Available online at www.sciencedirect.com
ScienceDirect
Molecular Phylogenetics and Evolution 46 (2008) 237–251
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Genetic and phenotypic variation among geographically isolated populations of the globally threatened Dupont's lark *Chersophilus duponti*

Jesús T. García [a,*], Francisco Suárez [b], Vicente Garza [b], María Calero-Riestra [a], Jorge Hernández [b], Javier Pérez-Tris [c]

[a] *Instituto de Investigación en Recursos Cinegéticos (IREC, CSIC-UCLM), Ronda de Toledo s/n, E-13005 Ciudad Real, Spain*
[b] *Departamento de Ecología, Facultad de Ciencias, Universidad Autónoma, c/Darwin 10, E-28049 Madrid, Spain*
[c] *Departamento de Zoología y Antropología Física, Facultad de Biología, Universidad Complutense, E-28040 Madrid, Spain*

Received 26 April 2007; revised 20 June 2007; accepted 22 June 2007
Available online 13 July 2007

Abstract

Identifying genetically and phenotypically distinct populations of threatened species is critical if we are to delineate appropriate plans for their conservation. We conducted an integrated analysis of population genetic structure, historical demographic events, current gene flow (all based on mtDNA sequences) and morphological variation of three geographically separated groups of populations of Dupont's lark *Chersophilus duponti*, located in the Iberian Peninsula (three populations), Morocco (two populations), and Tunisia (one population). Unusually, this lark species is the only one among the genus *Chersophilus*. Our results revealed the early historical divergence of an eastern Dupont's lark lineage (in Tunisia) and a western lineage (in Morocco and Spain), consistent with subspecies taxonomy and distribution. The western lineage subsequently split into two lineages, following the isolation of Iberian and African populations. Such pattern of historical differentiation caused great population genetic structure, with differences among geographic areas explaining more than 80% of total genetic variation. Mismatch distributions and coalescent estimates of divergence time showed that lineage divergence was associated with sudden population expansion events, which apparently took place during the last glaciation, when steppe habitats were widespread across the Mediterranean region. Extant populations from different geographic areas hardly shared any haplotype (only one out of 16 ND2 haplotypes was shared by Tunisian and Moroccan Dupont's larks), and consequently gene flow between geographic areas was found to be virtually absent. Apart from showing great genetic differentiation, Dupont's larks from different geographic areas were morphologically distinct, showing substantial variation in body size and feeding-related traits (length of feet and bill). We conclude that Dupont's lark populations isolated in the Iberian Peninsula, Morocco, and Tunisia are distinct evolutionary entities and should be considered as such in conservation plans. Such circumstance sets a daunting conservation challenge that exemplifies the need of incorporating knowledge of historical processes to our general understanding of the demography of threatened species.

Keywords: Biodiversity conservation; Gene flow; Geographic population structure; Mitochondrial DNA sequences; Morphological variation; NADH dehydrogenase subunit 2

1. Introduction

Biodiversity conservation has to be approached at different levels from ecosystems to intraspecific diversity (Bowen, 1999; Groom et al., 2006), because different populations within a species may show unique traits that could potentially assist the entire species in overcoming natural or manmade evolutionary challenges (Lande, 1988; Lande and Shannon, 1996; Crandall et al., 2000; Frankham et al., 2002). Within a species' range, diversity is seldom homogeneously distributed, but it is usually structured by the long-term effects of geography, historical climatic events, and

* Corresponding author. Fax: +34 926295451.
E-mail address: jesus.ggonzalez@uclm.es (J.T. García).

1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.06.022

evolutionary forces (Hewitt, 2000). The evolutionary and biogeographical consequences of historical events need to be fully understood if we are to delineate appropriate plans for preserving biodiversity globally. For example, a species with geographically isolated populations that are hardly connected by gene flow will necessarily require different conservation planning than a species with populations connected by gene flow in a continuous geographical range (Frankham et al., 2002; Groom et al., 2006).

The Mediterranean region is considered to be a priority area for conservation worldwide (Myers et al., 2000). Eurasian and African faunas merge in the Mediterranean area increasing species richness. Besides, the geographical configuration of the Mediterranean basin favoured the existence of peninsular glacial refugia, which acted as reservoirs of genetic diversity of many species during historical periods (Hewitt, 2000). In addition, climatic variation caused by mountains and plateaux intercepting the oceanic influence, together with centuries of human activity, has favoured the existence of a high environmental variability in the Mediterranean region (Alcaráz et al., 2006). Mediterranean habitat types are often interspersed forming a regional mosaic, in which different species may find different opportunities to occur and to interchange individuals among distantly located populations.

Geographical and climatic events have favoured the existence of many steppe bird species in the Mediterranean area, which cause much conservation concern because their habitat choice often conflicts with human activities (Santos and Suárez, 2005). Thus, most steppe bird species have suffered a severe decline in Europe during the last decades (Tucker and Heat, 1994). This problem might be accentuated in the future due to global climate change, which is expected to have a deep impact in the Mediterranean region (Sanz, 2002; Walther et al., 2002). Alarmingly, the conservation of Mediterranean steppe birds is hampered by variable degrees of between population isolation. Thus, many steppe bird species have small resident populations isolated in small remnants of suitable habitat, which are often distantly located from one another (Santos and Suárez, 2005). At a greater geographical scale, the Mediterranean sea keeps African and Iberian populations of many species separated (Guillaumet et al., 2006). Limited dispersal opportunities outline a worrying conservation situation that demands urgent management actions, and may require coordinated but largely independent conservation actions being implemented by African and European countries (Gärdenfors, 2001; Frankham et al., 2002). However, we know very little about the evolutionary history and current gene flow among populations of most steppe bird species (Donald, 2004; De Juana and Suárez, 2004), which seriously compromises our ability to design appropriate plans for their conservation.

Within this scenario of threatened and barely studied Mediterranean steppe avifauna, one case of particular concern is the lark genus *Chersophilus*, which comprises a single species (Dupont's lark, *Chersophilus duponti*). This species remained unnoticed until the 19th century, when two subspecies were described: *C. d. duponti* Viellot (1820) occurs in Spain, Morocco, northern Algeria, and northern Tunisia, and *C. d. margaritae* Koening (1888) occurs in southern Algeria, southeastern Tunisia, the north of Libya, and western Egypt (Cramp, 1988; De Juana and Suárez, 2004). Both subspecies are distinguished by subtle differences in plumage (*C. d. margaritae* is paler and shows more cinnamon reddish tinge than *C. d. duponti*) and morphology (*C. d. margaritae* has longer bill and shorter hind claw than *C. d. duponti*; Koening, 1888; Cramp, 1988). Beyond taxonomic considerations, the evolutionary history of Dupont's lark populations has been largely neglected, which is surprising given its unfavourable conservation status. Recent population trends of Dupont's larks reveal a concerning decline in Europe. Consequently, the species has been listed in the Annex I of the European Birds Directive, it has recently been included in the IUCN Red List of Threatened Species (IUCN, 2006), and it is listed as 'Near Threatened' in Europe (BirdLife International, 2006) and as 'Endangered' in Spain (Garza et al., 2004). The current European range of the Dupont's lark is restricted to the Iberian Peninsula, where the species occurs in small populations scattered among different regions, with a total population of around 1300–2000 territorial males (Garza et al., 2003; Garza et al., 2004; Tella et al., 2005). To further aggravate the situation, the size, and distribution of African Dupont's lark populations remain largely unknown, where research on the species has only recently started, in Morocco and Tunisia (García et al., in press; Suárez et al., unpublished data).

The population decline of Dupont's larks is attributed to habitat loss and deterioration associated with the intensification of agriculture, reforestation schemes, and other changes in land use (Garza et al., 2004). Such activities have contributed to increase fragmentation of Dupont's lark populations, arguably because the species has an inflexible pattern of habitat selection that keeps it from using buffer habitats (Garza et al., 2005; García et al., in press). In addition, African populations are particularly threatened by ongoing climate change (Sanz, 2002; Walther et al., 2002), which is causing the severe desertification of the habitat of African Dupont's larks (Le Houérou, 2000). Therefore, understanding the genetic structure, ecological divergence, and opportunities for gene flow among Dupont's lark populations is crucial for the conservation of the species. We studied the evolutionary history of Iberian and African populations of Dupont's lark (from Morocco and Tunisia) using a phylogeographic approach. In particular, we investigated (1) whether the phylogenetic relationships among Dupont's lark populations are consistent with phenotypic variation and taxonomical classification, (2) when during evolutionary history did the different Dupont's lark populations diverge from each other, (3) how much gene flow there is among extant populations within and between continents, and (4) to what extent such populations have diverged phenotypically. The outcome of

our integrated analysis of population genetic structure, current gene flow and phenotypic variation is expected to shed light on the actual status of Dupont's lark populations, helping us to establish management priorities for the conservation of this globally threatened species.

2. Materials and methods

2.1. Study area and populations sampled

In the Iberian Peninsula, we sampled Dupont's larks in 14 different sites distributed among three distantly located regions (Fig. 1), which show substantial variation in climate and floral composition (Suárez et al., 1992). One of these regions is located in northwestern Spain (SpNW), where the species maintains a small population located more than 120 km away from the nearest breeding area. We sampled one site in SpNW (Zamora: 41°30′N, 5°43′W). The largest part of the Iberian breeding range is located on the Spanish central plateau (SpC), an area which shows continental Mediterranean climate, where Dupont's larks occur scattered in patches of suitable habitat. We sampled five sites in SpC to broadly represent variation within the region (Soria, Segovia, Zaragoza, Burgos, and Cuenca; average location: 41°46′N, 2°28′W). Finally, Dupont's larks occur in southeastern Iberia, in areas with typical arid Mediterranean climate. We sampled four sites in the Mediterranean part of the Spanish range (SpME) to broadly represent variation within the region (Murcia, Granada, Almería, and Albacete; average location: 37°58′N, 1°08′W).

There is no general agreement about the exact range limits of the two Dupont's lark subspecies in the Maghreb region. According to the literature, *C. d. margaritae* occurs in Algeria (from the southern slopes of the Atlas Mountains to Biskra), the southeastern part of Tunisia, and the north of Libya, up to reach the west coast of Egypt. On the other hand, nominate *C. d. duponti* occurs in the Iberian Peninsula, Morocco, and the northern edge of the Maghreb region from Algeria to Tunisia (De Juana and Suárez, 2004). In order to include birds of both subspecies in our analysis, we sampled three sites in Morocco, which were located more than 100 km away from one another within the breeding range of *C. d. duponti* (García et al., in press). One of the Moroccan sites was located in the northeastern part of the country (MoNE; Aïn-BeniMathar: 34°0′N, 2°1′W), another one was located in the Moroccan central plateau (MoC: 32°41′N, 4°44′W), and the third one was located in the Anti-Atlas region, in the southwestern part of the country (MoSW: 30°41′N, 6°25′W). We also sampled one population located in the only place where the species is known to exist today in Tunisia (Tu: 34°40′N, 8°30'E). This population is located at the putative edge between the typical range of *C. d. duponti* and *C. d. margaritae* (Isenmann et al., 2005), and therefore it might include individuals of one or both subspecies.

Dupont's larks were captured by tape-luring them near clap-traps baited with mealworms, as described elsewhere (García et al., in press). In all, we captured 340 Dupont's larks between 2004 and 2007: 284 in Spain, 134 in Morocco, and 13 in Tunisia (Fig. 1). Although the species is a year-round resident, we were careful to sample birds during the breeding season only (starting 30 days before the first nests or clutches were recorded), which excluded any birds that could be dispersing from other areas. Capture periods ranged from February to June for African populations (Thévenot et al., 2003; Isenmann et al., 2005) and from April to July for Iberian populations (Cañadas et al., 1988; Herranz et al., 1994).

All larks were measured and ringed to avoid repetition (all birds were released at the site of capture). We measured tarsus length (tarsometatarsus) to the nearest 0.01 mm, and wing length (maximum chord), tail length (straightened) and bill length (from the bill tip to the skull), all to the

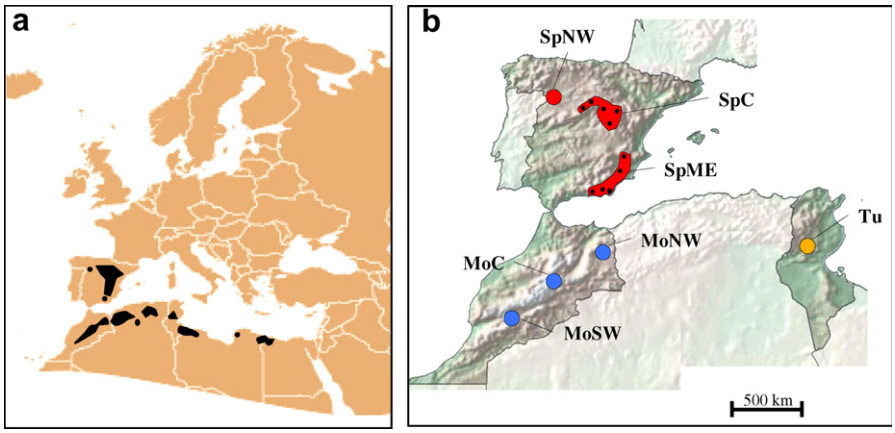

Fig. 1. (a) Global distribution range of the Dupont's lark (shaded in black) according to De Juana and Suárez (2004). (b) Sampling sites in the Iberian Peninsula (red), Morocco (blue), and Tunisia (orange). The seven populations analysed are labelled as SpNW (northwestern Spain), SpC (Spanish central plateau), SpME (Mediterranean Spain), MoNE (northeastern Morocco), MoC (Moroccan central plateau), MoSW (southwestern Morocco), and Tu (Tunisia). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nearest 0.5 mm (Svensson, 1992). We took a blood sample from each individual by venipuncture, and stored it in SET buffer or absolute ethanol until further analysis. We could not measure all morphological traits to all birds (for example during active moult), and therefore the sample sizes vary among analyses.

2.2. Molecular analyses

Total genomic DNA was extracted from blood using a standard ammonium acetate protocol. Purified DNA was diluted to a working concentration of 25 ng/ml.

We determined the sex of all individuals by amplifying a section of the CHD gene using the primers P2 and P8, which produced PCR products of different sizes for gene copies located on W and Z chromosomes (Griffiths et al., 1998). We set the annealing temperature to 48 °C, and separated the PCR products during 45-min in 2.5% agarose electrophoresis gels containing ethidium bromide. Using birds of known sex, we confirmed that all males produced a single band and all females produced two bands.

We used sequence variation in the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene to investigate population structure in Dupont's larks. The ND2 gene shows substantial neutral variation in other lark species, and it has been successfully used in phylogeographic analyses (Drovetski et al., 2005). We amplified two overlapping parts of the ND2 gene, which were sequenced from both ends and assembled to get the complete ND2 gene sequence (1041 bp). Given the amount of time and resources required to determine the complete gene sequence from all 340 birds, we decided to type only part of our sample. In order to reduce bias, we used males only, keeping all males from populations with small sample size, and randomly discarding males from the populations with the largest sample size. Our sample size for genetic analyses was thus reduced to 115 male larks.

We used two primer pairs (L5216-H5766 and L5758-H6313; Sorenson et al., 1999) to amplify two overlapping ND2 fragments from each end of the gene (525 and 572-bp long, respectively). PCR reactions were set up in 10 μl total volumes and included 50 ng of template DNA, 1× PCR buffer (Biotools), 0.125 mM of each nucleotide, 0.6 mM of each primer, 1.5 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (Biotools). Amplifications started with 3 min at 95 °C, which were followed by 35 cycles of 60 s at 95 °C, 60 s at 54 °C, and 60 s at 72 °C. Each reaction was terminated by a 5-min extension at 72 °C. We evaluated 2.5 μl of each reaction on a 2% agarose gel, using 0.5× TBE buffer and ethidium bromide. We used a Qiaquick PCR Purification Kit (QIAGEN) to purify PCR products, which were sequenced from both ends using a BigDye Terminator Kit. The sequencing reactions were cleaned on standard Sephadex columns, and DNA sequences were determined using an ABI 3100 automated sequencer (Applied Biosystems).

2.3. Genetic diversity and structure

We aligned and edited DNA sequences using Clustal W (Thompson et al., 1994) and Bioedit (Hall, 1999), using published ND2 sequences of other lark species as references (Drovetski et al., 2005; Guillaumet et al., 2005). Standard indices of haplotypes (h) and nucleotide (π) diversity were computed using Arlequin 2.0 (Schneider et al., 2000), which was also used to conduct Fu's F_S (Fu, 1997) and Tajima's D (Tajima, 1989) tests of selective neutrality. We also used Arlequin 2.0 to conduct analyses of molecular variance (AMOVA; Excoffier et al., 1992), using Tamura and Nei distance method (Tamura and Nei, 1993), under a gamma distribution with $\alpha = 0.57$, which was estimated from the data by Tree-Puzzle 5.0 (Schmidt et al., 2002). We first examined overall differences among populations, and then tested for genetic structure resulting from two alternative historical scenarios: (1) differentiation between African and Iberian populations due to restricted gene flow between both sides of the Mediterranean sea and (2) differentiation among the three geographic areas (the Iberian Peninsula, Morocco, and Tunisia). The statistical significance of different sources of genetic variation was estimated using Monte Carlo simulations with 5000 permutations (Excoffier et al., 1992). Finally, we analysed isolation-by-distance effects on population genetic structure, for which we computed correlations between pairwise geographic and genetic distances between populations, which were statistically analysed by means of a Mantel test using genetic distances derived from between population Φ_{ST} estimates computed by Arlequin 2.0 (Schneider et al., 2000). The statistical significance of correlations between distance matrices was obtained from 5000 random permutations of matrix elements.

2.4. Analyses of population history

We used PAUP* 4.0 (Swoford, 2002) to construct a maximum likelihood (ML) phylogenetic tree of all ND2 haplotypes, using sequences of *Eremophila alpestris* (GenBank Accession nos. DQ187487 and DQ187486), and *Alauda arvensis* (AY847059) as outgroups. We used the model of nucleotide substitution that best fitted the data (selected among 56 models tested by PAUP*), as determined using the Akaike information criterion implemented in Modeltest 3.7 (Posada and Crandall, 1998). To build the ML tree, we conducted a heuristic search starting from a neighbour-joining tree and using a tree bisection reconnection (TBR) algorithm for branch swapping, with random addition of sequences. The statistical support for internal branches of the tree was estimated by 1000 bootstrap replicates. We also used neighbour-joining and maximum parsimony tree reconstruction methods to ensure consistency of the results.

We calculated mismatch distributions of pairwise haplotype differences using Arlequin 2.0 and DnaSP 4.10 (Rozas et al., 2003), which produced the same results. Mismatch

distributions allow to infer whether populations have suddenly expanded or maintained a constant population size during historical periods, allowing to date population expansion events (Rogers and Harpending, 1992). A ragged, multimodal mismatch distribution is expected from long-term constant population size, while a smooth, unimodal distribution might be expected after sudden population expansion events. The fit of data to either model can be assessed by computing the raggedness of the distribution, the value of Fu's F_S statistics, and the associated P-values. We calculated θ, τ, and chi-square statistics to test whether the mismatch distributions observed in each population better fit to a model of constant population size or to one of changing population size (sudden expansion model; Rogers, 1995). Then, we used these estimates to date population divergence and expansion events. We assessed the validity of the molecular clock by using a likelihood ratio test comparing trees that were built either enforcing or not enforcing a pattern of clocklike evolution, for which we used Tree-Puzzle 5.0 (Schmidt et al., 2002). We excluded the MoSW population from the analysis of population expansion, because it only had two genotyped individuals.

Our estimates of time since population expansion or time since population divergence depend on the reliability of the assumed substitution rate for the ND2 gene. Recent studies on Galapagos mockingbirds *Nesomimus* spp., in which divergence times could be calibrated using island geological ages, suggest a minimum rate of 0.020–0.022 s/s/Myr for the ND2 gene (Arbogast et al., 2002, 2006; Drovetski et al., 2004), but comparative studies have estimated higher substitution rates (0.055 s/s/Myr; Warren et al., 2003; Drovetski et al., 2004; Peck and Congdon, 2004; Arbogast et al., 2006). We prudently assumed an average substitution rate for the ND2 gene of 0.04 s/s/Myr, which is intermediate between the minimum estimate of the avian mtDNA clock (0.02 s/s/Myr) and the maximum rate estimated in comparative studies (0.055 s/s/Myr; Warren et al., 2003; Drovetski et al., 2004; Peck and Congdon, 2004; Arbogast et al., 2006).

2.5. Divergence time vs. migration rate

Populations diverge genetically by the accumulation of mutations during long periods of independent evolutionary history, or by interrupted gene flow between recently diverged populations. To distinguish between these two alternatives, we obtained non-equilibrium coalescent estimates of migration rate and time since divergence between pairs of populations. We used MDIV (Nielsen and Wakeley, 2001), using 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, and m is the migration rate between the two populations (which is assumed to be symmetrical). Thus, coalescent methods allow obtaining scaled estimates of divergence time (T) and migration rate (M) for each pair of populations. To estimate θ, T and M, we ran the MCMC during 2,000,000 generations, removing the first 500,000 generations as a 'burn-in' period to make our estimates independent of initial conditions (Nielsen and Wakeley, 2001). 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). However, pairwise comparisons involving the Tunisian population only reached convergence when we expanded the parameter range to T $[0,60]$. Using the parameters thus estimated, we calculated divergence times (t) and migration rates (m) by assuming different rates of evolution of the ND2 gene (0.02, 0.04 or 0.055 s/s/Myr, as described above), which also allowed us to evaluate whether our previous analyses of population expansion and isolation could be affected by violation of the assumption that populations are at equilibrium. For calculations of divergence time and migration rate, we used a generation time of two years, which assumes that Dupont's larks reach maturity during their second year of life, and their average mortality equals 50% per year, as observed in other lark species (Donald, 2004; Cramp, 1988).

2.6. Phenotypic variation among populations

To avoid any bias related to age or sex of birds, we excluded all females ($n = 17$) and juvenile individuals ($n = 79$) from the analysis of morphological variation among Dupont's lark populations, which rendered a final sample size of 244 adult males. The MoSW population had less than five fully measured individuals, and was excluded from the analysis of morphological variation. We first conducted a principal component analysis (PCA) on the correlation matrix of all morphological variables measured (tarsus length, wing length, tail length and bill length, available for 179 individuals), which allowed us to obtain new variables that summarized variation in body dimensions (principal components, PCs). Then, we compared such PCs among populations by means of ANOVA. We tested for morphometric differences among the six populations included in the analysis, and among geographic areas (Iberian Peninsula, Morocco, and Tunisia) using a nested ANOVA, with the effect of population nested within the effect of geographic area. Given that the power to estimate between group effects with our nested design was very low (because the effect of differences among geographic areas had only 3 degrees of freedom for the error, calculated as an effect between six populations), we also conducted planned comparisons testing for differences among geographic areas using within-population variation as the error term to test for statistical significance of geographic effects. All analyses were done using Statistica 6.1 software (StatSoft, 2002).

3. Results

3.1. Genetic diversity

We did not find any indels or stop codons that could indicate amplification of nuclear copies of the mtDNA ND2 gene. The absence of nuclear pseudogenes in our analysis was further supported by exact matching of overlapping sequences independently amplified with different primers, and also by the nucleotide composition of ND2 sequences, which had typically high content of cytosine and low content of guanine (C = 36.2%, T = 22.8%, A = 28.8%, G = 12.1%), as observed in other species (Drovetski et al., 2004, 2005; Johnson et al., 2006).

Out of 115 genotyped male larks, we found 16 unique mtDNA haplotypes, which were distinguished by 73 polymorphic sites in the ND2 gene (Table 1). Two haplotypes found in Tunisia (H17 and H18) showed large differences from all other haplotypes (Table 1, Fig. 2). There was one dominant haplotype in each geographic area: H1 was the most common in Morocco, H3 in the Iberian Peninsula, and H18 in Tunisia (Table 2). The haplotype that was most frequently found in Morocco (H1) was also found in four Tunisian birds (33%; Table 2). We did not observe any haplotypes shared by Iberian and African populations (Fig. 2).

3.2. Population genetic structure

Differences among Dupont's lark populations accounted for 73% of total molecular variance (Table 3). Hierarchical analyses testing for differences between Iberian and African populations revealed nearly significant differences between both sides of the Mediterranean sea (27.3% of the total variance was explained by differences between populations from different continents; Table 3), but found larger and statistically significant genetic variation among populations within regions (48.4% of total genetic variance; Table 3). However, geographic structure among the Iberian Peninsula, Morocco, and Tunisia accounted for 81% of total genetic variation, leaving no substantial variation to be explained by differences among populations within geographic areas (Table 3). Such a strong geographic structure was also evident from the pattern of pairwise tests of population differentiation (Table 4), with nearly all comparisons of two populations from different geographic areas resulting in large and significant Φ_{ST} values. In general, the Iberian populations of Dupont's larks were more differentiated from African populations than the African populations were differentiated from each other (mean pairwise Φ_{ST}: Iberian vs. Moroccan populations = 0.91; Iberian vs. Tunisian populations = 0.70; Moroccan vs. Tunisian populations = 0.57; Table 4). However, differentiation between populations within geographic areas was extremely weak, as shown by negative Φ_{ST} estimates (which by definition are equivalent to $\Phi_{ST} = 0$; Table 4). Excluding the MoSW population ($n = 2$) from the analyses did not change the results of the AMOVA nor the between population comparisons.

The differentiation of three major groups of Dupont's lark populations (Iberian, Moroccan, and Tunisian) was also supported by the phylogenetic relationships between mtDNA haplotypes. We built a ML tree (Fig. 3) assuming a TIM+I model of nucleotide substitution (the model that best fitted the data among 56 models tested by PAUP*), which supported the existence of three major clades of haplotypes nearly endemic to Iberian, Moroccan, and Tunisian populations, respectively (only one haplotype was shared between Moroccan and Tunisian populations). Other tree reconstruction methods (neighbour-joining and maximum parsimony) produced the same topology.

We found a statistically significant correlation between genetic and geographic distance matrices (Mantel test:

Table 1
Sequence differences among the 16 haplotypes found in Dupont's larks

Haplotype	Variable sites
	1 1
	1 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 0 0
	2 5 9 9 9 6 0 1 1 2 2 4 5 7 3 3 4 4 5 5 6 6 9 0 1 2 4 5 6 7 8 9 3 3 4 5 0 1 1 2 3 3 4 5 5 5 9 9 0 0 1 2 3 0 1 1 2 3 4 5 5 6 7 0 2 3 4 6 6 7 9 0 3
	1 1 6 7 9 8 3 6 9 2 8 6 2 3 6 9 2 8 0 4 1 3 9 8 2 9 1 9 6 1 9 8 4 7 7 3 1 6 9 7 0 6 0 1 4 6 3 6 5 8 1 9 8 9 0 7 5 7 1 6 8 1 0 3 5 9 2 1 7 8 0 6 3
1	A T C C T A T C G A A G A T A A C C T T T A G A T G C C A G C G T A G A G A G A C C G G A T G G A T G C T C A A A C G C T C T A A C A G C G C G A
2	. . . . . A . . . . .
3	. . . . . G . . . . . A . . . T . . . . . C . . . . . A G
4	. . . . . G . . . . . A . . . T . . . . . C . . C . . . . . A G
5	. . . . . G . . . . . A . . A T . . . T . C . . . . . A G
6	. . . . . G . . . . . T . . . . . C . . . . . A G
7	C . . . . G . . . . . A . . . T . . . . . C . . . . . A G
9	. . . . . G . . . . . A . . . T . . . T . C . . . . . A G
10	. . . . . G . . . . . A . . . T . . . . . C . . . . . T . G G . . . . . A G
12	. . . . . G . . . . . A . . . T . . . . . C . . . . . A . . . . . A G
13	. . . . . G . . . . . A G . . T . . . . . C . . . . . A G
14	. . . . . G . . . G . . . . . A . . . T . . . . . C . . . . . A G
15	. . . . . G . . . . .
16	. . . . . G . . . . C . . . . .
17	. C T T A . C T A . G A G C . G T A C C C G . . G A . T C A . A C G A . A G A C T T . A . . A A G C A T C T G G G A . . C T C G . T C A T A A . G
18	. C T T A . C T A . G A G C . G T A C C C G . . G A . T C A . A C G A . A G A C T T . A . . A A G C A T C T G G G A . . C T C G . T C A . A A . G

Dots indicate identical bases. The complete 1041-bp sequence of haplotype H1 was deposited in Genbank with Accession no. EF488005.

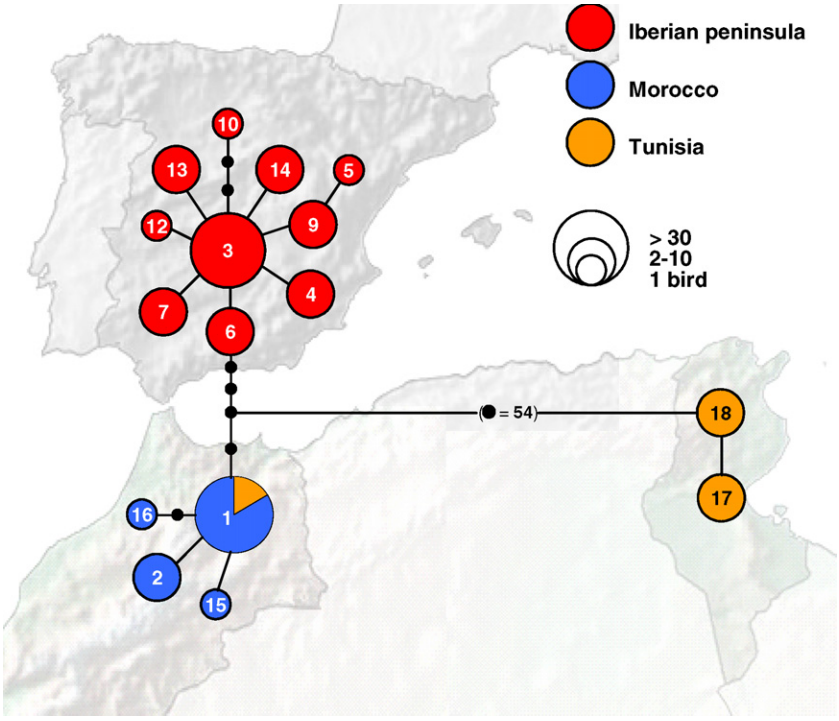

Fig. 2. Minimum spanning network of 16 Dupont's lark mtDNA haplotypes, built using TCS (Clement et al., 2000). Each link between two haplotype represents a unique mutational event, and black dots represent additional mutational changes. Two Tunisian haplotypes are distinguished from other haplotypes by many mutational changes (including a link with 54 changes). The size of circles indicates number of individuals carrying each haplotype (detailed in Table 2), and colours identify geographic areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Distribution of ND2 haplotypes among Dupont's lark populations (SpNW: northwestern Spain; SpC: Spanish central plateau; SPME: Mediterranean Spain; MoNE: northeastern Morocco; MoC: Moroccan central plateau; MoSW: southwestern Morocco; Tu: Tunisia)

Haplotype	Iberian Peninsula			Maghreb				Total
	SpNW	SpC	SpME	MoNE	MoC	MoSW	Tu	
1	—	—	—	12	13	2	4	31
2	—	—	—	1	1	—	—	2
3	8	32	7	—	—	—	—	47
4	—	2	1	—	—	—	—	3
5	—	1	—	—	—	—	—	1
6	—	7	2	—	—	—	—	9
7	—	1	1	—	—	—	—	2
9	—	2	1	—	—	—	—	3
10	—	1	—	—	—	—	—	1
12	—	1	—	—	—	—	—	1
13	1	—	1	—	—	—	—	2
14	—	3	—	—	—	—	—	3
15	—	—	—	1	—	—	—	1
16	—	—	—	—	1	—	—	1
17	—	—	—	—	—	—	2	2
18	—	—	—	—	—	—	6	6
n	9	50	13	14	15	2	12	115

$r = 0.54$, $P = 0.014$), revealing isolation-by-distance effects that were consistent with the above results. However, isolation-by-distance was much influenced by the deep divergence and distant location of the Tunisian population relative to all other populations. Thus, when the Tunisian population was excluded from the analysis, the isolation-by-distance effect remained but it was much smaller and did not reach statistical significance ($r = 0.71$, $P = 0.08$).

3.3. Historical demographic events

Mismatch distributions fitted to the expectations of a sudden expansion model in all populations but Tunisia, which significantly deviated from such expectation (Table 5). Fu's F_S tests of selective neutrality showed that Iberian populations (all populations pooled: $P = 0.002$) and Moroccan populations (all populations pooled:

Table 3
Results of AMOVA testing for differences among populations and geographic areas

Population structure tested	df.	SS	Var. comp.	% Var.	*P*
No grouping					
Among populations	6	449.35	5.08	73.03	<0.001
Within populations	108	202.81	1.88	26.97	
Maghreb vs. Iberian					
Among groups	1	200.91	2.12	27.33	0.07
Among populations	5	248.44	3.76	48.47	<0.001
Within populations	108	202.81	1.88	24.20	<0.001
Moroccan/Tunisian/Iberian					
Among groups	2	448.78	7.46	81.06	0.017
Among populations	4	0.568	−0.13	−1.48	0.89
Within populations	108	202.81	1.877	20.41	<0.001

The analyses partition out total molecular variance into different components, and statistical significance is obtained by randomization after 5000 permutations.

Table 4
Between population differentiation in Dupont's larks (population codes as in Table 2) and measures of genetic variability for each population (nucleotide diversity (π_n) times 1000, gene diversity (h), and observed values of θ)

	Pairwise between population differentiation							Genetic variability		
	Tu	MoC	MoNE	MoSW	SpC	SpME	SpNW	π_n	h	θ_s
Tu	—	***	***		***	***	***	31	0.66	18.87
MoC	0.662	—			***	***	***	0.25	0.14	0.92
MoNE	0.654	−0.038	—		***	***	***	0.14	0.13	0.31
MoSW	0.411	−0.329	−0.328	—	***	**	*	—	—	—
SpC	0.833	0.892	0.898	0.878	—			0.76	0.57	2.23
SpME	0.667	0.902	0.920	0.871	−0.028	—		0.87	0.72	1.61
SpNW	0.628	0.948	0.972	0.968	−0.005	−0.027	—	0.21	0.22	0.37

Below the diagonal, pairwise Φ_{ST} values (based on Tamura & Nei distance method) between haplotypes under a gamma distribution with $\alpha = 0.57$. Above the diagonal, *P*-values for significant Φ_{ST} values obtained after 5000 permutations.
Tunisian population excluding haplotype 1: $\pi_n = 0.41$; $h = 0.43$; $\theta_s = 0.38$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

$P = 0.003$) significantly deviated from population equilibrium, which is consistent with a scenario of population expansion (Fig. 4). The Tunisian population significantly deviated from the sudden expansion model (Table 5), due to a bimodal mismatch distribution caused by great sequence divergence between two haplotypes exclusive to Tunisia (H17 and H18) and the haplotype H1, which was also found in Morocco (Fig. 4).

Our data fitted to a model of clocklike evolution ($\log L_{clock} = -2394.23$; $\log L_{non\text{-}clock} = -2383.43$; $2\Delta\ln L = 21.61$, df. = 15; $P > 0.05$), which allowed us to reasonably derive the age of demographic events during population history from our parameter estimates. Using an intermediate rate of evolution for the ND2 gene of 0.04 s/s/Myr, the central Moroccan population (MoC) would have expanded around 36,000 years ago (range 26,000–72,000 years ago). The Mediterranean population of Iberian Dupont's lark (SpME) would have expanded around 14,000 years ago (range 10,000–28,000 years ago; Table 5). The most recent expansion event in the Iberian Peninsula would have occurred in the northwestern population around 3500 years ago (range 2500–7000 years ago), coinciding with the last expansion of the northeastern Moroccan population (Table 5). Contrasting with these recent events of population expansion in western populations, the Tunisian population would have maintained a more constant long-term population size, having expanded around 100,000 years ago (range 200,000–72,000 years ago; Table 5).

Our non-equilibrium coalescent estimates of divergence time and migration rate between pairs of populations were consistent with the above results (Table 6). Thus, assuming an intermediate substitution rate for the ND2 gene of 0.04 s/s/Myr, Moroccan and Iberian populations diverged from the Tunisian population around 350,000 years ago (Table 6), while the divergence between Iberian and Moroccan populations was much more recent, around 24,000 years ago. Such divergence would have coincided with the expansion of Moroccan populations (Table 5), and would have occurred some 10,000 years before the expansion of the Iberian populations (dates obtained from the sudden expansion model of SpME, see Table 5). Interestingly, our coalescent estimates reveal extremely reduced gene flow between Iberian and Moroccan populations,

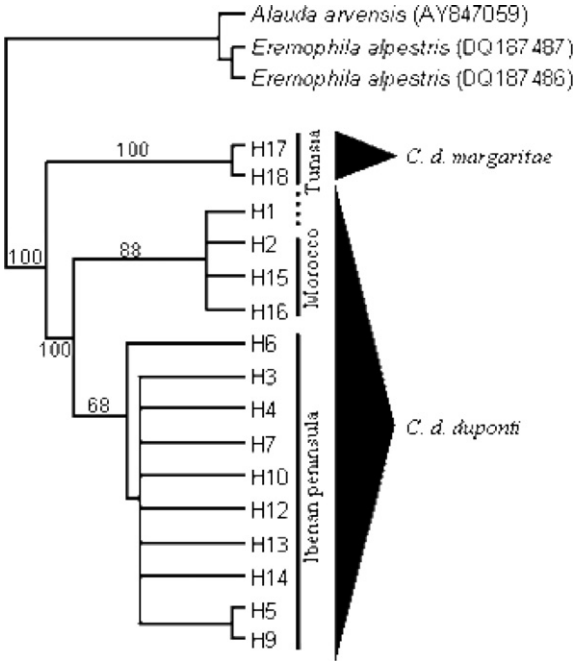

Fig. 3. Maximum likelihood phylogenetic tree of Dupont's lark mtDNA (ND2) haplotypes. DNA sequences of other lark species have been used as outgroups (Genbank accession numbers are indicated on the tree). Bootstrap support to internal branches is indicated by numbers (>50%; 1000 replicates). The haplotype H1 is shared by Moroccan and Tunisian populations.

with an estimated migration rate (m) ranging between 6×10^{-6} and 1.1×10^{-5} migrants per generation (computed on maximum and minimum estimates from all pairs of comparisons and assuming an intermediate substitution rate of 0.04 s/s/Myr). Such estimates are more than 60 times smaller than the migration rates estimated between pairs of Moroccan populations (0.008–0.02) or pairs of Iberian populations (0.0007–0.009). The analysis also reveal that current gene flow between Tunisian and Iberian populations ($m = 7 \times 10^{-6}$), or between Tunisian and Moroccan populations ($m = 9 \times 10^{-6}$) is extremely low.

3.4. Phenotypic differentiation among geographic areas

We found substantial morphological variation among Dupont's larks sampled in the Iberian Peninsula, Morocco, and Tunisia (Table 7). The PCA of body measurements generated two principal components, which together accounted for 71% of variation in the correlation matrix. The PC1 accounted for 42% of total variation (eigenvalue = 1.68; factor loadings: wing length = 0.83, $P < 0.001$; tail length = 0.72, $P < 0.001$; tarsus length = 0.24, $P < 0.001$; bill length = 0.65, $P < 0.001$). Individuals with large PC1 scores had large values for all body dimensions; therefore, the PC1 was a measure of structural body size. The PC2 accounted for 29% of variation in the correlation matrix (eigenvalue = 1.16; factor loadings: wing length = −0.14, $P = 0.08$; tail length = −0.49, $P < 0.001$; tarsus length = 0.85, $P < 0.001$; bill length = 0.41, $P < 0.001$). Individuals with large PC2 values had long tarsi, long bill, and a short tail for their body size. Given that Dupont's larks feed on the ground by walking and pecking out buried invertebrates (De Juana and Suárez, 2004), we interpreted variation in the relative size of legs, bill, and tail as possibly related to variable feeding habits. Our interpretation of principal components of morphological variation was supported by correlations between each component and body mass. In a multiple regression analysis, body mass was positively correlated with structural body size (PC1: $\beta = 0.38$, $P < 0.001$), but it was independent of feeding-related morphology (PC2: $\beta = 0.01$, $P = 0.87$).

We analysed variation in the principal components of morphological variation among geographic areas and among populations within geographic areas, using nested ANOVA. Despite of reduced power for geographic effects due to small sample size (six populations analysed), body size (PC1) showed significant differences between geographic areas ($F_{2,3} = 126.11$, $P = 0.0013$) but it did not vary significantly among populations within areas ($F_{3,170} = 0.20$, $P = 0.90$; Fig. 5). Planned comparisons showed strong differences between areas ($F_{2,170} = 24.83$, $P < 0.001$), and post-hoc Tukey HSD tests showed that variation was clinal from the Iberian Peninsula to Tunisia (Fig. 5). Feeding-related traits also varied significantly among geographic areas ($F_{2,3} = 30.14$, $P = 0.010$), but not among populations within areas ($F_{3,170} = 0.13$, $P = 0.94$; Fig. 5). Planned comparisons of geographic areas revealed the same regional effect ($F_{2,170} = 3.97$, $P = 0.021$). However, post-hoc Tukey HSD tests did not find clinal

Table 5
Statistics of expansion (τ, θ_0, θ_1, chi-square test and significance level) obtained from the sudden expansion model for the populations studied (population codes as in Table 2)

	Statistics of expansion					Years since expansion		
	τ	θ_0	θ_1	χ^2_1	P	Min	Average	Max
Tu	8.281	1.68	1.68	0.186	0.02	72,000	100,000	200,000
MoC	3	0	0.332	0.01	0.28	26,000	36,000	72,000
MoNE	0.289	0	0.239	0.00013	0.35	2500	3500	7000
MoSW	—	—	—	—	—	—	—	—
SpC	0.831	0	860.62	0.0057	0.18	7000	10,000	20,000
SpME	1.146	0	1582.5	0.032	0.09	10,000	14,000	28,000
SpNW	0.294	0	1.874	0.00052	0.29	2500	3500	7000

Estimates of time since expansion were calculated assuming different rates of evolution for the ND2 gene (minimum = 0.02 s/s/Myr; average = 0.04 s/s/Myr; maximum = 0.055 s/s/Myr).

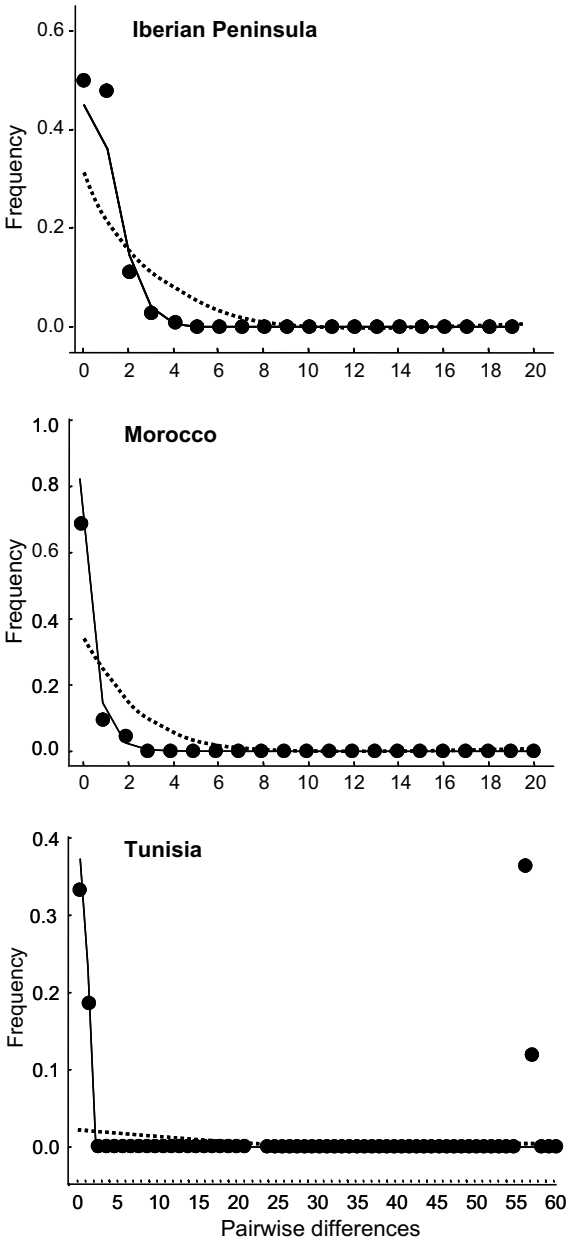

Fig. 4. Mismatch distribution (frequency of pairwise nucleotide differences between haplotypes) estimated in each geographic area (Iberian Peninsula, Morocco, and Tunisia). Dashed lines represent expected frequencies under a constant population size model, and solid lines expected frequencies under a sudden expansion model. The dots are observed frequencies.

variation in feeding-related traits (as observed for body size), but revealed a characteristic morphology of Tunisian Dupont's larks, which were separated from the other populations in the morphological space (Fig. 5). We repeated the same analysis using genotyped birds only, and our results did not change qualitatively.

4. Discussion

The phylogenetic relationships among Dupont's lark mtDNA haplotypes support an early divergence between an eastern lineage (Tunisia) and a western lineage (Morocco and the Iberian Peninsula). According to morphological differences between populations, such lineages probably correspond to the subspecies *C. d. margaritae* (Tunisia) and *C. d. duponti* (western Mediterranean), although the observation of one mtDNA haplotype shared by Tunisian and Moroccan populations (H1) suggests that *C. d. duponti* and *C. d. margaritae* have come into secondary contact sometime during recent history. This is consistent with the recent view that southern Tunisian Dupont's larks have shifted their range northwards (Suárez et al., unpublished data). Following the divergence between eastern and western lineages, the western lineage split into two, apparently when Moroccan and Iberian populations became isolated from each other. Our results regarding population genetic structure, demographic expansion, time since divergence and current gene flow among populations helped us to infer major historical events occurred during the evolutionary history of Dupont's larks.

4.1. Population genetic structure and current gene flow

The geographic structure of genetic variation among Dupont's lark populations in the Iberian Peninsula, Morocco, and Tunisia indicates a high degree of genetic isolation among birds living in different parts of the species' range. The observed phylogeographic pattern supports the idea that the Mediterranean sea acts as a major barrier against gene flow in this species. This is not surprising, because the Dupont's lark is a strict resident, and there are not suitable habitats on the coastal sectors around the Strait of Gibraltar, which are dominated by forests (Costa et al., 1990). Long-term isolation between African and European Dupont's lark populations is the most convincing explanation for the existence of an endemic Iberian lineage with several haplotypes, and the absence of all African haplotypes from Iberian populations (Fig. 2).

However, genetic structure in Dupont's larks could not be explained by geographic isolation between African and European populations alone. In fact, we found more genetic divergence between the two African populations than between Moroccan and Iberian populations, although the latter two populations did not share any haplotypes. Such a discrepancy between geography (i.e. the position of the most obvious geographic barrier against dispersal) and population differentiation was caused by strong divergence of the Tunisian population from both Iberian and Moroccan populations. Such a pattern of geographic structure of genetic variation is consistent with the assumed distribution of the two subspecies recognised by classical taxonomy (*C. d. duponti* in Morocco and the Iberian Peninsula, and *C. d. margaritae* in Tunisia). The correlation between genetic and geographic pairwise distances supported the same idea, revealing an isolation-by-distance effect that was primarily due to the strong divergence of the Tunisian population from the other two groups of populations. In fact, the isolation-by-distance effect vanished

Table 6
Coalescent estimates of divergence time (t) and migration rate (m) between pairs of populations of Dupont's lark (codes as in Table 2), scaled by effective population size

	SpNW	SpC	SpME	MoNE	MoC	MoSW	Tu
SpNW	—	2.47	3.80	0.02	0.02	0.02	0.04
SpC	0.06	—	29.94	0.04	0.02	0.04	0.02
SpME	0.19	0.01	—	0.02	0.02	0.02	0.04
MoNE	6.8	2.40	4.16	—	29.76	8.12	0.03
MoC	6.9	2.58	3.40	0.10	—	9.96	0.04
MoSW	7.8	1.36	1.66	0.02	0.04	—	0.04
Tu	34.68	34.2	30.24	59.2	46.8	57.7	—

Values above the diagonal are estimates of M (=$2Nm$), and values below the diagonal are estimates of T (=$t/2N$).

Table 7
Summary of Dupont's lark morphometrics in Morocco, the Iberian Peninsula, and Tunisia (populations within the same geographic area have been pooled)

Geographic area	Tarsus length	Bill length	Tail length	Wing length
Iberian Peninsula				
n	187	187	109	188
Mean	24.00	23.70	63.30	102.60
Min	22.10	19.69	55.00	94.00
Max	26.40	26.70	71.00	108.00
SD	0.74	1.07	2.94	2.23
Morocco				
n	63	63	60	63
Mean	24.08	24.82	66.46	104.51
Min	20.04	21.04	58.00	99.00
Max	26.20	28.91	76.00	110.00
SD	1.10	1.24	3.40	2.37
Tunisia				
n	12	12	12	12
Mean	24.60	26.22	65.71	105.33
Min	22.90	23.26	62.00	103.00
Max	26.02	28.16	69.50	109.00
SD	0.81	1.66	2.43	1.90

All measurements are in mm.

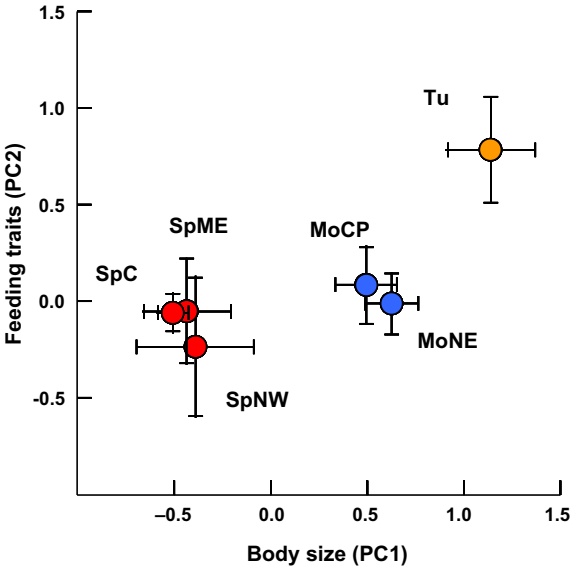

Fig. 5. Variation in Dupont's lark body size (PC1 of body dimensions) and feeding-related morphology (PC2) between populations located in different geographic areas (Means ± 1SE). Iberian populations are labelled in red, Moroccan populations in blue, and the Tunisian population in orange (population codes as in Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

when the Tunisian population was excluded from the analysis, and our estimates of pairwise Φ_{ST} revealed abundant genetic exchange between populations within the Iberian Peninsula or within the western part of the Maghreb, the most apparent differentiation taking place between pairs of populations from different continents (Table 4).

Our non-equilibrium coalescent estimates of divergence time and migration rate between pairs of populations produced the same conclusions derived from the above results. These estimates indicated that Iberian and Moroccan populations had a much younger (24,000 years old) most recent common ancestor (MRCA) than the MRCA of all three populations (our average estimate for time of divergence between Tunisian and Iberian or Moroccan populations was 350,000 years ago). Such a result is also consistent with the age of demographic expansions of Dupont's lark populations inferred from mismatch distributions (see below). In addition, our coalescent estimates of gene flow also support the view that the Mediterranean sea acts as a major barrier against dispersal between Dupont's lark populations. Thus, such estimates indicate that current gene flow is relatively high among populations within geographic areas, whereas migration between populations on different continents is virtually absent.

4.2. Historical demographic events in Dupont's larks

Mismatch distributions provided evidence for sudden population expansion events occurred during the recent evolutionary history of Moroccan and Iberian Dupont's lark populations, but they suggested more constant long-term effective population size for the Tunisian population (Table 5). Demographic expansion events in the western part of the species' range apparently happened in two waves. The first expansion took place some 36,000 years ago in Morocco and around 14,000 years ago in the Iberian Peninsula, which is consistent with coalescent estimates of divergence time between the two groups of populations (24,000 years ago). A more recent expansion event (around

3500 years ago) seemed to take place in northwestern Iberia and in northeastern Morocco (Table 5). Recent expansion of these populations could be related with changes in human land use, such as the substitution of forests by arable land, which dramatically changed the Mediterranean landscape during the last 5000 years (Puigdefábregas and Mendizábal, 1998; Pantaleón-Cano et al., 2003). In fact, most populations of steppe birds, in particular the Iberian ones, are found today in agricultural pseudosteppes, which have become priority areas for conservation of steppe avifauna in European countries (Santos and Suárez, 2005).

Mismatch distributions and coalescent estimates of time of divergence between pairs of populations supported the idea that Dupont's larks recently colonised the Iberian Peninsula from northwestern Africa. Although we assumed a wide range of possible substitution rates for mtDNA, our results consistently indicated that Iberian and Moroccan populations diverged from each other during the last glacial period, around 24,000 years ago. During the last glaciation, many areas in southern Europe and northern Africa became more arid (Goudie, 1979). As a consequence, environmental moisture was largely restricted to the slopes of Mediterranean mountains (Tzedakis et al., 2002), favouring the spread of steppes, which were much better represented in the Mediterranean area 40,000 to 18,000 years ago than they are represented today (Jolly et al., 1998; Allen et al., 1999). Increased range of suitable habitat during cold Pleistocene periods should have increased connectivity among populations, thereby favouring the spread of steppe birds over larger geographic areas, and perhaps also the dispersal of Dupont's larks between northern Africa and the Iberian Peninsula.

Since the last glacial maximum 18,000 years ago, the Mediterranean region has become progressively warmer, initially causing the contraction of areas occupied by steppe habitats due to increased overall moisture (Costa et al., 1990; Blondel and Aronson, 1999), which probably caused population bottlenecks that would contribute to explain the intense sorting of ancestral genetic variation observed among geographic areas. Since 7000 years ago until present time, steppes and other open dry habitats expanded up to reach their current range, primarily due to increased environmental xericity (Costa et al., 1990; Blondel and Aronson, 1999; Pantaleón-Cano et al., 2003), but also due to the manmade opening of agricultural landscapes in otherwise forested areas (Puigdefábregas and Mendizábal, 1998). In turn, these environmental changes probably determined the current configuration of Dupont's lark's geographic distribution.

4.3. Phenotypic variation among populations

Our analysis of morphological variation showed that Dupont's lark populations are not only genetically distinct, but also phenotypically differentiated. Gradual differences in body size (Iberian birds are comparatively small, whereas Tunisian birds are comparatively large) overlap with a clear differentiation of the Tunisian population from the other two populations. Unique morphological traits of Tunisian Dupont's larks are long feet and bill relative to body size, which may be related with particular feeding habits (Carrascal et al., 1990; Durell, 2000; Zeffer and Norberg, 2003). In general, the observed morphological differences are consistent with subspecies differences reported in the literature, with putative *C. d. margaritae* having longer bill than putative nominate *C. d. duponti* (cf. Koening, 1888). Morphological differentiation observed in this study may be due to different natural selection forces acting in each region. For example, coexistence with other lark species in the Maghreb could have favoured character displacement and increased body size in this region, as it has apparently happened in other lark species (Guillaumet et al., 2006). In relation to feeding traits, Tunisian population inhabit particularly dry habitat, where insects may need to be pulled out from deeper in the ground, which might explain the comparatively long bill of Tunisian Dupont's larks (similar variation in bill size has been found in other lark species; Guillaumet et al., 2005). As an alternative to adaptive explanations for the observed morphological differences, Dupont's larks might have diverged phenotypically as a consequence of genetic drift during long periods of independent evolution in separated geographic areas, which indeed are hardly connected by gene flow (Guillaumet et al., 2005, 2006).

An important conclusion of our integrated analysis of genetic and phenotypic variation among Dupont's lark populations is that the species has apparently faced long-term limitation to dispersal among populations. This may seem an odd result because most passerine birds can fly long distances, and overcome geographic barriers that typically restrict gene flow in other animals (Hewitt, 2000). In addition, although all extant populations of a bird species are resident, climate change during glaciations may have triggered rapid evolutionary changes of migratory behaviour, which could have favoured long-distance dispersal in the past (Pérez-Tris et al., 2004). Therefore, we conclude that Dupont's larks have maintained strict sedentary habits and very restrictive habitat selection during historical periods, in the same way as they behave today (Thévenot et al., 2003; De Juana et al., 2004; Garza et al., 2005 , Isenmann et al., 2005; García et al., in press). If Dupont's larks do have reduced evolutionary flexibility, such circumstance would further compromise the long-term preservation of the species.

4.4. Implications for Dupont's lark conservation

Historical vicissitudes related to the expansion and contraction of steppe habitat, together with extreme sedentary behaviour, has rendered Dupont's larks subdivided into genetically and phenotypically distinct populations. When considered separately, such populations have restricted

geographic ranges, which apparently are not connected by gene flow. The population genetic structure observed in Dupont's larks compares to the observed in species with different populations isolated on oceanic islands, which are doubly threatened by small population size and reduced connectivity (Manne et al., 1999). Such a scenario represents a daunting challenge for the conservation of Dupont's lark, because the fact that the species' genetic variation is strongly subdivided into discrete units means that the loss of one population would be irreversible in genetic terms.

Today, gene flow between European and African Dupont's lark populations is extremely low, meaning that Iberian populations, which represent unique genetic variants within the range of *C. d. duponti* of classical taxonomy, need specific conservation plans that cannot rely on the existence of African populations of the same subspecies flourishing in more continuous, natural habitat. The urge to protect Iberian Dupont's larks increases because natural habitats occupied by the species are becoming increasingly scarce and fragmented (Laiolo and Tella, 2005; García et al., in press). Reciprocally, in the light of our results the genetic heritage of African populations becomes more threatened than previously thought, which demands urgent decisions to protect the habitat of African Dupont's larks. For example, our results illustrate the critical situation of Dupont's larks in Tunisia. In this country, less than 500 males remain restricted to a single area of less than 90 km^2, where habitat loss or degradation due to agricultural development, overgrazing and increased aridity is pushing the population to the verge of extinction (Suárez et al., unpublished data). Worryingly, the situation is expected to get worse as a consequence of ongoing global warming and the associated desertification of steppes used by Dupont's larks (Le Houérou, 2000). Within such scenario of general habitat loss and deterioration, our results reveal independent evolutionary history, phenotypic divergence and reduced gene flow among Iberian, Moroccan, and Tunisian Dupont's lark populations, which should be considered as different management units (Moritz, 1994). The situation is probably not better for other populations scattered across northern Africa, for which we hardly know the size and distribution—let alone their genetic or phenotypic characteristics—, which stands for the urgency of conservation policies being put into action to protect the Dupont's lark and its habitat.

Acknowledgment

We thank A. Ramírez, J. Oñate, J. Viñuela, I. Hervás, R. del Pozo, E. Juarez, E.L. García, and Mohammed Alouí for their help during fieldtrips, P. Ozenda for the great book *Flore du Sahara*, and one anonymous referee for their comments on the manuscript. All samples were collected under license from the corresponding national and regional authorities. This project was partially financed by the authors, the Spanish Ministry of Environment (MMA), the Spanish Agency for International Cooperation (AECI), the Spanish Ministry of Science and Technology (Ramón y Cajal Programme, JP-T) and the CSIC-I3P programme (J.T.G).

References

Alcaráz, D., Paruelo, J.M., Cabello, J., 2006. Identification of current ecosystem functional types in the Iberian Peninsula. Global Ecol. Biogeogr. 15, 200–212.

Allen, J.R.M., Brandt, U., Brauer, A., et al., 1999. Rapid environmental changes in southern Europe during the last glacial period. Nature 400, 740–743.

Arbogast, B.S., Edwards, S.V., Wakely, J., Beerli, P., Slowinski, J.B., 2002. Estimating divergence times from molecular data on phylogenetic, population genetic timescales. Ann. Rev. Ecol. Sys. 33, 707–740.

Arbogast, B.S., Drovetski, S.V., Curry, R.L., et al., 2006. The origin, diversification of Galapagos mockingbirds. Evolution 60, 370–382.

BirdLife International, 2006. Species factsheet: *Chersophilus duponti*. Birdlife International, Cambridge, UK <http://www.birdlife.org>, (accessed 20 February 2006).

Blondel, J., Aronson, J., 1999. Biology, Wildlife of the Mediterranean Region. Oxford University Press, New York.

Bowen, B.W., 1999. Preserving genes, species, or ecosystems? Healing the fractured foundations of conservation policy. Mol. Ecol. 8, S5–S10.

Cañadas, S., Castro, H., Manrique, J., Miralles, J.M., 1988. datos sobre la reproducción de la Alondra de Dupont *Chersophilus duponti* en Almería. Ardeola 35, 158–162.

Carrascal, L.M., Moreno, E., Tellería, J.L., 1990. Ecomorphological relationships in a group of insectivorous birds of temperate forest in winter. Holarctic Ecol. 13, 105–111.

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1660.

Costa, M., García, M., Morla, C., Sainz, H., 1990. La evolución de los bosques de la Península Ibérica : una interpretación basada en datos paleobiogeográficos. Ecología 1, 31–58.

Cramp, S., 1988The Birds of the Western Palearctic, vol. 5. Oxford University Press, Oxford, UK.

Crandall, K.A., Bininda-Emonds, O.R.P., Mace, G.M., Wayne, R.K., 2000. Considering evolutionary processes in conservation biology. Trends Ecol. Evol. 15, 290–295.

De Juana, E., Suárez, F., 2004. Dupont's lark *Chersophilus duponti*. In: Del Hoyo, J., Elliott, A., Christie, D. (Eds.), Handbook of the Birds of the World, vol. 9. Lynx Edicions, Barcelona, p. 568.

De Juana, E., Suárez, F., Ryan, P., 2004. Family Alaudidae larks. In: Del Hoyo, J., Elliott, A., Christie, D. (Eds.), Handbook of the Birds of the World, vol. 9. Lynx Edicions, Barcelona, pp. 496–541.

Donald, P.F., 2004. The Skylark. T and AD Poyser, London.

Drovetski, S.V., Zink, R.M., Fadeev, I.V., et al., 2004. Mitochondrial phylogeny of *Locustella*, related genera. J. Avian Biol. 5, 105–110.

Drovetski, S.V., Pearson, S.F., Rohwer, S., 2005. Streaked horned lark *Eremophila alpestris strigata* has distinct mitochondrial DNA. Conserv. Genet. 6, 875–883.

Durell, S., 2000. Individual feeding specialization in shorebirds. Population consequences, conservation implications. Biol. Rev. 75, 503–518.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Frankham, R., Ballou, J.D., Briscoe, D.A., 2002. Introduction to conservation genetics. Cambridge University Press, Cambridge.

Fu, Y.-X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking, background selection. Genetics 147, 915–925.

García, J.T., Suárez, F., Garza, V. et al., in press. Distribution, population size of the threatened Dupont's lark *Chersophilus duponti* in Morocco. Oryx 41.

Gärdenfors, U., 2001. Classifying threatened species at national versus global levels. Trends Ecol. Evol. 16, 511–516.

Garza, V., Traba, J., Suárez, F., 2003. Is the European population of Dupont's lark *Chersophilus duponti* adequately estimated? Bird Study 50, 309–311.

Garza, V., Suárez, F., Tella, J.L., (2004). Alondra de Dupont, *Chersophilus duponti*. In: Madroño, A., González, C., Atienza, J.C. (Eds.), Libro Rojo de las Aves de España. Dirección General para la Biodiversidad-SEO/BirdLife, Madrid, Spain, pp. 309–312.

Garza, V., Suárez, F., Herranz, J., et al., 2005. Home range, territoriality, habitat selection of the endangered Dupont's lark *Chersophilus duponti*: the breeding, postbreeding periods. Ardeola 52, 133–146.

Goudie, A.S., 1979. Environmental change. Clarendon Press, Oxford.

Griffiths, R., Double, M.C., Orr, K., Dawson, R.J.G., 1998. A DNA test to sex most birds. Mol. Ecol. 7, 1071–1075.

Groom, M.J., Meffe, G.K., Carroll, C.R., 2006. Principles of Conservation Biology, third edition. Sinauer.

Guillaumet, A., Crochet, P.A., Godelle, B., 2005. Phenotypic variation in Galerida larks in Morocco: the role of history, natural selection. Mol. Ecol. 14, 3809–3821.

Guillaumet, A., Pons, J.-M., Godelle, B., Crochet, P.-A., 2006. History of Crested lark in the Mediterranean region as revealed by mtDNA sequences, morphology. Mol. Phylogenet. Evol. 39, 645–656.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor, analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Herranz, J., Manrique, J., Yanes, M., Suárez, F., 1994. The breeding biology of Dupont's lark, *Chersophilus duponti*, in Europe. Avocetta 18, 141–146.

Hewitt, G., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913.

Isenmann, P., Gaultier, R., El Hili, A., Azafzaf, H., Dlensi, H., Smart, M., 2005. Oiseaux de Tunisie. Birds of Tunisia. Société d'Études Ornithologiques de France, Paris.

IUCN, 2006. 2006 IUCN Red List of Threatened Species, IUCN, Cambridge, UK <http://www.iucnredlist.com>, (accessed 29 May 2006).

Johnson, A.J., Lerner, H.R.L., Rasmussen, P.C., Mindell, D.P., 2006. Systematics within Gyps vultures: a clade at risk. BMC Evol. Biol. 6, 65.

Jolly, D., Harrison, S.P., Damnati, B., Bonnefille, R., 1998. Simulated climate, biomes of Africa during the late Quaternary: comparison with pollen, lake status data. Quaternary Sci. Rev. 17, 629–657.

Koening, A., 1888. Avifauna von Tunis. J. Ornithol. 183, 9–298.

Laiolo, P., Tella, J.L., 2005. Habitat fragmentation affects culture transmission: patterns of song matching in Dupont's lark. J. Appl. Ecol. 42, 1183–1193.

Lande, R., 1988. Genetics, demography in biological conservation. Science 241, 1455–1460.

Lande, R., Shannon, S., 1996. The role of genetic variation in adaptation, population persistence in a changing environment. Evolution 50, 434–437.

Le Houérou, H.N., 2000. Restoration, rehabilitation of arid, semiarid Mediterranean ecosystems in North Africa, West Asia: a review. Arid Soil Res. Rehab. 14, 3–14.

Manne, L.L., Brooks, T.M., Pimm, S.L., 1999. Relative risk of extinction of passerine birds on continents, islands. Nature 399, 258–261.

Moritz, C., 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. Mol. Ecol. 3, 401–411.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858.

Nielsen, R., Wakeley, J., 2001. Distinguishing migration from isolation: a Markov Chain Monte Carlo approach. Genetics 158, 885–896.

Pantaleón-Cano, J., Yll, E.I., Pérez-Obiol, R., Roure, J.N., 2003. Palynological evidence for vegetational history in semi-arid areas of the western Mediterranean Almería, Spain. Holocene 13, 109–119.

Peck, Dr., Congdon, B.C., 2004. Reconciling historical processes, population structure in the sooty tern *Sterna fuscata*. J. Avian Biol. 35, 327–335.

Pérez-Tris, J., Bensch, S., Carbonell, R., Helbig, A.J., Tellería, J.L., 2004. Historical diversification of migration patterns in a passerine bird. Evolution 58, 1819–1832.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Puigdefábregas, J., Mendizábal, T., 1998. Perspectives on desertification: western Mediterranean. J. Arid Environ. 39, 209–224.

Rogers, A.R., 1995. Genetic evidence for a Pleistocene population explosion. Evolution 49, 608–615.

Rogers, A.R., Harpending, H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9, 552–569.

Rozas, J., Sanchez-DelBarrio, J.C., Messequer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analysis by the coalescent, other methods. Bioinformatics 19, 2496–2497.

Santos, T., Suárez, F., 2005. Biogeography, population trends of Iberian steppe birds. In: Bota, G., Morales, M.B., Mañosa, S., Camprodon, J. (Eds.), Ecology, Conservation of Steppe-land Birds. Lynx Edicions and centre Tecnològic Forestal de Catalunya, Barcelona, pp. 69–102.

Sanz, J.J., 2002. Climate change, birds: have their ecological consequences already been detected in the Mediterranean region? Ardeola 49, 109–120.

Schmidt, H.A., Strimmer, K., Vingron, M., von Haeseler, A., 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets, parallel computing. Bioinformatics 18, 502–504.

Schneider, S., Roessli, D., Excoffier, L., 2000. Arlequin version 2000: a software for population genetics data analysis. Genetics, Biometry Laboratory, University of Geneva, Switzerland.

Sorenson, M.D., Ast, J.C., Dimcheff, D.E., Yuri, T., Mindell, D.P., 1999. Primers for a PCR-based approach to mitochondrial genome sequencing in birds, other vertebrates. Mol. Phylogenet. Evol. 12, 105–114.

StatSoft, Inc., 2002. STATISTICA data analysis software system, version 6. <www.statsoft.com>.

Suárez, F., Sainz-Ollero, H., Santos, T., González-Bernáldez, F., 1992. Las estepas ibéricas. M.O.P.T., Unidades Temáticas de la Secretaría de Estado para las Políticas del Agua y el Medio Ambiente, Madrid.

Svensson, L., 1992. Identification guide to European passerines. L. Svensson, Stockholm.

Swoford, D.L., 2002. PAUP*: phylogenetic analysis using parsimony, other methods., version 4.0 b10. Sinauer, Sunderland, MA.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Tella, J.L., Vögeli, M., Serrano, D., Carrete, M., 2005. Status of the threatened Dupont's lark in Spain: overestimation, decline, extinction of local populations. Oryx 39, 1–5.

Thévenot, M., Vernon, R., Bergier, P., 2003. The Birds of Morocco. An annotated checklist. British Ornithologists' Union Checklist N°20, Tring.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment trough sequence weighting, position-specific gap penalties, weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tucker, G.M., Heat, M.F., 1994. Birds in Europe: their conservation status. Birdlife International Conservation Series N°3. Cambridge, UK.

Tzedakis, P.C., Lawson, I.T., Frogley, M.R., Hewitt, G.M., Preece, R.C., 2002. Buffered tree population changes in a Quaternary refugium: evolutionary implications. Science 297, 2044–2047.

Viellot, L.J.P., 1820. Faune française ou Histoire naturelle, générale et particulière des animaux qui se trouvent en France. Le Vrault & Rapet, Paris, Strasburg, Brüssel, 1820–30.

Walther, G.R., Post, E., Convey, P., et al., 2002. Ecological responses to recent climate change. Nature 416, 389–395.

Warren, B.H., Bermingham, E.B., Bowie, R.C.K., Prys-Jones, R.P., Thébaud, C., 2003. Molecular phylogeography reveals island colonization history, diversification of western Indian Ocean sunbirds Nectarinia: Nectariniidae. Mol. Phylogenet. Evol. 29, 67–85.

Zeffer, A., Norberg, U.M., 2003. Leg morphology, locomotion in birds: requirements for force, speed during ankle flexion. J. Exp. Biol. 206, 1085–1097.