THE ROYAL SOCIETY

Received 13 July 2001
Accepted 5 September 2001
Published online 21 December 2001

Great bustard population structure in central Spain: concordant results from genetic analysis and dispersal study

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We found significant sex differences in the mtDNA genetic structure and dispersal patterns of great bustards in a population of 11 breeding groups, 'leks', in central Spain. The analysis of genetic distances showed that the female population was divided into three groups of leks separated by *ca.* 50 km, whereas male haplotypes were randomly distributed among leks. Genetic distances among pairs of leks were positively correlated with geographical distances in females but not in males. While female haplotype distributions were homogeneous among leks at close distances, differences in male genetic structure were highly variable even between two close leks. These results from genetic analyses were concordant with those from a radiotracking study on natal dispersal. Natal dispersal distances were higher in males than in females. Also, the frequency of movement of a female between two leks was positively correlated with their genetic affinity and geographical proximity. In males, the frequency of movement was correlated with geographical proximity but not with genetic affinity. Males dispersed among genetically unrelated leks, contributing to keep nuclear genetic diversity in the population, whereas females tended to be philopatric. These results suggest that isolation-by-distance influences the distribution of maternal lineages at a regional level.

Keywords: great bustard; mtDNA; population structure; dispersal

1. INTRODUCTION

Great bustards (*Otis tarda*) are large, sexually dimorphic birds living in dry grassland habitats from the Iberian peninsula and northwestern Morocco to eastern Asia (del Hoyo *et al.* 1996). The species shifted habitat use from the primeval natural steppes to cereal agrosteppes, reaching maximum range and largest total population during the 18th century, when bustards benefited from agricultural expansion in Europe (Glutz *et al.* 1973). Since then, most great bustard populations have declined, due mainly to habitat loss and hunting. The species is currently categorized as globally threatened (Collar *et al.* 1994), with a last stronghold of about 20 000 individuals still surviving in the Iberian Peninsula (Alonso & Alonso 1996). Although widely distributed, their range is highly fragmented and the species now occurs in distinct units in which it is common to find only a few tens of birds.

Landscape fragmentation due to rapidly expanding human development is one of the major threats, causing habitat deterioration and endangering the survival of many vulnerable species (Murphy 1988). The ability of these species to move among patches of suitable habitat depends on the physical distance between such patches but also on the dispersal behaviour of the species. Although management of species threatened by habitat fragmentation processes has been traditionally based on demographic studies, the use of mtDNA analysis has been increasingly accepted as a powerful tool for describing genetic diversity and to assist demographic studies in designing conservation strategies. Ideally, both genetic and demographic studies should be tightly linked and would provide concordant or complementary results, enhancing their potential conservation relevance (Moritz 1994*a*; Avise 1995).

To develop adequate conservation strategies for great bustards it is important to understand their phylogeographical structure, so that they can be managed on the basis of historical partitions and current genetic differentiation (Avise *et al.* 1987; Moritz 1994*a*). A recent study based on nuclear and mtDNA analyses showed that in Europe the species is subdivided into two different phylogeographical units (evolutionary significant units (ESUs)) (Moritz 1994*a*,*b*), respectively in the European mainland and the Iberian Peninsula (Pitra *et al.* 2000). The existence of such macro-geographical partitioning at a continental level is probably explained by the presence of a significant topographic barrier, the Pyrenean mountains. However, studies at a micro-geographical scale (regional level) are also necessary to detect additional significant genetic differentiation. An ESU may include various demographically independent breeding populations, which should be managed as separate units (Management Units (MUs) (Moritz 1994*a*,*b*)). Genetic differentiation between populations may be enhanced in species with strong female philopatry and hence matrilineal population structure. The great bustard might be such a species, since females are highly philopatric, returning as a rule to their

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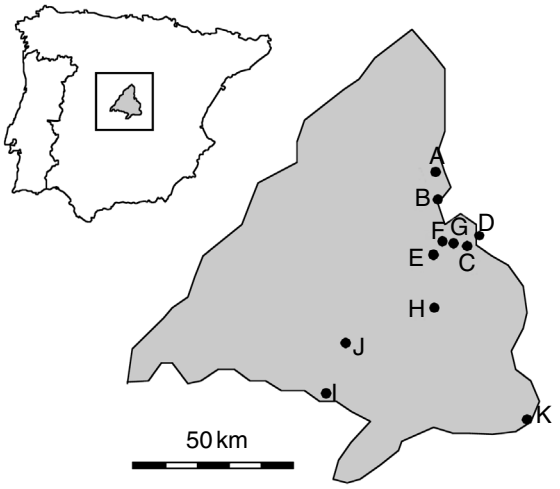

Figure 1. Map of the Iberian Peninsula showing the location of Madrid Province and the great bustard leks where blood samples were collected.

natal sites after juvenile dispersal, while males usually establish as adults outside their natal areas (Martín 1997; Alonso *et al.* 1998).

In the present study we examined the genetic structure of a great bustard population in central Spain using nucleotide sequence variation within the mitochondrial control region. Our main hypothesis was that sex bias in natal dispersal (Alonso *et al.* 1998) would result in a geographical structuring of the population even at a small regional scale (see Avise 1995). Such genetic structure should have produced independent demographic units (Moritz 1994*a*,*b*), which would be of potential conservation relevance. 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). We also investigated the natal dispersal patterns of radiotagged individuals among different subpopulations within our study area. Most authors have highlighted the advantages of using both genetic and demographic approaches simultaneously in assessing population structure and drawing relevant conclusions about conservation (Moritz 1994*a*; Avise 1995; Bossart & Prowell 1998; Haig 1998).

2. MATERIAL AND METHODS

(a) *Study area and species*

Blood samples and radiomarked birds were collected in 11 leks of Madrid Province (figure 1), which holds around 1000 individuals (Martín 2001), representing *ca*. 2% of the estimated total world population. Great bustards are distributed in flat to slightly undulating areas of the northeastern and southern parts of the province. Their main habitat is extensive, 2 year rotation cereal cultures (wheat and barley), crops such as legumes, olive trees and vineyards.

Great bustards are highly sexual dimorphic polygynous birds that gather for mating at traditional display areas known as 'leks'. Females select their mates among displaying males in March–April, and nest generally close to the lek where they copulated. They take over all brood-caring duties, usually raising a single chick. For the rest of the year, flocks containing both male and female adult birds are very rare.

(b) *Sample collection and sequencing of mtDNA*

Between 1995 and 1999 we captured 210 chicks at their natal sites when they were still dependent on their mothers at ages of 4–10 weeks. In the winter of the same years 59 adult males were captured at their breeding leks. We withdrew 0.4 ml of blood from their brachial veins and stored the samples in Queen's buffer (Seutin *et al.* 1991) at −70 °C. All adult males and 190 chicks were also provided with radiotransmitters. Total genomic DNA was isolated using the QIAamp blood and tissue kit (Qiagen; http://www.qiagen.com). We used PCR (Saiki *et al.* 1985) to amplify a 657 bp fragment from the variable parts of control regions I and II of the mtDNA. Primers were CtrIaL 5′-ATA-TCG-TGC-ATA-CAT-TTA-TAT-TCC-C-3′ and H772 (*Calidris alpina* (Wenink *et al.* 1993)). PCR reaction mixtures contained 0.8 U AmpliTaq DNA Polymerase (Perkin Elmer; http://www.perkinelmer.com), 10 mM tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTPs, 10–50 pmol of each primer and 100–500 ng of DNA in a final volume of 50 μl. Reaction mixtures were subjected to the following PCR cycling protocol on a GeneAmp 2400 cycler (Perkin Elmer): initial denaturation (94 °C, 3 min), 30 cycles (94 °C, 15 s; 53 °C, 20 s; 72 °C, 45 s) and final extension (72 °C, 7 min). PCR products were purified using the QIAquick PCR purification kit (Qiagen), and directly sequenced with automated ABI 373/310 sequencers using the fluorescent Prism BigDye Terminator Cycle Sequencing kit (Perkin Elmer). The amplified fragment was very long, therefore, it was necessary to include two nested primers in the sequencing procedure: CtrIIoH 5′-AAA-GAA-TGG-GCC-TGA-AGC-TAG-3′ and L438 (*Calidris alpina* (Wenink *et al.* 1993)). We analysed all blood samples from adult males ($n = 59$) and those from chicks that we knew with complete certainty were not directly related ($n = 85$). Sequences were submitted to the GenBank database (accession nos AF421964–AF422107).

(c) *Statistical analyses*

The mean number of nucleotide differences between haplotypes, overall nucleotide diversity and gene flow (Nm) values were computed using the program DnaSP 3.0 (Rozas & Rozas 1999). Following Roff & Bentzen's (1989) recommendations for small sample sizes, heterogeneity of haplotype distribution among leks was tested for significance using a Monte Carlo χ^2-test and 10 000 randomizations. This test has been shown to be appropriate for datasets in which many elements (in our case, haplotypes) occur less than five times (Roff & Bentzen 1989).

Two approaches were used to measure the degree of genetic differentiation among leks. First we calculated ϕ_{ST}, an analogue of the standard *F*-statistic (Wright 1951; Cockerham 1969), using the program Amova (Excoffier *et al.* 1992): ϕ_{ST} was calculated both with and without weighting the haplotype frequencies with pairwise Euclidean distances of nucleotide differences between haplotypes. The second parameter used to measure the degree of genetic differentiation among leks was *Dxy*, the mean number of nucleotide substitutions per site among populations *x* and *y* (Nei 1987), using the program DnaSP 3.0 (Rozas & Rozas 1999). *Dxy* always takes positive values and hence shows a clearer relation with other variables. To calculate ϕst and *Dxy* we used those leks with more than three samples sequenced.

A multidimensional scaling analysis was performed using SPSS for Windows (SPSS Inc. 1999), and χ^2 from haplotype

Table 1. Percentages of the 10 mtDNA haplotypes obtained from the 11 leks sampled (young birds, adult males). (Last line shows the number of samples sequenced in each lek, which are named as in figure 1.)

	leks											
haplotype	A	B	C	D	E	F	G	H	I	J	K	total A–K (%)
1	7,	, 33	27,	13,	9	11,	25		9, 20	, 25	25,	11, 10
2	7, 38	29,	9, 38	13, 25	9	11,		29, 25	36,	33,		16, 14
3	64, 38	57, 67	64, 50	75, 50	64	67, 80	50	71, 75	9, 20	67, 38	25, 71	56, 53
4	, 25											0, 3
5	14,	14,	, 13	, 25	18	11, 20	25		, 40	, 13	25, 29	8, 15
6	7,											1, 0
7									18,	, 25	25,	4, 3
8									9,			1, 0
9									18,			2, 0
10									, 20			0, 2
total (*n*)	14, 8	7, 6	11, 8	8, 4	11, 0	9, 5	0, 4	7, 4	11, 5	3, 8	4, 7	85, 59

distribution as a dissimilarity measure, to group the leks by their haplotype composition. 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 leks and the logarithms (log) of inter-lek geographical distances was investigated. To estimate the significance of this relationship, we conducted a Mantel (1967) test using MANTEL FOR WINDOWS (Cavalcanti 1999), with 10 000 randomizations.

(d) *Dispersal study*

We radiotracked 32 individuals (16 males and 16 females) caught as chicks at their natal leks throughout their dispersal phase until their settlement as adult birds at an age of 3–5 years. All radiotagged individuals were located from aircraft or ground vehicles and by subsequent visual observation at least once per month. Natal dispersal distance was defined as the straight line between the natal site and the site selected for the first breeding attempt. To study sex differences in natal dispersal the Mann–Whitney test was used. With all radiolocation data we calculated the frequency of inter-lek movements for all pairs of leks using the percentage of males or females marked at one of them and later located at a different lek. Finally, we investigated the relationship between pairwise frequency of inter-lek movements and pairwise genetic distances among leks.

3. RESULTS

(a) *Frequency and diversity of haplotypes*

Sequence analysis of 657 bp of the mtDNA control region revealed 11 variable sites defining 10 haplotypes among 85 young and 59 adult male great bustards from 11 leks (see electronic Appendix A, available on The Royal Society's Publications Web site). The mean number of nucleotide differences between haplotypes was 3.008, and overall nucleotide diversity was 0.004 75, i.e. within the range of 0–0.025 found by Moore (1995) for 34 bird species. Total haplotype diversity was 0.641 ± 0.050 for chicks and 0.681 ± 0.055 for adult males.

Haplotype 3 was the most abundant, and the only one present in all leks (table 1). Haplotypes 1, 2 and 5 were also relatively frequent, only absent in one or two leks. Haplotype 7 was only found in leks I, J and K, and haplotypes 8, 9 and 10, only in lek F. Finally, haplotype 4 appeared only in two adult males in lek A1 and haplotype 10 in just one male in lek F.

(b) *Genetic differences among leks*

Within the sample of young birds, overall ϕ_{ST} was 0.036, indicating a low degree of genetic differentiation among leks. This ϕ_{ST} estimate is equivalent to a gene flow (Nm) of 13 females migrating among leks per generation. Comparisons among leks A–H resulted in negative values of ϕ_{ST}, indicating genetic homogeneity within this group of leks (see electronic Appendix B*a*). However, all comparisons of leks A–H with lek I, and most comparisons with lek K, gave positive and much higher values of genetic distance. Monte Carlo χ^2-tests of haplotype frequencies only revealed significant differences ($p < 0.05$) between I and A, C, D, E, F and H. Finally, multidimensional scaling analysis based on haplotype composition also distinguished three groups of leks, A–H, I and K (figure 2*a*). A hierarchical subdivision analysis was performed based on these groups, resulting in a lek divergence $\phi_{ST} = 0.181$ (Nm = 2.3). The 23% genetic variance among groups indicated a clear divergence between lek groups A–H, I and K.

A similar analysis was performed with adult males. We found no genetic differentiation in their haplotype composition among leks (overall $\phi_{ST} = 0.013$, pairwise comparisons ranging from -0.212 to 0.159; see electronic Appendix B*b*), and no clear grouping of leks (figure 2*b*).

(c) *Patterns of dispersal*

Breeding dispersal, i.e. the change in breeding site between consecutive breeding attempts, is rare in adult great bustards (Alonso *et al.* 2000, 2001; Morales *et al.* 2000). Thus, gene flow among leks occurs mostly through natal dispersal, i.e. movements between natal and breeding sites. Seventy per cent of the 16 males radiotracked throughout their dispersal phase settled as adults at different leks from their natal leks, while 75% of the 16 females of our sample returned to breed at their natal leks. The median natal dispersal distance was significantly higher in

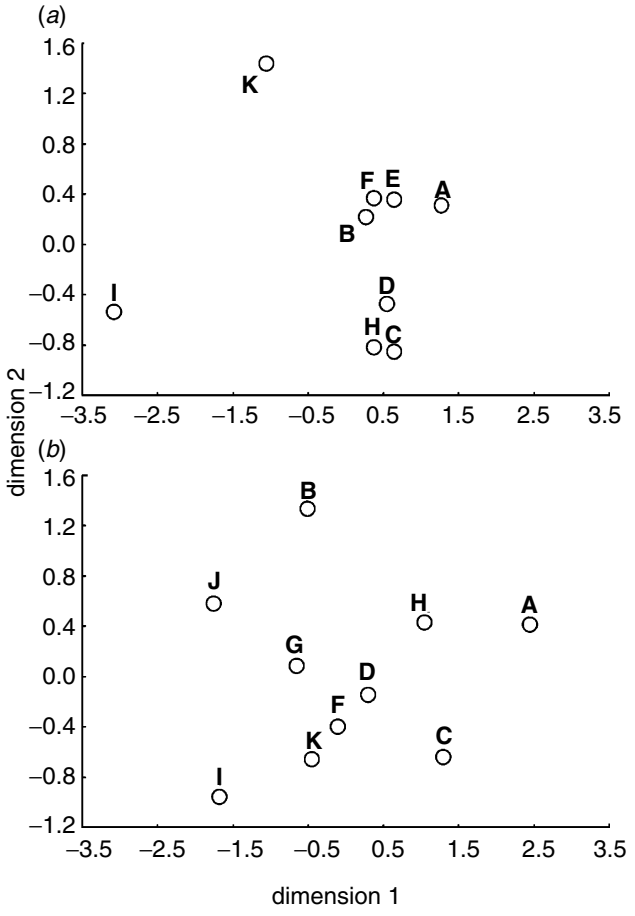

Figure 2. Results of the multidimensional scaling analysis based on the haplotype composition of the leks studied for (*a*) young birds (which represent the female population) and (*b*) adult males.

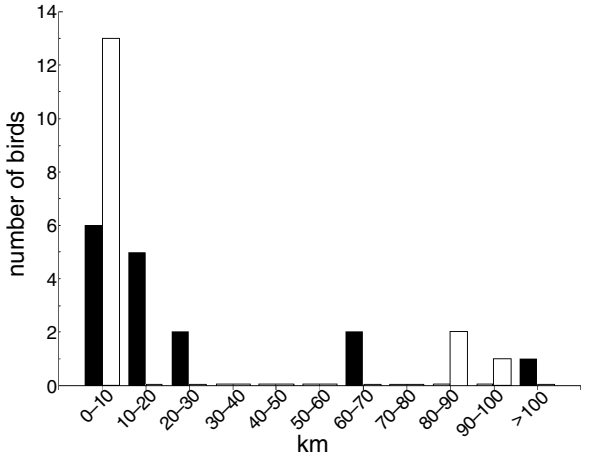

Figure 3. Frequency distribution of natal dispersal distance in males (black bars) and females (white bars).

males than in females (16 vs 2 km, $Z = 2.223$, $p = 0.026$, Mann–Whitney test; figure 3).

(d) *Geographical distance and genetic differentiation among leks*

We found that pairwise genetic differences between leks increased with geographical distance in young birds ($r = 0.593$, $p < 0.000\,1$, $n = 36$; Mantel test, $t = 2.154$, $p = 0.023$; figure 4*a*). This result suggests an isolation-by-distance effect in the genetic structure of females in the lek system studied. However, in adult males, genetic differences between leks were not correlated with geographical distance ($r = -0.065$, $p = 0.67$, $n = 45$; Mantel test, $t = -0.289$, $p = 0.386$; figure 4*b*). The average and variance of pairwise genetic differentiation values between close leks was much higher in male adults than in young birds.

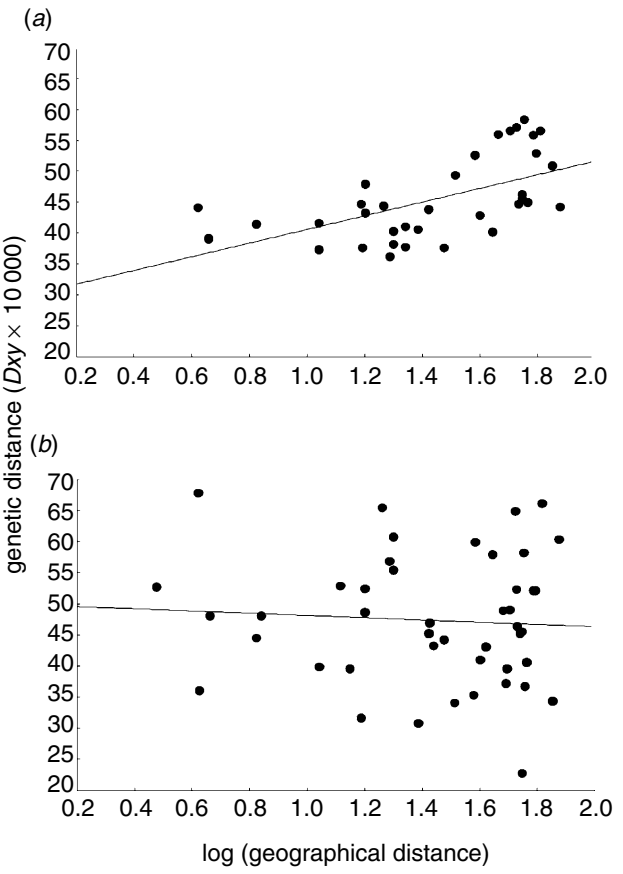

Figure 4. Relationship between pairwise comparisons of genetic distance and geographical distance among leks for (*a*) young birds (which represent the female population) and (*b*) adult males.

Pairwise frequencies of movements between leks were negatively correlated with geographical distance in both sexes ($r = -0.553$, $p < 0.001$, $n = 36$, for females; $r = -0.742$, $p < 0.001$, $n = 28$, for males). However, the frequency of movements observed between two leks in female and male young birds was negatively correlated with pairwise genetic differentiation values among leks only in females but not in males (figure 5; females: $r_s = -0.454$, $p = 0.005$, $n = 36$, Mantel test, $t = -1.744$, $p = 0.040$; males: $r_s = -0.072$, $p = 0.638$, $n = 44$, Mantel test, $t = 0.286$, $p = 0.612$).

4. DISCUSSION

Although we had described male-biased dispersal in great bustards based on radiotracking data of a different population (Alonso & Alonso 1992; Alonso *et al.* 1998), we believe the present study is the first to show concordant results from genetic analysis and direct field observations in this species, and one of the few published examples

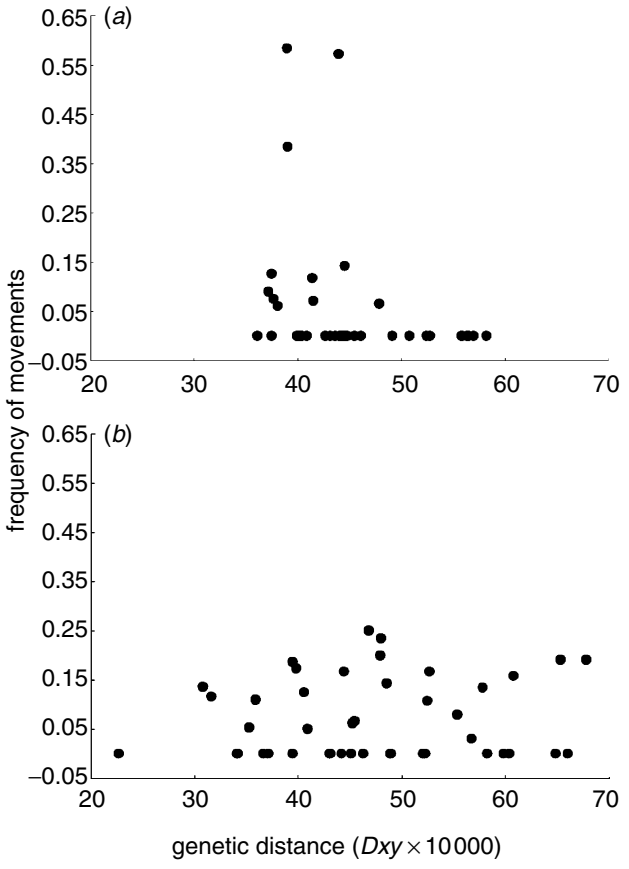

Figure 5. Relationship between genetic distance ($Dxy \times 10\,000$) and the frequency of inter-lek movements based on radiotracking data (see § 2 for a definition) in (*a*) females and (*b*) males during the period of immature dispersal (1–4 years). In figure 4*a* we used inter-lek genetic distances from young birds (which represent the female population), while in figure 4*b* we used genetic distances from the adult male sample.

showing connections between genetic structure and population demography (see Avise 1995). Our results showed significant sex differences in the genetic structure and dispersal patterns of adult male and adult female populations. Below we suggest that this sex difference is probably related to the breeding system and life strategy of the species.

The genetic variability of the species was low, with only 10 haplotypes identified among 144 samples. However, the analysis of genetic distances in the sample of young birds, which in our mtDNA study represent the female population, showed a significant separation between the group of leks A–H in the north and leks I and K in the south of our study area. By contrast, in the sample of adult males we found no equivalent genetic difference between lek groups, and their overall genetic differentiation (ϕ_{ST}) was much smaller than that of females (0.030 vs 0.181).

Although the location of Madrid city between groups of leks A–H and I may represent today a significant barrier to dispersal of females between them, it cannot explain the genetic differences observed, since the barrier effects caused by the city have acted only during the last century. Earlier, no marked habitat discontinuity existed between both areas. Furthermore, both male and female great bustards are capable of much longer movements than the actual distance separating both lek groups (Alonso *et al.* 2000; Morales *et al.* 2000). Indeed, many females breeding in leks A–H spend part of the winter in areas further south, and some females from leks A–H, I and K even share the same wintering areas south of our study area (C. A. Martín *et al.*, unpublished data).

With no obvious geographical barrier between these two groups of leks, the observed changes in their female genetic composition may have been originated by recent events such as colonization of females with different haplotype composition (founder effects), demographic fluctuations, bottlenecks, or a combination of these. The observed differences may have been maintained afterwards by a low level of gene flow due to an isolation-by-distance effect (Wright 1931), which is absent in the male genetic structure. The positive correlation found between geographical distances and female, but not male, genetic distances among pairs of leks agrees with this conclusion. While female haplotype distributions were homogeneous among leks at close distances (< 50 km), differences in the male genetic structure were quite variable even between two close leks. This means that adult males of a lek have more diverse origins than adult females of the same lek. In fact, the percentage of adult male haplotypes not present in the chick sample of a given lek was relatively high (average 23%, range 0–63%), suggesting external origin of many males. This is further confirmed by the presence of two individuals with haplotypes 4 among adult males in lek A and one individual with haplotype 10 in lek I, whereas these haplotypes were not found in the whole sample of chicks. These males were probably hatched at leks outside the study area, from which they dispersed to settle at lek A or I as breeding adults. Moreover, haplotypes 4 and 10 have not yet been found in any samples from various other Spanish regions (C. A. Martín *et al.*, unpublished data). Male dispersal is thus the cause of the sex differences in the relationship between genetic distances and geographical distances shown in figure 4.

Two pieces of evidence from the dispersal study support these genetic results. First, we found a strong correlation between frequency of movement of radiotagged females between lek pairs and their genetic affinity and geographical proximity. In males, the frequency of movement was correlated with geographical proximity but not with genetic affinity. This result suggests that the genetic data do reflect contemporary movement patterns only in the case of females, and that these female movements are less probable the further apart the leks are. By contrast, although male movement patterns are also related to inter-lek geographical proximity, the genetic distances between male groups at different leks are independent of the current movement patterns of young males. This means that male movements are not directed to genetically related leks. Another study comparing movements of radiotagged individuals with genetic distances also found a negative correlation (Paetkau *et al.* 1999). Although our analysis of frequency of movement was also based on a small sample size, we agree with these authors that such a comparison is important to allow the interpretation of genetic distances in terms of actual rates of movement.

Second, there was a strong sex-bias in natal dispersal patterns. After juvenile dispersal, most males settled to breed at leks that were different from their natal leks, while

most females returned to their maternal leks after a short juvenile dispersal, or did not perform such dispersal at all (3 out of 16 females). The haplotype structure observed agrees with the limited female dispersal observed, since male dispersal has no influence on mtDNA distribution. The strong female philopatry is not enough, however, to produce differences in allelic frequencies among leks separated by small distances (leks A–H). The few females dispersing to non-natal leks are enough to homogenize the haplotype diversity among close leks. Finally, three of the marked females performed a long natal dispersal (80 km), which shows the potential of female dispersal (although so far breeding success has not been confirmed for these three females, and two of them unfortunately died after their first unsuccessful breeding attempt).

In conclusion, our genetic results represent evidence for isolation-by-distance of maternal lineages at a regional level. This genetic differentiation was probably driven by recent events, and maintained by limited gene flow during many years due to the geographical distance between them. Regional isolation-by-distance is consistent with the social structure of the great bustard, which favours a much closer maternal link with daughters than with sons. Female chicks remain in their natal areas much longer than male chicks (Alonso *et al.* 1998). In spite of their ability to undertake long seasonal movements (Alonso *et al.* (2000); C. A. Martín *et al.*, unpublished data for the present study area), females are highly philopatric, returning as a rule to breed in their natal areas. Early, young male dispersal is favoured by increased maternal care at early stages of development. Increased maternal care results in faster growth rates, which probably enable males to integrate at an earlier stage into male flocks and helps them to reach a more competitive status in the lek (Alonso *et al.* 1998). Female philopatry thus plays a significant role in structuring great bustard populations, whereas male dispersal probably contributes to keeping nuclear DNA diversity. At *ca.* 50 km distance between leks, ϕ_{ST} is already high enough to show some mtDNA genetic structure among female groups; below that distance there is no genetic differentiation among leks.

The mtDNA structure described in this study has important implications for management. The groups of leks identified (A–H, I and K) should be regarded as three different management units that have diverged regardless of the phylogeny of their alleles, since allele frequencies respond to population isolation more rapidly than the phylogeographic patterns (Moritz 1994*a*). Our results suggest that their female populations are demographically independent and that these leks are only connected by male dispersal. Thus these groups of leks should be considered as important conservation units within any regional conservation programme to guarantee their survival and the persistence of the male patterns of dispersal observed. The mtDNA structure and the strong female philopatry also suggest that females are unlikely to rapidly recolonize an area after a local population crash. Even small decreases in the number of females in one of these demographic units would hardly be compensated by recruitment from other leks.

We thank S. J. Lane, M. Magaña, E. Martín, M. B. Morales and C. Palacín for their help during fieldwork, the farmers of Madrid Province for allowing us to work on their lands, and the Dirección General del Medio Natural of the Madrid Community for permits to capture great bustards. We thank the 42 Group of the Spanish Airforces for their generous collaboration in locating the radiotagged birds, and S. Auls and S. Schmidt for laboratory assistance. The study was funded by Dirección General de Enseñanza Superior e Investigación Científica (DGESIC) project PB97-1252, Spanish (DGESIC)–German (Deutscher Akademischer Austauschdienst) cooperative project HA97-0046, and project PI265/2-1 of the Deutsche Forschungsgemeinschaft. C.A.M. held a PhD fellowship from the Community of Madrid.

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