal dispersal distance in females was 91 km). The proportion of philopatric individuals increases in geographically isolated groups (Martín et al. 2008). On the other hand, great bustards are partial migrants, and many adults of both sexes perform seasonal post-breeding migrations to summering and wintering areas far from their breeding sites (up to 250 km in males, 120 km in females). However, they typically exhibit fidelity to their breeding sites.

The genetic structure and patterns of population subdivision in Iberia are still unknown, except for a slight genetic structuring found among breeding groups in Madrid region (Martín et al. 2002). As for Morocco, a recent study of 834 bp of combined control region and cytochrome-*b* mtDNA fragments found there a single haplotype that was shared with Iberian birds, and claimed there was moderate gene flow between both populations across the Straits of Gibraltar (Broderick et al. 2003). However, the low-variable cytochrome-*b* mtDNA fragment used in that study, and the very small sample size taken in Spain for comparison limit the value of these conclusions.

In the present study we describe the distribution of genotypic variation of the Afro-Iberian great bustard population based on a large sample collected at several sites over its distribution range in Iberia and Morocco. Our aims were to investigate the spatial structure of the existing genetic variation, and re-examine the relationships between Iberian and African birds. The results are used to highlight the most urgent conservation actions necessary to keep current levels of genetic diversity of this endangered species within the Afro-Iberian region. In particular, we identify haplotypes that may be currently threatened with extinction, which could determine a decrease in overall genomic diversity. We also evaluate inter-continental divergence, assess genetic relationships between populations and propose scenarios explaining the observed patterns of diversity at both contemporary and evolutionary timescales.

Methods

Sample collection

Between 1991 and 2005 we collected 327 samples at 67 breeding groups or leks covering most of the distribution range of the species in the Iberian Peninsula and Morocco (Fig. 1, Table 1). The samples were blood (63.0%), feathers (30.3%) or faeces (0.6%) of free-living birds, muscle tissues of birds found dead (5.5%) or unfertilized eggs (0.6%). To collect blood or feather samples, birds were captured as 4–10 weeks-old chicks by running after them (43%) or as adults with rocket nets at their breeding sites (57%). The age and sex distribution of the sample was 50.76% adult males, 6.12% adult females and 43.12% chicks. Only in region Madrid we took samples from chicks for several consecutive years (1995–2005), although the possibility of sampling overlapping generations was very small due to the large population size and low breeding success (ca. 1,400 birds, and one young recruited into the adult population per 8 females, unpubl. data). We drew 0.4 ml of blood from the brachial vein and stored it in Queen's buffer (Seutin et al. 1991). Alternatively, we plucked a growing undercover feather from the wing and stored it dry in a plastic bag at 5°C. In Morocco, where the population is severely endangered (Hellmich and

Fig. 1 Map of the study area (Iberian Peninsula and Morocco) showing the present distribution of great bustards (grey colour), sampling localities (black circles) and predefined regions (ellipses): Andalusia (1), Aragón (2), Castilla y León (3), Castilla-La Mancha (4), Extremadura (5), Madrid-North (6), Madrid-South (7), Navarra (8) and Morocco (9)

Idaghdour 2002; Alonso et al. 2003, 2005a), and also in some areas of Andalusia where breeding groups are very small, we only collected feathers from the ground or faeces. Both feathers and faeces have been used successfully as sources for DNA extraction in birds (Taberlet 1991; Idaghdour et al. 2003). To minimize the probability of taking more than one sample from the same individual we collected only fresh faeces after having spent some time observing the birds with telescopes to fix their foraging paths. The faeces were preserved in 70% ethanol and later stored at −20°C. The UTM coordinates were determined for each sample using a GARMIN-12 GPS. Each sample was assigned to one of nine predefined populations/regions (Fig. 1, Table 1), which were delimited according to the main discontinuities in both the distribution of the species and the areas of steppe-like habitat, and considering the movements of a wide sample of birds individually marked and radio-tracked in most of these regions (over 400 birds in total, 1991–2007; Martín 1997, 2001, Alonso et al. 2000, 2001, 2005b; Morales et al. 2000; Martín et al. 2008; Magaña 2007; Palacín 2007). The partition among regions 4, 6 and 7 was based on the presence of Madrid city, nearby suburban areas and infrastructures, and the river Tajo valley, which probably limit dispersal between them. We repeated all analyses dividing our sample into 8, 17 and 19 populations based on slightly different interpretations of habitat discontinuities, and obtained similar results to those with nine populations.

Laboratory analysis

DNA extraction, polymerase chain reaction (PCR) amplification of a 657 base pair (bp) fragment of the mitochondrial control region (Domain I = 283 bp, Domain II = 374 bp), and DNA sequencing were performed using the protocols outlined in Martín et al. (2002). The rapid genetic differentiation and rapid pace of sequence evolution within this mtDNA region (Stoneking et al. 1991), together with its maternal inheritance, makes it a useful tool for the study of the phylogenetic relationships among mtDNA haplotypes and the current genetic structure of the population (Wilson et al. 1985; Avise et al. 1987; Moritz et al. 1987; Avise 1994). Sequences were aligned manually and all variable sites were confirmed by visual inspection of the chromatograms. New haplotype sequences were submitted to the GenBank database under the accession numbers shown in Table 1.

Analysis of molecular data

Initial sequence comparisons, diversity measures of the mtDNA control region -both haplotype diversity (h) and nucleotide diversity (π; Nei 1987)-, Tajima's D, Fu's F and mismatch distributions to assess population size trends across all regions were estimated using DNASP 3.0 (Rozas and Rozas 1999; Rozas et al. 2003). Haplotype diversity was measured as the total number of differences recorded in all possible pairwise comparisons of the haplotypes, and nucleotide diversity is the average proportion of nucleotide differences between all possible pairs of sequences in the sample. These measures provide information about the level of variation in the population and can thereby give indications of demographic history, effective population size, and mutation rates. Sequences of the same haplotype were identified using the computer program Collapse 1.1 (Posada and Crandall 1998). In order to standardize estimates of diversity for sample size we used POPTOOLS (Hood 2005) to randomly resample individuals, creating 100 synthetic populations of equal size (four individuals, corresponding to the smallest population size, Aragón). After boostrapping we used MICROSATELLITE TOOLKIT for Excel (Park 2001) to calculate unbiased expected diversity values for each population. The genealogical relationship between haplotypes was examined by using the median-joining algorithm of Bandelt et al. (1999) implemented in NETWORK 3.0 (http://www.fluxus-engineering.com) to depict phylogenetic, geographical, and potential ancestor–descendant relationships among the identified mtDNA haplotypes.

The presence of genetic structure at a regional level (predefined subpopulations, Table 1) was assessed with the analysis of molecular variance (AMOVA, Excoffier et al. 1992)

Table 1 Geographic distribution of mtDNA haplotypes, number of individuals (N), haplotype diversity (h), and nucleotide diversity (π) within great bustard populations sampled in Iberia and Africa using a 657 bp fragment of the mitochondrial control region

Haplotype	Variable sites[a] 58111112222222234455 70233371444569933926 335607456247863580	Accession numbers	Andalusia	Aragón	Castilla y León	Castilla-La Mancha	Extremadura	Madrid-North	Madrid-South	Navarra	Morocco	*N*
H1	CTTGCCTACCTACAAACGGT	AF421964	4			4		10	4			22
H2	CCTGTCTACTCACAAACGAT	AF421966			10	2	2	18	7			39
H3	CCTGCCTACCTACAGATAAT	AF421967	1		4	2		70	16	11		104
H4	TCTGCCCACTCACAAACGAT	AF421974				1		3				4
H5	CCTGCCCACCTACAAACGAT	AF422106	4	4	9	11		11	6			45
H6	CCTGCCTACCTACAGACGGT	AF422007	1					1				2
H7	CCTGTCTACTTACAAACGAT	DQ445296	5		2		2					9
H8	CCTGCCTACCCACAGATAAT	AF422095	1		8	1	4		5			19
H9	CCTGCCTACCTACAAACGGT	DQ445297	3		5	2	2					12
H10	CCTGCCTACCTGCAAACGAT	DQ445298					3					3
H11	CCTGCCTACCTACAAACGAT	DQ445299	1		1		1					3
H12	CCTGTCTACTCACAAATAAT	DQ445300					1					1
H13	CCTGCCCACCTACAGATAAT	AF422064			5				1			6
H14	CCTGCCTACCCACAAACGAT	AF422060						1	2			3
H15	CTTGCCTACCTACAAACGAT	DQ445301							1			1
H16	CCTGCCTATCTACAAACGAT	DQ445302	2									2
H17	CCTGCCTACCTACAGATGAT	DQ445303	27									27
H18	CCCGCCTACCTACAAACGAT	EU232174									2	2
H19	CCCGCCTACCTACAAATGAT	EU232175									16	16
H20	CCTGCTCACCTACAAACGAT	AF422100							1			1
H21	CCTGCCCACCTATAAACGAT	DQ445304	5									5
H22	CCTACCTACCTACAAACGAT	DQ445305				1						1
N			54	4	44	24	15	114	43	11	18	327
π (%)			0.36	0.0	0.52	0.42	0.53	0.45	0.50	0.0	0.03	
h			0.730	0.000	0.856	0.768	0.886	0.585	0.808	0.000	0.209	
h corrected[b]			0.699	–	0.838	0.750	0.822	0.531	0.788	0.000	0.201	

[a] The sequenced fragments correspond to positions 280–945 in control region in the complete *Ciconia ciconia* mitochondrial genome (AB026818). Letters designate base substitutions. Nucleotide position numbers of the great bustard mtDNA correspond to positions in the alignment

[b] To correct for unequal sample sizes, diversity values standardized to sample size of $n = 4$, corresponding to the number for Aragón, are also given

algorithm and exact tests as implemented by ARLEQUIN version 2.0 (Schneider et al. 2000). In the AMOVA analysis, F_{ST} analogs (Φ_{ST}) were estimated both with and without weighting the haplotype frequencies with pairwise Euclidean distances of nucleotide differences between haplotypes. Both approaches gave similar results and we present only those based on haplotype frequency distributions since it has been suggested that they give more accurate estimates of population subdivision when dealing with numerous haplotypes separated by small numbers of substitutions, and when populations are distinguished by differences in haplotype frequencies rather than clear geographic subdivision and interhaplotypic differences (O'Corry-Crowe et al. 1997). Statistical significance was assessed by generating 1,024 replicate datasets by permutation and determining the proportion of occurrences with values greater than the observed Φ-statistics. The samples were grouped in analyses of molecular variance to find the grouping that maximized the proportion of variation due to differences among groups (F_{CT}) and minimized the proportion of variation due to differences among populations within groups (F_{SC}; Stanley et al. 1992). Exact tests of spatial differentiation were used to test the null hypothesis of the random distribution of haplotypes among populations (Raymond and Rousset 1995). In this analysis, statistical significance was assessed by determining the proportion of contingency tables that have an equal or lower probability of occurrence than the observed distribution of haplotypes as determined by 10,000 iterations of a Markov chain Monte Carlo algorithm (Schneider et al. 2000).

Numbers of migrants per generation (N_em, where m equals the fraction of effective migrants per generation) were inferred from the pairwise F_{ST}'s according to Slatkin (1991) and assuming equilibrium between migration and drift during the time following the coalescence of two populations. Because this assumption is most likely violated in the case of natural populations, non-equilibrium-based estimates of migration rates were also obtained using the program MDIV (Nielsen and Wakeley 2001). We run each simulation 2×10^6 times with a 25% burn-in period, as recommended by Nielsen (2002). Likelihood values for M were plotted and the value with the highest likelihood accepted as the best estimate. A finite-sites (HKY) model was used in all analyses. We compared the non-equilibrium-based estimates of the M-mode with the equilibrium-based values produced in Arlequin, restricting comparisons to regions with more than 10 samples.

To visually inspect genetic differentiation among regions and clusters of genetically related populations we performed both a neighbour joining tree and a multidimensional scaling analysis with estimates of F_{ST} from all possible pairwise comparisons of populations as dissimilarity measure, using STATISTICA V.6 (StatSoft 2001). Both analyses gave similar results, and here we only present those of the second. Finally we examined the data for evidence of isolation-by-distance in our study area. To do this, the relationship between genetic distance among all pairs of populations (F_{ST}) and the logarithms (log) of the corresponding geographic (straight-line) distances between the central coordinates of all pairs of populations was investigated. To estimate the significance of this relationship, we conducted a Mantel test (Mantel 1967) using Mantel for Windows (Cavalcanti 1999), with 1,000 randomizations. To investigate whether males and females followed different patterns we performed the Mantel test separately for each sex.

Results

mtDNA sequence variation

The 657-bp mtDNA fragment revealed 20 variable sites defining 22 haplotypes in our sample of 327 individuals (Table 1, Fig. 2). Twenty of these haplotypes were present in the Iberian Peninsula and two in Morocco (H18 and H19). The overall nucleotide diversity (π) was 0.48%, while the haplotype diversity (h) was 0.85. Diversity was highly variable between populations, ranging from 0.00 in Aragón and Navarra (respectively fixed for haplotypes H5 and H3) to high values in Extremadura (h = 0.886, π = 0.53%, Table 1). These figures were very similar to expected diversity values corrected for inter-population variation in sample size (0.000 to 0.838, see Table 1).

The four most frequent haplotypes (H3, H5, H17 and H2) were present in 66% of the birds, although none was found

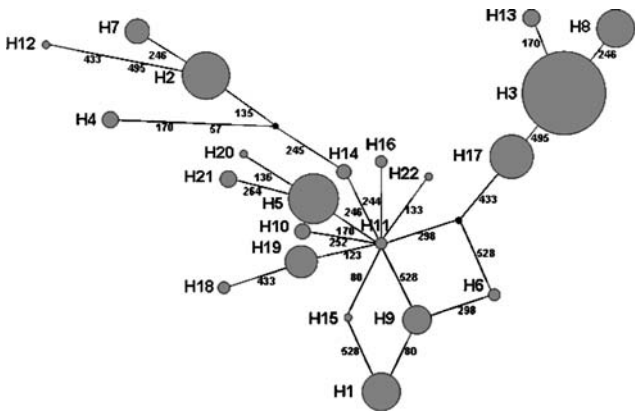

Fig. 2 Median-joining network of 22 haplotypes found among 327 great bustard mtDNA sequences (657 bp). The labels beside each circle indicate the haplotype designations as given in Table 1. The areas of the circles are proportional to the overall abundance of the different haplotypes. Branches between haplotypes represent mutations labelled by position as indicated in Table 1

in all populations (Table 1). Five regions had unique haplotypes: Morocco (H19, H20), Andalusia (H16, H17, H21), Extremadura (H10, H12), Madrid-South (H15, H20) and Castilla-La Mancha (H22). Eleven haplotypes occurred only in one region. However, unique haplotypes were generally rare, only occurring in one, two or three individuals (four, two, and one haplotypes respectively). Exceptions were H19, the most frequent in Morocco, and H17 and H21, widespread in Andalusia (Table 1).

The median-joining network showed that most haplotypes appeared to be related and removed by one or two substitutions from their nearest neighbour (Fig. 2). Moreover, 64% of them were within two mutational events of H11, which was central in the network. This star-like pattern is characteristic of a past population expansion from that, most probably ancestral, haplotype H11 found in three regions. Several branches, however, protrude significantly beyond this limit, suggesting that most of these branches are not derived from H11 but are the result of more ancient expansions from other roots.

Fu's (Fu 1997) F_s statistic (−3.23, $P = 0.017$) but not Tajima's D (0.66, $P > 0.1$) were significantly different from zero, suggesting either recent population expansion or purifying selection in the Afro-Iberian population. In addition, the distribution of the number of pairwise differences between sequences (the mismatch distribution) revealed a smooth and unimodal mismatch curve, consistent with exponential population growth (Fig. 3). Harpending's raggedness index (Harpending 1994) was 0.452, also suggesting a very good fit of the empirical data to a sudden expansion model.

Our samples from Morocco and Iberia shared no haplotypes according to the rapid evolving mtDNA fragment sequenced (Table 1). In Morocco we only found two haplotypes, one more widespread (H19), and a second one found only in a male in the North and a female in the southern limit of the species' distribution range in this country. It is also apparent that the haplotype and nucleotide diversity is substantially higher in Iberia than in Morocco (Iberian populations combined: $\pi = 0.48\%$, $h = 83.2\%$, $n = 309$; Morocco: $\pi = 0.03\%$, $h = 20.9\%$, $n = 18$).

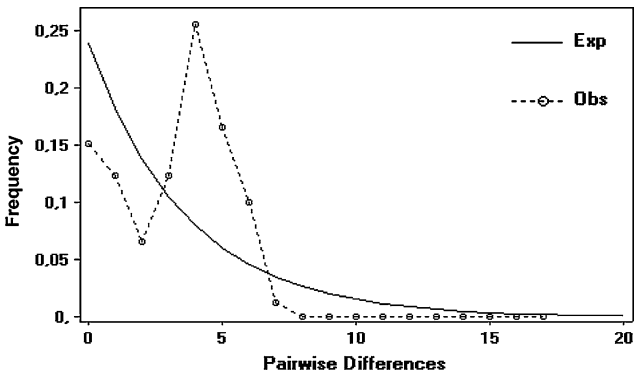

Fig. 3 Mismatch distribution for sequences from the entire sample showing consistency with sudden population expansion. The expected distribution is based on model of constant population size

Population structure

Pairwise comparisons between populations were used to determine the level of genetic differentiation among regions (Table 2). *F*-statistics yielded significant values ranging from 0.042 to 1.000. Population comparisons all showed significant partitioning except for: Aragón versus Castilla-La Mancha, and Castilla y León versus Extremadura. Better resolution might be possible with larger sample sizes from Aragón. Exact test of spatial differentiation yielded similar results (Table 2).

The multidimensional scaling analysis based on F_{ST} values as dissimilarity measure showed a clear division between Morocco and the Iberian Peninsula (Fig. 4). Furthermore, the haplotype composition in Aragón, Navarra and Andalucía, the regions holding the most peripheral populations within the Iberian Peninsula, also differed from the main populations in central Spain.

The results of AMOVA revealed that a large percentage of the total mtDNA variation was distributed within populations (73.6%) and a lower but significant percentage among populations (26.4%; $P < 10^{-5}$). Grouping our nine populations in different ways in AMOVAs resulted in an increase of global F_{ST} (see Table 3). Maximum F_{CT} values were obtained considering two populations, one in the Iberian Peninsula and one in Morocco. The subdivision in five populations (Morocco, Navarra, Aragón, Andalusia, and rest of Iberia) minimized the F_{SC} values keeping reasonably high F_{CT} values. The AMOVA with only Iberian samples giving highest F_{CT} values (0.162) and lowest F_{SC} values (0.146) was the one considering four groups: Navarra, Aragón, Andalusia, and rest of Iberia (Table 3).

About 57% of the compared population pairs have classical Nm estimates greater than 1.0 to above 15.0, which indicate that migration is approaching levels sufficient to overcome genetic divergence among populations caused by drift (Table 4, below diagonal). High levels of migration were estimated among populations on the Iberian Peninsula.

As expected, estimates of migration between the populations on the Iberian Peninsula and the relict population in Morocco were quite low. However, point estimates of migration rates between populations were lower in 24 of 28 comparisons using the coalescent-based method in MDIV that does not make assumptions about equilibrium (Table 4, above diagonal). This is probably because the process of genetic drift within the Iberian populations has not reached equilibrium yet, which would violate the assumptions of F_{ST} analysis but not of coalescent analysis.

Table 2 Pairwise comparisons between Afro-Iberian great bustard populations

	Andalusia	Aragón	Castilla y León	Castilla-La Mancha	Extrem.	Madrid-N	Madrid-S	Navarra	Morocco
Andalusia	–	*	***	***	***	***	***	***	***
Aragón	0.408***	–	–	–	***	**	–	***	***
Castilla y León	0.183***	0.251**	–	**	**	***	***	***	***
Castilla-La Mancha	0.211***	0.099	0.057**	–	***	***	**	***	***
Extrem.	0.183***	0.392***	0.032	0.148***	–	***	***	***	***
Madrid-N	0.335***	0.486***	0.205***	0.247***	0.290***	–	***	–	***
Madrid-S	0.211***	0.325**	0.051**	0.094**	0.110**	0.042*	–	–	***
Navarra	0.490***	1.000***	0.394***	0.489***	0.514***	0.114*	0.231***	–	***
Morocco	0.463***	0.831***	0.404***	0.489***	0.470***	0.526***	0.431***	0.871***	–

Below the diagonal are estimates of F_{ST} and above the diagonal the results of the exact test of spatial differentiation. Significance values: *$P < 0.05$, **$P < 0.01$, ***$P < 0.0014$ (significance value after Bonferroni correction)

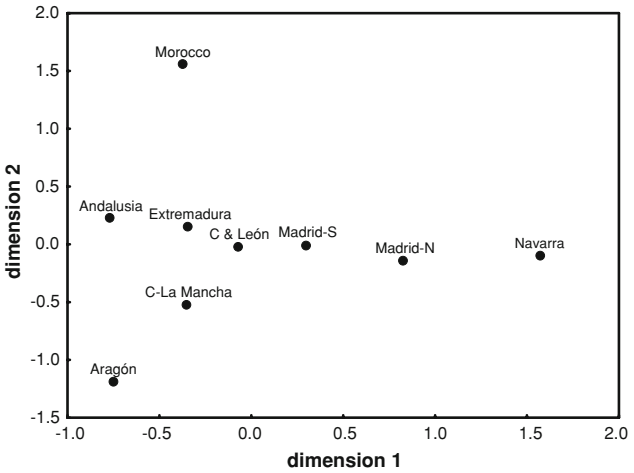

Fig. 4 Results of the multidimensional scaling analyses based on the genetic distances (F_{ST}) obtained from the haplotype composition of the populations studied. The stress value for the two-dimension representation was 0.069

Geographical distance and genetic differentiation among populations

We found that pairwise genetic differences between the nine populations increased with geographical distance (Mantel test $r = 0.469$, $t = 1.912$, $P = 0.028$). However, the increase became insignificant when we excluded Morocco ($r = 0.205$, $t = 0.811$, $P = 0.209$). The same results were obtained for each sex separately and using only populations with ≥ 3 samples (males: $r = 0.561$, $t = 1.882$, $P = 0.030$; excluding Morocco, $r = 0.336$, $t = 1.287$, $P = 0.099$; females: $r = 0.628$, $t = 2.240$, $P = 0.013$; excluding Morocco, $r = 0.432$, $t = 1.369$, $P = 0.086$). These results suggest a weak isolation-by-distance effect in the genetic structure of great bustards in the study area.

Discussion

In spite of our large and widely distributed sample, only 22 haplotypes were identified and the four most frequent ones

Table 3 Results of subdivision analyses of molecular variance with different populations groupings ranked according to F_{CT} values

Group composition	F_{SC}	F_{ST}	F_{CT}
2 groups: Morocco, Península	0.22429	0.45157	0.29300
4 groups: Morocco, Navarra, Aragón, rest of Iberia	0.20584	0.40631	0.25244
3 groups: Morocco, Navarra, rest	0.21405	0.40833	0.24719
5 groups: Morocco, Navarra, Aragón, Andalusia, rest of Iberia	0.15191	0.33962	0.22133
4 groups: Morocco, Navarra, Andalusia, rest of Iberia	0.16623	0.33757	0.20550
3 groups: Morocco, Andalusia, rest of Iberia	0.17940	0.34603	0.20305 ns
2 groups: Andalusia, rest of Iberia	0.24134	0.30323	0.08159 ns
Group composition excluding Morocco	**F_{SC}**	**F_{ST}**	**F_{CT}**
4 groups: Andalusia, Navarra, Aragón, rest of Iberia	0.14646	0.28513	0.16247
3 groups: Navarra, Aragón, rest of Iberia	0.19920	0.32839	0.16133
2 groups: Navarra, rest of Iberia	0.20715	0.30780	0.12694 ns
2 groups: Andalusia, rest of Iberia	0.17309	0.28273	0.13259 ns

All F_{SC}, F_{ST} and F_{CT} P-values were <0.05 except when indicated by ns (not significant)

Table 4 Classical estimates of migration rates (N_em) among great bustard populations with $n \geq 11$ samples based on N_{ST} (below diagonal)

	Andalusia	Navarra	Morocco	Madrid-S	Madrid-N	Extremadura	C-La Mancha	Castilla y León
Andalusia	–	0.30	0.02	1.36	0.82	2.04	3.36	5.64
Navarra	0.52	–	0.01	0.42	3.32	0.13	0.26	0.42
Morocco	0.58	0.07	–	0.01	0.02	0.03	0.02	0.02
Madrid-S	1.87	1.67	0.66	–	5.54	1.02	3.52	3.57
Madrid-N	0.99	3.89	0.45	11.31	–	4.02	2.38	1.46
Extremadura	2.27	0.47	0.56	4.06	1.23	–	1.23	3.08
C-La Mancha	1.87	0.52	0.52	4.81	1.53	2.88	–	3.86
Castilla y León	2.23	0.77	0.74	9.35	1.94	15.33	8.31	–

Values above the diagonal are nonequilibrium.based estimates of migration rates (M = Nm for mtDNA) by MDIV

were common (present in a 66% of the birds) and three of them geographically widespread (found respectively in 6, 6 and 5 of the nine regions defined). Overall, nucleotide diversity (π) values for populations within this species were comparable with published values on other bird populations (Milot et al. 2000; Zink et al. 2001; Sgariglia and Burns 2003; Shepherd and Burns 2007).

Phylogeographic structure

A previous study showed that gene flow between Iberian and central European great bustard populations might have been restricted by the Pyrenean mountains during the last glacial period (Pitra et al. 2000). We proposed that these two populations should be considered separate evolutionary significant units (ESUs, Avise et al. 1987), and emphasized the need to study the genetic structure at a lower geographical scale in order to identify further unique local populations which could be managed as separate units (Moritz 1994). The results of the present study also show clear differences between Moroccan and Iberian bustards, which are separated by the Strait of Gibraltar, and below we discuss to what extent this could constitute a geographical barrier limiting gene flow. Within the Iberian Peninsula all sampled populations were differentiated mainly by shifts in haplotype frequency, which probably indicates extensive post-glacial exchange. Only the most peripheral populations (Navarra, Aragón and Andalusia) differed significantly from the main ones found in central Spain. Although some mountain chains could also limit dispersal in central Spain (e.g., Sistema Central between Castilla y León and Madrid, and Sierra Morena between Extremadura and Andalusia), these mountains are not as high as the Pyrenees, and there are observations of bustards crossing the Sistema Central, whereas adult males fly regularly over Sierra Morena during their seasonal migrations (Palacín 2007; Palacín et al. 2007). Thus, gene flow has been possible among all these regions over historical time scales via intermediate populations.

We conclude that there are no obvious geographical barriers that might have contributed to the separation of bustard populations within the Iberian Peninsula, and suggest that population structure has probably evolved through different processes such as demographic fluctuations, bottlenecks, founder effects (i.e. colonization of females with different haplotype composition), or a combination of these. The observed differences may have been maintained afterwards by a low level of gene flow due to isolation-by distance (Wright 1943; Avise 1996). For example, on the one hand recent extinction of local breeding groups could have contributed to the genetic divergence of some marginal populations. In fact in Andalusia and Aragón the number of extinct breeding groups recorded during the last 50 years has been proportionally higher than in other Iberian regions (14 in Andalusia, 4 in Aragón, 11 in all other Spanish regions, with current breeding population sizes of, respectively, 360, 130 and >23,000 birds, Alonso et al. 2003). On the other hand, the populations in Morocco, Navarra and probably Aragón show extremely low levels of genetic variation, although in the latter region both our sample size and the population size are small. They are also very small and geographically separated from other bustard groups by long distances. These results suggest that these populations might have been colonized in historical times by few birds (isolated founder events) or have undergone historical bottleneck events. In contrast, the high genetic diversity and small bird numbers in Andalusia nowadays could be interpreted as remnant of a larger ancestral population size (Allard et al. 1994; Lahanas et al. 1994). This population was indeed much larger in a recent past, and in spite of having been decimated during the second half of the twentieth century, it has probably retained much of its former genetic diversity.

In sum, a plausible scenario for the evolution of the observed genetic structure of Afro-Iberian great bustards could be as follows. The star-like pattern centred on H11 suggests this could have been the ancestral haplotype,

which possibly originated in central Spain. The genealogical relationships between haplotypes suggest that Moroccan great bustards diverged relatively early from Spanish birds, and that radiation of Moroccan haplotypes is probably a recent event (see Fig. 2). Birds could have remained in a glacial refuge in souhwestern Iberia (Extremadura, Andalusia), and later a postglacial colonization of the central Iberian region would have taken place by accidental immigration of haplotypes. Finally, a recent colonization probably occurred in Navarra and Aragón. Inferences about population history derived from analyses in Arlequin, such as $F_{ST,}$ are based on the assumption that populations are at equilibrium between drift and migration. Given that the mismatch distribution for the entire sample is unimodal, we expect that populations have recently undergone expansions and range shifts. Use of the MDIV program provides alternative estimates of gene flow to the equilibrium methods in Arlequin. The general agreement in this study between results derived from MDIV and Arlequin suggests that departures from equilibrium were not sufficiently large to bias results.

Gene flow Iberia-Morocco

A recent study reported moderate gene flow between Spanish and Moroccan great bustard populations (Broderick et al. 2003). These authors combined mitochondrial control region domain II (Ctr II) and cytochrome *b* gene (cyt *b*) fragments. However, since cyt *b* genes are known to evolve slowly, they should not be expected to offer much resolution for populations that have diverged very recently. In contrast, the most rapidly evolving segments of the mitochondrial genome can be very informative when traditional mtDNA markers show low or inadequate levels of genetic variability. Based on the distribution of the variable nucleotide positions and differential nucleotide frequencies in different parts of the control region, it is divided into three domains (Brown et al. 1979; Moritz et al. 1987). In most bird species studied so far, intraspecific variability, expressed as both nucleotide substitutions and deletions/insertions, has been largely found in the flanking domains I and III, whereas the central domain II has been the least variable (e.g. Wenink et al. 1993, 1994; Edwards 1993; Marshall and Baker 1997; Zink and Blackwell 1998).

In our study we have re-examined the relationships between Spanish and Moroccan populations using a fragment of the mitochondrial control region including the domain I to increase the number of informative characters and the resolution power in population differentiation compared with more conservative Ctr II and cyt *b* markers used by Broderick et al. (2003). We found no haplotypes shared by both populations, and thus no evidence of current genetic flow between them. However, our study was conducted with a single mtDNA locus, and current evidences of genetic structure based on haplotype frequencies should be confirmed with a study of nuclear DNA variation. Our results compared to those of Broderick et al. (2003) illustrate the importance of selecting an appropriate molecular marker in studies dealing with genetic variation within species. The variability exhibited by the molecular marker will determine the results and therefore highly variable molecular markers are needed in studies assessing the presence of genetic structure and gene flow between populations.

Our finding of exclusive haplotyes in Morocco is in line with the lack of recent observations of birds crossing the Strait of Gibraltar, in spite of frequent and intensive migration studies that have been carried out by many ornithologists in this area (SEO/BirdLife 2001). Ornithological reports from the late nineteenth and early twentieth centuries indeed suggest that some individuals from the Andalusian population could have crossed the Strait of Gibraltar (Saunders 1871; Irby 1895; Chapman and Buck 1893; Hartert and Jourdain 1923). It is reasonable to assume that at that time the Andalusian population was much larger than today (only 35 years ago it was estimated at more than 1,000 birds, Trigo de Yarto 1971).

In summary, our results suggest that Morocco was colonized from the Iberian Peninsula thousands of years ago, probably by birds belonging to few matrilines close to H11 which later originated new and exclusive African haplotypes (i.e. H18 and H19). Although a male-biased dispersal across the Strait of Gibraltar cannot be excluded, long-term isolation of the two mtDNA gene pools is likely to have occurred, possibly as a consequence of the high philopatry of females, and the barrier effect of the Strait of Gibraltar. After a long period of genetic divergence, the isolation between them has been enhanced in recent times due to the population declines at both sides of the Strait (Alonso et al. 2003, 2005a, b).

Implications for conservation

The genetic structure described in this study has important implications for management. Based on distribution and demographic criteria, the four most endangered populations within our study area were Morocco, Andalusia, Aragón and Navarra. All of them were small and peripheral with respect to the main population in central Spain. Together they comprised only some 550 birds, i.e. 2% of the ca. 25,500 birds estimated in Iberia plus Morocco. However, 5 of the 22 haplotypes identified in our study were exclusive of two of these populations (2 in Morocco, 3 in Andalusia). Although further sampling could lead to the finding of these haplotypes elsewhere, current data suggest that unique Andalusian and Moroccan haplotypes

represent a significant fraction of the genetic pool identified in Afro-Iberia, and thus these populations deserve the highest conservation effort.

The populations breeding in Navarra and Aragón are indeed very small (respectively, ca. 40 and ca. 130 birds, Alonso et al. 2003) and show extremely low genetic diversity values, with only one haplotype identified in each of them, which could increase their extinction probabilities in the near future (Frankham 1995). Geographically they are not very far from nearby bustard regions, and at least one young from Navarra established in Castilla y León and one from Aragón visited the region Madrid during its juvenile dispersal, showing that current gene flow is possible. However, we had no records of our large sample of radio-tagged birds from the nearby larger regions (Castilla y León, Castilla-La Mancha, Madrid) establishing in Navarra or Aragón as breeding adults (unpubl. data). In earlier studies (Alonso et al. 2004) we have shown that one of the factors determining population dynamics and natal dispersal in great bustards is conspecific attraction, by which dispersing individuals use the number of conspecifics as indicators of habitat quality to select breeding sites (Stamps 1988; Smith and Peacock 1990; Reed and Dobson 1993; Danchin and Wagner 1997). If small breeding groups in Navarra and Aragón do not attract dispersing birds from neighbour regions, their genetic diversity will remain low and the inbreeding depression risk high.

The Andalusian subpopulation living in the Guadalquivir river basin (Córdoba, Jaén, Sevilla and Huelva) is estimated at ca. 250 birds (Alonso et al. 2005b) and the species is considered here critically endangered with extinction (Junta de Andalucía 2001). As for the Moroccan population, it is estimated at some 100 birds and considered one of the most endangered worldwide (Hellmich and Idaghdour 2002; Alonso et al. 2005a). The extinction of these two populations (only ca. 1.5% of the birds in the Afro-Iberian region) would cause a loss of 23% of the 22 existing matrilineages from the species' current genetic pool in this geographic area. Therefore, special conservation efforts should be directed towards protecting them in order to preserve the current biodiversity and metapopulation structure.

In spite of low numbers, the genetic diversity in Andalusia was not low. We found there 11 of the 20 haplotypes identified in Iberia, suggesting that Andalusian bustards share a common origin with other Iberian bustards and that in recent times there has been at least some gene flow between them. On the other hand, 90% of a sample of radiotagged adult males breeding in Andalusia abandoned their leks after mating and most migrated in summer to very distant (up to 250 km) areas in Castilla-La Mancha or Extremadura (Palacín et al. 2007). This was the case of e.g. H8 and H11, found in Andalusia only in two adult males that migrated in summer respectively to Extremadura and Castilla-La Mancha, which suggests they could have hatched in these regions and established as breeding adults in Andalusia. However, three haplotypes were exclusive to Andalusia, showing a certain degree of isolation from other Iberian populations. Our radiotracking studies have shown that natal dispersal is significantly reduced when a population is isolated from nearby breeding groups (Martín et al. 2008). Indeed, none of the young birds marked in Andalusia visited other regions during their juvenile dispersal period, and all established in Andalusia as breeding adults, probably due to the disappearance of stepping-stone groups between this and neighbour regions. This situation contrasts with that mentioned above for Navarra and Aragón, where connectivity with neighbour populations has been demonstrated through radiotracking.

As for Morocco, the two haplotypes found there were also exclusive to this population. We could not mark birds in this country, but given that this population is even more isolated from other Iberian groups than Andalusian birds, natal dispersal events between Iberia and Morocco should be considered at present an extremely rare event. The closest breeding groups in southern Andalusia have the lowest annual productivity values recorded for any bustard population (Alonso 2007). Thus, it is unlikely that the Moroccan population would be re-colonised by immigration from Spain should it once go extinct.

In conclusion, Andalusia and Morocco hold a significant proportion of the great bustard current genetic diversity. Furthermore, bustards living in these two regions could be regarded as separate genetic management units (sensu Moritz 1994). Given their extremely threatened status, both populations deserve the highest conservation efforts in order to safeguard the biodiversity of the Afro-Iberian metapopulation, which represents the last stronghold of this globally endangered species.

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