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Genetic consequences of interglacial isolation in a steppe bird

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ABSTRACT

In response to climate changes that have occurred during Pleistocene glacial cycles, taxa associated to steppe vegetation might have followed a pattern of historical evolution in which isolation and fragmentation of populations occurred during the short interglacials and expansion events occurred during the long glacial periods, in contrast to the pattern described for temperate species. Here, we use molecular genetic data to evaluate this idea in a steppe bird with Palaearctic distribution, the little bustard (*Tetrax tetrax*). Overall, extremely low genetic diversity and differentiation was observed among eight little bustard populations distributed in Spain and France. Mismatch distribution analyses showed that most little bustard populations expanded during cooling periods previous to, and just after, the last interglacial period (127,000–111,000 years before present), when steppe habitats were widespread across Europe. Coalescent-based methods suggested that glacial expansions have resulted in substantial admixture in Western Europe due to the existence of different interglacial refugia. Our results are consistent with a model of evolution and genetic consequences of Pleistocene cycles with low between-population genetic differentiation as a result of short-term isolation periods during interglacials and long-term exchange during glacial periods.

1. Introduction

Past climate changes that occurred in the Palearctic are argued to have sculpted the patterns of genetic diversification of many plants and animals, including most existing avian species (Avise, 2000; Hewitt, 2000). Over the last 400,000 years, the environmental conditions of major glacial periods are thought to have reduced available habitat for temperate species and compressed it southwards, promoting the fragmentation and isolation of most species into southern refugia and resulting in genetic divergence (Dawson, 1992; Hewitt, 2004). Following postglacial climate warming and consequent vegetation shift, species expanded their ranges over deglaciated areas often resulting in the contact of genetically differentiated populations (Taberlet et al., 1998; Hewitt, 1999). While this phylogeographic pattern is globally accepted for temperate species, the model does not fit well in arctic-boreal faunas (Hamill et al., 2006), which often show little genetic differentiation among populations (Hewitt, 2004).

In contrast to the large body of literature exploring phylogeographic patterns in temperate or arctic species, little is known about such patterns in species associated to steppe habitats. These species, with features intermediate between temperate and arctic-boreal fauna, currently experience dramatic population declines due to the anthropogenic habitat perturbations taking place in vast areas of the European continent (Santos and Suárez, 2005). It is possible to postulate a scenario in which steppe species remained isolated during the interglacials while reached their maximum extent during glacial periods (Santos and Suárez, 2005) when steppe-like communities expanded their ranges over vast extensions dominated by Gramineae, *Artemisia spp.* and Chenopodiaceae (e.g. Allen et al., 1999). According to this scenario, the genetic consequences of Pleistocene cycles, in terms of genetic structuring and diversification, must have necessarily been different for temperate and steppe species. A crucial issue is the relative importance of time in isolation and time of connection among populations. During the Pleistocene, glacial periods were much longer (≈100,000 years) than interglacials (≈15,000 years), and therefore the genetic consequences of being isolated in one or the other should be very different, since the time of isolation determines the accumulation of genetic differentiation. Therefore, we might predict that steppe species, contrary to the expected for temperate species (Taberlet et al., 1998; Hewitt, 2000) will show low genetic differentiation because of the short time of isolation during the interglacial periods.

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In the present study we used molecular genetic data to evaluate these predictions (low genetic differentiation together with expansion events restricted principally to glacial periods rather than to interglacials) in a steppe bird: the little bustard (*Tetrax tetrax*).

2. Material and methods

2.1. Model species and sample collection

The species is a medium-sized partially migrant bird belonging to the *Otididae* family that inhabits flat arid plains, natural steppe habitats, and agricultural landscapes in Eurasia (Del Hoyo et al., 1996). It is one of the few monotypic genera among the bustards whose taxonomic status is currently under debate (Pitra et al., 2002). Molecular analyses have revealed an African origin and subsequent colonisation of Eurasia (Pitra et al., 2002), but the genetic structure of the European populations and extent of connectivity among them remains completely unknown. Two subspecies or races are recognised (Del Hoyo et al., 1996), with *T. tetrax* formerly distributed over most Western Europe and now restricted to Iberia (where its stronghold is found), France and Italy. Between 2001 and 2007 we collected 136 samples from eight breeding populations across the Iberian Peninsula and France (Fig. 1) covering most of the distribution range of the species in western Europe.

2.2. Amplification and sequencing

Total genomic DNA was extracted using a standard NH_4Ac protocol. We used sequence variation in the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) to investigate population structure in little bustards. We chose ND2 gene because its substitution rate has been estimated with some precision and it has been successfully used in phylogeographic analyses of other avian species (Drovetski et al., 2005; Arbogast et al., 2006; García et al., 2008). Primers L5216–H5766 and L5758–H6313 were used for the complete amplification of ND2 gene (Sorenson et al., 1999). PCR reactions were performed following García et al. (2008). All samples were sequenced using BigDye Terminator Kit and an ABI 3100 automated sequencer (Applied Biosystems).

2.3. Genetic diversity and structure

We edited and aligned DNA sequences using Bioedit (Hall, 1999) and Clustal W (Thompson et al., 1994). Population haplotype (h) and nucleotide (π) diversity were computed using Arlequin 3.5 (Excoffier and Lischer, 2010), which was also used to conduct Tajima's D (Tajima, 1989) tests of selective neutrality.

The genetic structure and differentiation of little bustard populations was examined with an analysis of molecular variance (AMOVA) and pairwise Φ_{ST} statistics as implemented in Arlequin 3.5 Two possible scenarios were tested: (1) differentiation between geographic areas (Spain vs. France) due to potential restricted gene flow between both sides of the Pyrenees, and (2) differentiation between migratory and sedentary populations (Fig. 1). For this, Tamura–Nei distances (Tamura and Nei, 1993) were used, selecting the best fit model among those available in Arlequin as estimated in ModelTest 3.7 (Posada and Crandall, 1998) under the AIC criterion. A Mantel test was performed with 5000 random permutations in Arlequin 3.5 to test for a significant correlation between geographic and genetic distances among populations.

To test whether the populations underwent population expansion we plotted the mismatch distribution, using the observed number of differences between pairs of haplotypes. Then goodness-of-fit to the theoretical distribution under a sudden expansion model was tested with the sum of squared deviations (SSD) between the observed and expected mismatch distributions, using Arlequin 3.5. We converted the parameter-value (τ) of the mismatch distribution to an estimate of time since expansion assuming different rates of evolution of the ND2 gene (min 0.02 s/s/Myr; mean 0.04 s/s/Myr; max 0.055 s/s/Myr) that encompass a range of values from the minimum estimate of the avian mtDNA clock (0.02 s/s/Myr) to the maximum rate estimated in comparative studies (0.055 s/s/Myr; Warren et al., 2003; Drovetski et al., 2005; Arbogast et al., 2006; García et al., 2008).

2.4. Phylogeographic analyses and gene flow

We used the program TCS 1.21 (Clement et al., 2000) to build the most parsimonious haplotype network that would illustrate the mutation step between sequences. To distinguish gene flow from retention of ancestral polymorphism we obtained non-equilibrium coalescent estimates of migration rate and time since population splitting with MDIV (Nielsen and Wakeley, 2001). The software uses a Markov chain Monte Carlo (MCMC) simulation to estimate the parameters $\theta = 2uN$, $T = t/2N$, and $M = 2Nm$, where N is the female effective population size (which is assumed to be equal for the two populations compared), t is the divergence time in generations, which was calculated to be 4.61 years (based on life history data for the species obtained from Morales et al., 2005), and m is the migration rate between the two populations (which is assumed to be symmetrical). Thus, coalescent methods produce scaled estimates of divergence time (T) and migration rate (M) for each pair of populations. Multiple MCMC with different random seed values were run to ensure convergence. Each MCMC consisted of 5×10^6 steps and a burn-in of 1.25×10^6 steps. We constrained the parameter space within the ranges θ $[0, \infty]$, M $[0, 30]$ and T $[0, 30]$, which should encompass most biologically realistic scenarios (Nielsen and Wakeley, 2001).

3. Results

We obtained 1042 base pairs (bp) of the mtDNA ND2 gene for 136 little bustards from eight populations. The ND2 sequence alignment showed only four different haplotypes defined by five polymorphic sites (GenBank accession numbers: GU055933, GU055934, GU055935, GU055936). All populations showed some proportion of haplotypes shared with the others (Fig. 1), indicating the existence of relatively recent gene flow. However, one haplotype (Tetet2) is restricted to Spanish populations. Haplotype Tetet3 was the most frequent haplotype, shared by 121 (88.9%) individuals in Spanish and French populations (Fig. 1), and occupied a central position in the haplotype network (Fig. 1). All haplotypes are closely related, differing between one and three mutations (Fig. 1). Further, Haplotype Tetet1 (more frequent in France than in Spain) and Tetet2 (exclusively found in Spain) appeared to be more closely related between them than to haplotype Tetet4, collected only in SE France (FRSE) and NE Iberia (CAT) (Fig. 1).

3.1. Population genetic structure and gene flow

The AMOVA results revealed low but significant genetic differentiation between populations located at each side of the Pyrenees (12% of variance), and low genetic variation among populations within groups (<2% of variance, Table 2). No evidence for differentiation was observed between resident and migratory populations (Table 1). Genetic differences between little bustard populations did not depend on the geographic distances among them (Mantel tests: $r = 0.04$, $P = 0.37$). The lack of evidence for strong genetic

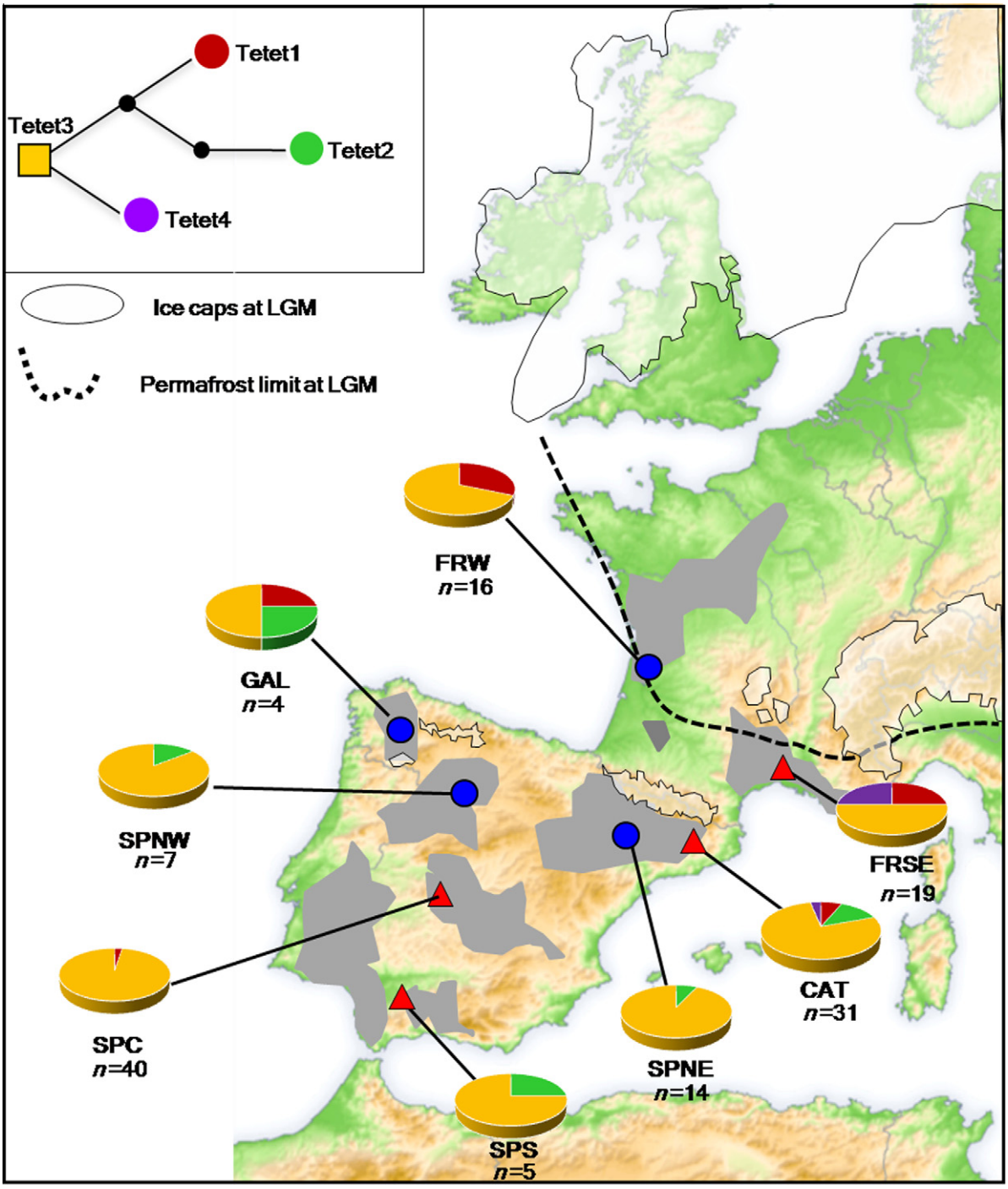

Fig. 1. Geographical sampling. Distribution of the little bustard is represented in gray (drawn after Del Hoyo et al., 1996). For each population (blue circle = migratory population, red triangle = sedentary population), we give the number of specimens sampled and the distribution of ND2 haplotypes. The figure shows the expected situation of ice caps and southern permafrost limit at Last Maximum Glacial, 20,000–18,000 years ago (redrawn from Taberlet et al., 1998; Ehlers and Gibbard, 2004). The inset shows the most parsimonious haplotype network of little bustards. Each link between haplotypes represents a unique mutational event, and black dots represent additional mutational changes. The square represents the most probable ancestral haplotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure was corroborated by the pairwise tests of population differentiation (Table 2), with only 9 out of 20 pairwise comparisons resulting in significant Φ_{ST} values (Table 2).

Analysis of the mitochondrial ND2 data indicated more "new" mutations than would be expected from the number of segregating sites (see negative Tajima's *D* values in Table 3). Evidence for population expansions is confirmed by goodness-of-fit measures used to evaluate mismatch distributions (Table 3). Exceptions are FRW, SPC and SPNW that fit better a model of constant population size, indicating stable long-term population numbers. Assuming a constant mtND2 gene molecular clock FRSE would have expanded around 108,000 years ago (78,000–216,000 years BP). The most ancient population correspond to SPS, which would have expanded at the beginning of the Riss glacial period, around 228,000 years BP (166,000–456,000 years BP). The remaining Iberian populations would have expanded also during the Riss glacial period (between 166,000 and 171,000 years BP), as well as FRW (~157000 years BP) (Table 3).

Table 1
Results of AMOVA testing for differences among little bustard populations and geographic areas. The analyses partition out total molecular variance into different components, and statistical significance is obtained by randomisation after 5000 permutations.

Population structure tested	*d.f.*	SS	Var. comp.	% Var.	*P*
No grouping					
Among populations	7	6.62	0.04	9.54	0.005
Within populations	127	45.34	0.36	90.46	
Spain vs. France					
Among groups	1	3.12	0.05	12.1	0.022
Among populations	6	3.59	0.07	1.69	0.012
Within populations	127	45.28	0.36	86.22	0.015
Resident vs. migrant					
Among groups	1	0.62	−0.01	−2.53	0.716
Among populations	6	6.01	0.04	11.03	0.004
Within populations	127	45.38	0.36	91.5	0.0009

Table 2
Between-population differentiation in little bustards. Bellow the diagonal, pairwise F_{ST} values (based on Tamura and Nei distances between haplotypes under a gamma distribution of $\alpha = 0.98$). Above the diagonal, *p*-values for significant F_{ST} values obtained after 5000 permutations ($^{*}P < 0.05$; $^{**}0.05 > P < 0.01$, $^{***}0.01 > P < 0.001$). Population codes as in Fig. 1.

	CAT	FRSE	FRW	SPC	SPNE	SPNW	SPS	GAL
CAT	–	**	**	**				
FRSE	0.084	–		***	***			
FRW	0.099	0.010	–	**	*			
SPC	0.119	0.264	0.303	–			**	***
SPNE	−0.012	0.116	0.150	0.022	–			
SPNW	−0.089	0.055	0.084	0.161	−0.087	–		
SPS	−0.137	0.042	0.073	0.427	−0.008	−0.196	–	
GAL	0.000	0.027	−0.001	0.597	0.199	−0.033	−0.222	–

Finally, the gene flow varied substantially between pairs of populations (Table 4). Our coalescent estimates reveal high current gene flow between some pairs of populations (e.g. SPNW–SPNE: $m = 18.7 \times 10^{-4}$), whereas the gene flow was more than 50 times less between some pairs of populations (e.g. FRSE–SPNW: $m = 0.33 \times 10^{-4}$). Gene flow between extant French and Spanish populations was overall low (several $m < 1.0 \times 10^{-4}$; Max = 2.33×10^{-4}). Among the Spanish populations, gene flow was low in a few cases ($n = 3$ comparisons with $m < 1.0 \times 10^{-4}$), but mostly intermediate ($n = 6$ comparisons with $1.0 \times 10^{-4} > m < 5.0 \times 10^{-4}$) or high ($n = 8$ comparisons with $m > 5.0 \times 10^{-4}$; Max = 18.7×10^{-4}).

4. Discussion

We documented an astonishing low genetic diversity and a lack of strong genetic structure across little bustard populations throughout the core of the little bustard range in Western Europe. Therefore, our results differ with the classical picture drawn from temperate avian species, but are similar to those found in a closely related species, the Great bustard (*Otis tarda*) in Western Europe (Broderick et al., 2003).

Table 4
Estimates (scaled by effective population size, *N*) of divergence time (*t*) and migration rate (*m*) between pairs of populations of little bustards (Nielsen and Wakeley, 2001). Values below the diagonal are estimates of M (=2 N*m*), and those above the diagonal are estimates of T (=*t*/2*N*). Population codes as in Fig. 1.

	CAT	FRSE	FRW	SPC	SPNE	SPNW	SPS	GAL
CAT	–	7.34	0.42	0.27	0.06	0.04	5.55	0.03
FRSE	1.86	–	0.21	0.21	0.84	0.48	0.6	0.3
FRW	1.22	1.62	–	8.61	0.42	11.49	10.35	0.69
SPC	3.57	0.9	1.05	–	0.06	0.57	3.33	2.04
SPNE	8.7	0.48	0.63	0.93	–	0.03	9.3	11.19
SPNW	9.22	0.48	0.75	10.17	13.5	–	9.48	0.12
SPS	10.56	1.38	0.6	0.45	3.66	13.11	–	7.7
GAL	4.56	4.08	1.53	0.42	2.67	8.94	15.25	–

4.1. Patterns of genetic diversity

Several lines of evidence converge to support the role of pre-human events rather than recent, human-related impacts, to explain our low diversity results. Firstly, the little bustard, as well as other steppe bird species (Geroudet, 1961; Santos and Suárez, 2005), has been favoured in Europe by the transformation of vast areas to agrarian substrates since the early Neolithic (c. 7000 years ago), whereas in the absence of human-created habitats, their current populations would be naturally restricted to small relicts of steppe vegetation (Bennett and Provan, 2008). Secondly, mitochondrial ND2 data showed clear evidence for Pleistocene expansion events for all populations except for FRW, SPC and SPNW (Table 3), which showed stable long-term population numbers. During the successive expansion–contraction of ice sheets in the Pleistocene, the area of land occupied by steppe vegetation should have declined repeatedly during deglaciation times and expanded during glacial periods (e.g. Allen et al., 1999). Tracking habitat suitability, the steppe-associated fauna would have been much more widespread during cold periods than during warm periods (interglacials), in which the advance of temperate forests reduced the extent of suitable habitats. Therefore, the observed pattern of expansion agrees with the idea that reduction in numbers of little bustards should have occurred during warm, steppe-contraction periods, such as interglacials. However, we cannot rule out the possibility of very recent reductions in genetic diversity due to changes in land use, at least for certain populations such as FRW, for which drastic population reductions (95% loss) have been documented over the last 25 years (Bretagnolle and Inchausti, 2005).

Table 3
Indices of genetic diversity (H) and nucleotide diversity (π_n), Tajima's D value (D) and test for population expansion (τ, θ_0, θ_1, SSD and significance level) in populations of little bustard *Tetrax tetrax*. Estimates of the years since expansion for the populations studied (labelled as in Fig. 1) were calculated assuming different rates of evolution of the ND2 gene (min 0.02 s/s/Myr; mean 0.04 s/s/Myr; max 0.055 s/s/Myr).

				Statistics of expansion					Years since expansion		
	H	π_n	*D*	τ	θ_0	θ_1	SSD	*P*	Min	Mean	Max
CAT	0.3849	0.00097	−0.52	3.08	0.608	0.609	0.091	0.1	341,000	171,000	124,000
FRSE	0.0500	0.00009	0.95	1.95	0	2.968	0.004	0.7	216,000	108,000	78,000
FRW	0.1429	0.00041	1.42	2.84	0	1.262	0.17	0.01	314,000	157,000	114,000
SPC	0.2857	0.00082	−1.49	3.0	0.021	0.028	0.003	0.04	332,000	166,000	121,000
SPNE	0.5000	0.00144	−1.67	3.0	0.087	0.089	0.03	0.07	332,000	166,000	121,000
SPNW	0.8333	0.00208	−1.36	3.0	0.227	0.259	0.11	0.03	332,000	166,000	121,000
SPS	0.4561	0.00087	−0.75	4.12	0	1.877	0.25	0.10	456,000	228,000	166,000
GAL	0.6667	0.00115	−0.06	3.03	0	3278.7	0.12	0.25	335,000	168,000	122,000

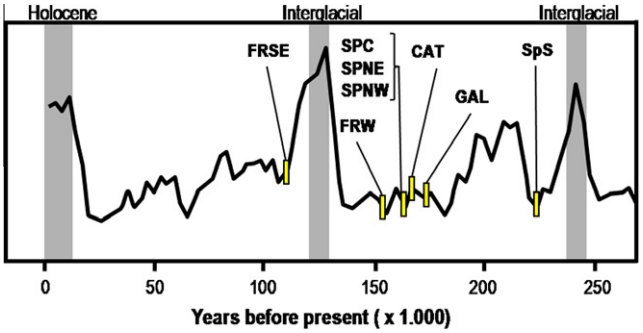

Fig. 2. Timeline of expansion events for each population of little bustards across Spain and France (assuming an intermediate rate of substitution of 0.04 s/s/Myr). Black line represents a proxy of temperature (δD, the ratio of deuterium to hydrogen) changes during the last 300,000 years (redrawn from Cheddadi et al., 2005). Population codes as in Fig. 1.

4.2. Inferring historical glacial–postglacial events

Considering an intermediate rate of evolution of the ND2 gene, demographic expansions should have occurred in at least three waves, coinciding with the end of rapid cooling episodes (Fig. 2) during the two last glacial periods. The first expansion took place during the middle Pleistocene (≈228,000 years BP) in southern Iberia. Around 165,000–170,000 years BP it seemed to take place the expansion of most Iberian populations. The last expansion event seemed to take place in south-eastern France about 108,000 years ago. However, the temporal pattern of expansions is not so clear when extreme rates of evolution for ND2 (minimum or maximum rates found in comparative studies) were applied.

The estimated expansion dates under intermediate rates of evolution of ND2 gene are in full agreement with the fossil record of Eurasia (Sanchez Marco, 2004). Fossil remains of little bustard and other steppe birds were well represented in Iberia and France during glacial periods (Sanchez Marco, 2004), when many areas over southern Europe were covered by steppe formations (Pons and Reille, 1988) and exchange of genes between populations should have been larger. When the advance of temperate vegetation from southern refugia replaced steppe formations, it is probable that most steppe species became adversely affected and were forced to take refuge in the coldest areas within their range, where gene exchange was restricted. Such a pattern consisting of isolation during the (short) interglacials and expansion during the (long) glacial periods would explain the lack of strong phylogeographic structure across such a broad region.

The concept of interglacial or postglacial refugia is not much considered in the literature (but see Hewitt, 2001, 2004), and specific studies on the identification of those refugia have been largely ignored to date (but see e.g. Schmitt and Hewitt, 2004; Schmitt et al., 2006; Dalén et al., 2007; Bennett and Provan, 2008). The identification of genetic refugia may be inferred from phylogeographic data concerning the level and pattern of genetic diversity in refugial vs. recolonised areas (Hewitt, 1996). In general, recolonised areas are composed of a subset of the diversity present in the original source (refugial) population, thus harbouring less genetic diversity. However, other factors, such as the genetic admixture in contact zones due to recolonisation from separate refugia could also lead to high genetic diversity areas (Petit et al., 2003). To discern alternative scenarios it is necessary to explore the composition of haplotypes: while colonised areas will be composed entirely of haplotypes present in the source populations, refugial areas will contain some haplotypes absent in the genetic pool participating in the recolonisation process, and therefore exclusive of refugial populations (Provan and Bennett, 2008). Our results are in clear agreement with the second scenario and suggest the existence of two different contact zones between France and Spain. The more diverse bustard populations through the range occur in GAL (Northwest Iberia) and CAT (Northeast Iberia). These two populations hold the Iberian Tetet2 haplotype together with haplotype Tetet1, more abundant in French than in Iberian samples. Furthermore, CAT samples also shared with southern French samples (FRSE) haplotype Tetet4, which is not found elsewhere. Finally, the frequency of the two haplotypes shared between North Iberian and French populations (Tetet1 and Tetet4) increased north-eastwards, suggesting an expansion from a refugium located somewhere outside Iberia. In contrast, private haplotype Tetet2 supports an Iberian origin. Under these considerations the two northern Iberian populations (CAT and GAL) fall better under the concept of contact populations rather than refugial.

Little bustard results are thus consistent with a picture of northern Iberian populations as admixture between the Iberian genetic pool and haplotypes coming from a refugium located somewhere in central or Eastern Europe. This pattern probably reflects ancestral expansion events during glacial periods and also dispersal behavior in more recent times (Villers et al., 2010).

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