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Population decline is accompanied by loss of genetic diversity in the Lesser Grey Shrike *Lanius minor*

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The Lesser Grey Shrike has suffered successive declines in population size and a marked contraction of its breeding range since the early 20th century, largely because of long-term agricultural intensification. This has resulted in a severely fragmented distribution in Western Europe, with isolated breeding nuclei in Spain, France and Italy and a more continuous distribution in Eastern Europe and Asia. Using a combination of nuclear and mitochondrial markers, we assessed the genetic structure and diversity of Lesser Grey Shrike populations from Western Europe, Central Europe and Asia. There was significant genetic differentiation among three major regional groups, one European and two Asian. Genetic diversity measures were lowest in the smallest and most marginal Spanish population. Limited genetic diversity, combined with rapid population decline, suggests the Spanish population may face extinction in the near future.

Keywords: endangered species, genetic structure, inbreeding, peripheral population, subspecies.

Increasingly, conservation biologists are successfully combining population ecology and population genetics in studies focused on the management of endangered and declining species (Frankham *et al.* 2002). Whereas population ecology focuses on processes affecting population demographics, such as habitat deterioration and population growth rates (Sinclair *et al.* 2006), conservation genetics examines geographical structure and temporal changes in genetic variation. Genetic drift and inbreeding are the major phenomena causing loss of genetic variation in small populations (Amos & Harwood 1998). In fragmented habitats, population sizes tend to decline and populations become isolated from each other. This can lead to increased genetic differentiation between populations via the fixation of alleles. Knowledge of the alteration of genetic makeup and/or loss of variation are of interest in conservation biology, because a population is considered to have greater fitness if genetic variation is sufficient to allow for adaptive response to environmental changes, particularly in the face of climate change.

Changes in demography and genetics are often more marked at the edges of a species' range, where numbers are lower and where often fragmented populations may occupy suboptimal habitats (Lesica & Allendorf 1995, Kvist *et al.* 2007). Many species with broad geographical distributions thrive at the centre of the range but decline at the periphery, where they are often considered regionally endangered (Hoffmann & Blows 1994). These peripheral populations are often genetically divergent from the central population and may hold genetic variation that does not exist elsewhere. Thus, their value for future adaptations and evolution can be substantial. Genetic analyses of such

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species include tests for the loss of genetic variation and definition of management units (MUs) and evolutionarily significant units (ESUs; Moritz 1994, Crandall *et al.* 2000) using population genetic, phylogenetic and ecological tools. Genetic data can provide objective measures to pass on to decision-makers.

The Lesser Grey Shrike *Lanius minor* is a socially monogamous long-distance migratory passerine breeding in Eurasia, and has a range extending over 6000 km from east (Kazakhstan) to west (northern Spain) and over 2000 km from south (Turkey) to north (Russia) (Lefranc & Worfolk 1997). Within this range, breeding localities are patchily distributed (especially in Europe) and limited to warm, flat areas with predominantly steppe and/or farmland habitats. The wintering area is centred on the Kalahari basin in southern Africa (Newton 1995, Herremans 1998). All populations migrate between southern Africa and Eurasia through the Middle East and southeastern Europe, with the western and easternmost populations covering about 10 000 km twice a year, making it one of the longest migratory movements among passerines (Lefranc & Worfolk 1997). This shrike is often considered to include two subspecies (Vaurie 1955, Clancey 1980), the smaller and slightly darker *minor* (from Spain to western Russia), and the larger *turanicus* (from the Ural Mountains to central Asia). Some authors consider the species to be monotypic (see Lefranc & Worfolk 1997 for a review) with just slight and clinal morphological variation across the range.

The Lesser Grey Shrike was a fairly abundant bird in central and southern Europe during the 19th century (Lefranc & Worfolk 1997). Thereafter, its population and range sharply declined and contracted, especially in the western half of Europe, where it became extinct in many countries in the 1930s. Because of agricultural intensification, most other European populations underwent moderate decline between 1970 and 2000, with the exception of some central and eastern European countries where the species' demography remains stable (e.g. Hungary; BirdLife International 2004). Currently, the western European populations are restricted to small isolated nuclei in northeastern Spain (fewer than 20 pairs, Giralt & Bota 2003, Giralt & Valera 2007), southeastern France (20–40 pairs, Rufray & Rousseau 2004) and Italy (1000–2500 pairs), whereas the species remains widespread in Eastern Europe (BirdLife International 2004). Data on Asian population trends are scarce, although the species is apparently stable and locally abundant, at least in southern Russia, Georgia (BirdLife International 2004) and Kazakhstan (A. Gavrilov pers. comm.).

The aims of this study were to assess genetic structure among Lesser Grey Shrike populations to identify management units and examine whether the defined races are genetically distinct, to analyse genetic variation within populations to determine whether the decline of the Spanish population has had any genetic effects, and to suggest guidelines for genetic management of the species.

METHODS

Sampling

Studied populations included two breeding nuclei in Spain (Catalonia, 41°34′N, 0°41′E; Aragón, 41°43′N, 0°12′E), two populations from central Europe (Slovakia, 48°36′N, 19°20′E; Hungary, 46°32′N, 20°05′E) and two from Asia (Georgia, 41°45′N, 44°50′E; Kazakhstan, 42°56′N, 70°37′E) (Fig. 1). Spanish birds were sampled during the 2004–2006 breeding seasons, Georgian and Kazakh birds in 2006, Slovakian birds during the 1999 breeding season and Hungarian birds in 1998. Adult birds were caught with clap-nets and mist-nets during nesting. A few nestlings were also sampled in Spain, Slovakia and Hungary. We sampled either blood or feathers.

Laboratory procedures

DNA was extracted from blood samples using the standard phenol-chloroform method and from feather quills using the method described in Kvist *et al.* (2003). Blood stored on FTA cards was prepared for PCR-amplification according to the manufacturer's protocol (Whatman).

Initially, the whole mitochondrial control region was amplified from 10 individuals using primers ND6L (5′-CTAAACAGCCCGAATCGCCC-3′; designed for this study to match a conserved region in the passerine ND6 gene) and laniusftph2 (5′-TCTTGACATCTTCAGTGCCATGC-3′; designed to match the tRNA-Phe gene using available sequences of *Lanius* on GenBank). Based on the obtained sequences, primers NDL6 and STH411 (5′-AAATAACCAGGTTCTCTGGCTTG-3′, originally designed for the Blue Tit *Cyanistes caeruleus*

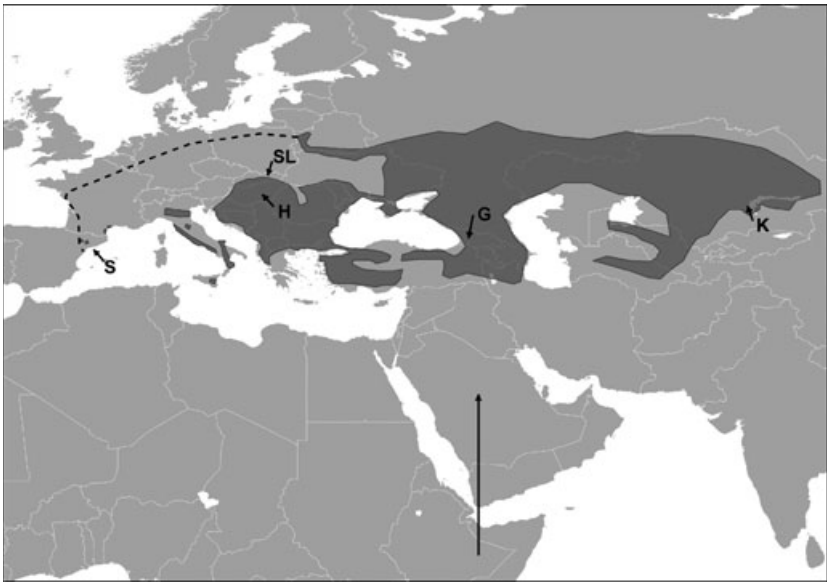

Figure 1. Current breeding range (dark shaded area) of the Lesser Grey Shrike (del Hoyo *et al.* 2008) and the species' western limit at the beginning of the 20th century (broken line; Lefranc & Worfolk 1997). Sampled populations are marked with small arrows (S, Spain; SL, Slovakia; H, Hungary; G, Georgia; K, Kazakhstan). The long arrow in Saudi Arabia represents the last section of the spring migration route before reaching the breeding areas.

(Kvist *et al.* 2005)) were used to amplify a shorter portion of the mitochondrial control region (including the highly variable first domain and a portion of the central domain). The following PCR profile was used 94 °C for 2 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s, with a final extension at 72 °C for 2 min. Sequencing was performed with BigDye™ v3.0 (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol using primer STH411. Sequencing was performed in one direction only and products were run on an ABI 3730 automatic sequencer.

Eight microsatellite loci – *LM1* (T_m = 47 °C), *LM2* (T_m = 47 °C), *LM3* (T_m = 53 °C), *LM4* (T_m = 53 °C), *STG4* (T_m = 55 °C), *Ppi2* (T_m = 50 °C), *Lox1* (T_m = 50 °C) and *Pocc6* (T_m = 50 °C) – were amplified with the following PCR profile: 94 °C for 2 min followed by 35 cycles of 94 °C for 45 s, 47–55 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 2–10 min. The first five loci were developed from the Loggerhead Shrike *Lanius ludovicianus* (Mundy & Woodruff 1996); *Ppi2* was developed from the Eurasian Magpie *Pica pica* (Martínez *et al.* 1999); *Lox1* is from the Scottish Crossbill *Loxia scotica* (Piertney *et al.* 1998); and *Pocc6* is from the Western Crowned Warbler *Phylloscopus occipitalis* (Bensch *et al.* 1997). The amplified microsatellite alleles were screened on an ABI 3730 automated DNA sequencer and scored using GENEMAPPER v3.7 (Applied Biosystems).

Data analysis

Prior to data analysis, all closely related birds were removed from the dataset. Only one nestling was included per nest and only one parent or nestling was included in the microsatellite data (we did not sample any mother–nestling pairs but fathers were included in the mitochondrial sequence data). Thus, our sample sizes for mitochondrial/microsatellite analyses were: Catalonia n = 13/15, Aragón n = 4/3, Slovakia n = 13/15, Hungary n = 9/9, Kazakhstan n = 14/16, Georgia n = 8/8.

From the mitochondrial data, the nucleotide diversity, theta and haplotype diversity for each population were estimated using DNASP v4.0 (Rozas & Rozas 1999); differences in parameter values among populations were tested with ANOVA. To identify populations that differed from each other, the least-square difference *post hoc* test was applied. DNASP was also used to calculate mismatch distributions, neutrality indices (Tajima's D and Fu's Fs) and the raggedness index to test for signs of demographic change in

the study populations. Smooth unimodal mismatch distributions (tested with raggedness statistics) with significantly negative Tajima's *D* and Fu's *Fs* values are consistent with demographic change, possibly as a consequence of population expansion (Rogers & Harpending 1992). In addition, the software LAMARC v2.1.3 (Kuhner 2006) was used to estimate of the growth rate with a 95% confidence interval based on the coalescent. The default parameters, 10 initial chains (10 000 steps) and two final chains (200 000 steps) were used with the MCMC algorithm, sampling every 20th tree. ARLEQUIN v3.1 (Excoffier *et al.* 1992) was used to estimate pairwise Φ_{ST} values among populations (AMOVA), using Tamura–Nei distances. We also used SAMOVA (Dupanloup *et al.* 2002) to group genetically similar populations and then recalculated Φ_{ST} values based on these groupings. Significance of the pairwise Φ_{ST} values was determined by comparison with a null distribution derived from permuting haplotypes between populations and recalculating Φ_{ST} values 1000 times. The Tamura–Nei substitution model was chosen based on results from MODELGENERATOR v0.83 (Keane *et al.* 2006). Tamura–Nei was the best-fit model based on the Akaike information criteria (AIC) and was the second best model using the Bayesian information criterion (BIC). As it was simpler than the alternative (HKY) and is implemented in most population genetic software, we used the Tamura–Nei model for our analyses. Correlations between genetic and geographical distances were calculated using a Mantel test as implemented in ARLEQUIN (Slatkin's linearized F_{ST} was used for genetic distance and geographical distances were converted into natural logarithms). A parsimony network was constructed with TCS (Clement *et al.* 2000).

The microsatellite data were tested for null alleles, scoring errors and large allele dropout using MICROCHECKER v2.2.3 (Van Oosterhout *et al.* 2004). Hardy–Weinberg (HW) equilibrium was calculated using GENEPOP 4.0 (Raymond & Rousset 1995) and linkage disequilibrium was estimated with FSTAT v2.9.3.2 (Goudet 2001). A Bonferroni correction for multiple tests was applied to *P*-values as implemented in FSTAT. ARLEQUIN was used to calculate observed and expected heterozygosities per locus and population, conduct a Mantel test (as detailed above for mitochondrial data) and sample assignment likelihoods for each of the predefined populations. Assignment likelihoods were computed as log-likelihoods of individual genotypes belonging to a population based on allele frequencies in the population. The inbreeding coefficient F_{IS}, allelic richness and allele distributions were calculated with FSTAT (Goudet 2001) using the rarefaction method to correct for differences in sample sizes. Between-population differences in heterozygosity and allelic richness were tested with ANOVA.

STRUCTURE v2.2 (Pritchard *et al.* 2000) was used to test for clustering of the genotypes into genetically differentiated populations. The number of populations (*k*) was varied from one to six and the assignment probabilities for each individual were estimated using the admixture model with a burn-in of 100 000, 200 000 MCMC replicates and two iterations. In addition, a factorial analysis of correspondence, as implemented in the program GENETIX v4.05 (Belkhir *et al.* 2004), was used to represent genetic variation graphically within and among populations. Pairwise F_{ST} values between populations were calculated with ARLEQUIN and SAMOVA, as explained above for mitochondrial data. Effective population sizes were estimated for the three populations with sample sizes larger than 10 (Spain, Slovakia and Kazakhstan) using the linkage disequilibrium method implemented in NeEstimator v1.3 (Peel *et al.* 2004). BOTTLENECK v1.2.02 (Cornuet & Luikart 1996) was used to test for recent bottlenecks in each population. Bottlenecked populations lose rare alleles faster as heterozygosity decreases, leading to a situation where heterozygosity is higher than expected for a given number of alleles. The distribution of allele frequencies is also affected, shifting towards larger proportions of low-frequency alleles. The program was run with three different mutation models: infinite allele, stepwise mutation and two-phase models (70% stepwise and 30% infinite) with 1000 iterations. To test whether the extent of genetic diversity retained in relation to the number of loci was greater than expected we made use of the Wilcoxon signed-rank test due to our small sample sizes. In addition, the M-ratio (Garza & Williamson 2001) was estimated for each population, again using ARLEQUIN. This index estimates the ratio between the number of alleles and allelic range. A bottleneck reduces the number of alleles faster than the range, and therefore a small ratio indicates a recent bottleneck.

RESULTS

Mitochondrial DNA

The obtained sequences contained no double peaks and no systematic differences could be related to the tissue from which DNA was isolated. Therefore, the possibility of amplifying a nuclear copy of a mitochondrial gene was considered very unlikely.

Table 1. Mitochondrial nucleotide diversity (π), theta (θ), haplotype diversity ($\hat{h}$), number of haplotypes and sample sizes (*n*) per sampled shrike population.

Population	π	θ	$\hat{h}$	No. of haplotypes	*n*
Spain	0.00061	0.00154	0.228	3	17
Catalonia	0.00080	0.00168	0.295	3	13
Aragón	0	0	0	1	4
Slovakia	0.00240	0.00168	0.718	3	13
Hungary	0.00289	0.00287	0.750	4	9
Kazakhstan	0.00524	0.00573	0.868	8	14
Georgia	0.00177	0.00201	0.607	3	8

The 385-bp alignment contained 11 segregating sites and 12 haplotypes. The population from Kazakhstan possessed the most mitochondrial variation, with a nucleotide diversity of 0.00524 and eight haplotypes (Table 1). The Spanish population possessed the least variation (subpopulations Aragón and Catalonia combined), with a nucleotide diversity of 0.00061 and three haplotypes. Differences between populations were highly significant for all these parameters (ANOVA for nucleotide diversity: $F = 111.76$, df = 4, $P < 0.01$; for theta: $F = 11.98$, df = 4, $P < 0.01$; for haplotype diversity: $F = 78.27$, df = 4, $P < 0.01$; subpopulations from Spain combined, Table 2). Only one haplotype was found in the small subpopulation from Aragón. The most common haplotype (W1) was shared by 31 individuals and was found in all populations (Table 3, Fig. 2). The second most common type (W2) was shared by eight individuals and was found in Slovakia, Hungary and Spain. Two more haplotypes were found in several populations; haplotype E8 occurred in both the

Table 2. Population pairwise Φ_{ST} values below the diagonal and F_{ST} values above.

Population	Spain	Slovakia	Hungary	Kazakhstan	Georgia
Spain		0.01238	0.01017	0.02592*	0.10154***
Slovakia	0.18849^{**}_{AC}		0.01192	0.02437***	0.05599***
Hungary	0.15282^{**}_{AC}	0.06004		0.05281**	0.12371***
Kazakhstan	0.17516^{***}_{ABC}	0.10981_{ABC}	0.09528_{AB}		0.03586***
Georgia	0.59890^{***}_{AC}	0.45216***	0.25590^{*}_{A}	0.15372^{*}_{ABC}	

Subscripts below the diagonal represent significant differences in the least-square comparison *post hoc* test following ANOVA ($P < 0.05$).
A, nucleotide diversity; B, theta; C, haplotype diversity.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3. Distribution of haplotypes among sampled populations. Haplotype designation follows Figure 2.

Haplotype	Catalonia	Aragón	Slovakia	Hungary	Kazakhstan	Georgia	Total
W1	11	4	5	4	5	2	31
W2	1	0	4	3	0	0	8
W3	1	0	0	0	0	0	1
E1	0	0	0	1	1	5	7
E2	0	0	0	0	1	0	1
E3	0	0	0	1	0	0	1
E4	0	0	0	0	0	1	1
E5	0	0	0	0	1	0	1
E6	0	0	0	0	1	0	1
E7	0	0	0	0	1	0	1
E8	0	0	4	0	2	0	6
E9	0	0	0	0	2	0	2
Total	13	4	13	9	14	8	61

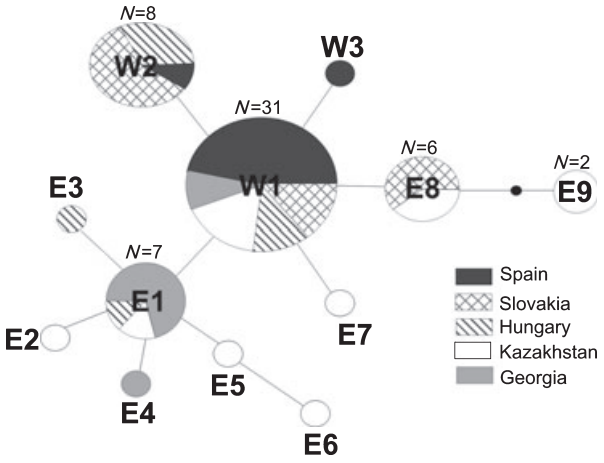

Figure 2. A parsimony network of mtDNA haplotypes. Each circle represents a haplotype with its size proportional to the number of birds sharing that haplotype. The bars connecting the haplotypes represent one nucleotide substitution. The small black circle represents an unsampled or extinct haplotype.

Slovakian and Kazakh populations, whereas E1 was shared among the Hungarian, Kazakh and Georgian populations. The remaining eight haplotypes were rare, as each was found in only one population.

Pairwise Φ_{ST} values (Table 2) were initially estimated between all sample locations. Because pairwise Φ_{ST} values (Table 2) between Catalonia and Aragón were not statistically different, they were combined into one 'Spanish' population. The divergence between the five remaining populations varied from $\Phi_{ST} = 0.060$ (Slovakia and Hungary) to $\Phi_{ST} = 0.599$ (Georgia and Spain). The Spanish population was significantly different from all the other populations, whereas the comparisons involving the Georgian population showed the highest Φ_{ST} values. The overall Φ_{ST} was 0.20 ($P < 0.001$). If populations were grouped according to assumed subspecies (Spain, Hungary, Slovakia representing *L. m. minor,* and Kazakhstan and Georgia representing *L. m. turanicus*), 13.97% of the genetic variation was attributable to between subspecies, 8.89% to between populations within subspecies and 77.15% was within populations, increasing the overall Φ_{ST} to 0.23 ($P < 0.001$). The total Φ_{ST} increased to 0.24 when European populations were grouped together and populations from Georgia and Kazakhstan were considered as separate groups. The highest total Φ_{ST} (0.323, $P < 0.001$) was obtained when the Georgian population was considered separate and all others were grouped into one, the same grouping obtained when using SAMOVA. This, however, increased the among-population within-group variation to 10.75%. A Mantel test suggested a weak trend between geographical and genetic distances ($r = 0.357$, $P = 0.102$).

Tajima's *D* and Fu' *Fs* values were negative for the Spanish population (−1.404 and −2.097), although not significantly so. The mismatch distribution followed the expected distribution of an expanding population and the raggedness index *r* was 0.3496 (ns, 95% CI 0.1289–0.7048). Other populations with a sample size over 10 (Slovakia, Kazakhstan) did not clearly follow either of the expected mismatch distributions, and their Tajima's or Fu's tests were not significant (Fig. 3). However, the combined Lesser Grey Shrike population followed rather closely the expected distribution of a growing population: it had significantly negative Tajima's *D* (−2.614, $P < 0.001$) and Fu's *Fs* (−5.926, $P < 0.02$) values, and a fairly low, but not significant raggedness index ($r = 0.0934$, 95% CI 0.0272–0.4748). LAMARC estimated a high positive growth rate (maximum likelihood estimate for $G = 2732.16$; 95% CI = 2051.96–3418.76), indicating historical expansion of the species' population size. Although we do not know the substitution rate of the Lesser Grey Shrike control region, an estimate of the expansion date of the western populations based on a nucleotide diversity of 0.00189 and the commonly used mitochondrial DNA (mtDNA) substitution rate of 2% sequence divergence per Mya would lead to an expansion date of about 100 000 years ago.

Microsatellites

There was no evidence of null alleles or scoring errors. We found between two and five private alleles per population (Supporting Information Tables S1 & S2). None of the populations showed significant heterozygote deficiency, but significant heterozygote excess was observed in the Georgian population ($P < 0.03$) when estimated across all eight loci. Some individual loci showed significant deviations from HW equilibrium (locus *LS2* in the Spanish and Hungarian populations, locus *Lox1* in the Slovakian population, and *LS3* in the Kazakh population). None of these remained significant after the Bonferroni correction was applied. In general, heterozygosity per locus and population varied from 0 to 1. *LS4* was monomorphic in the

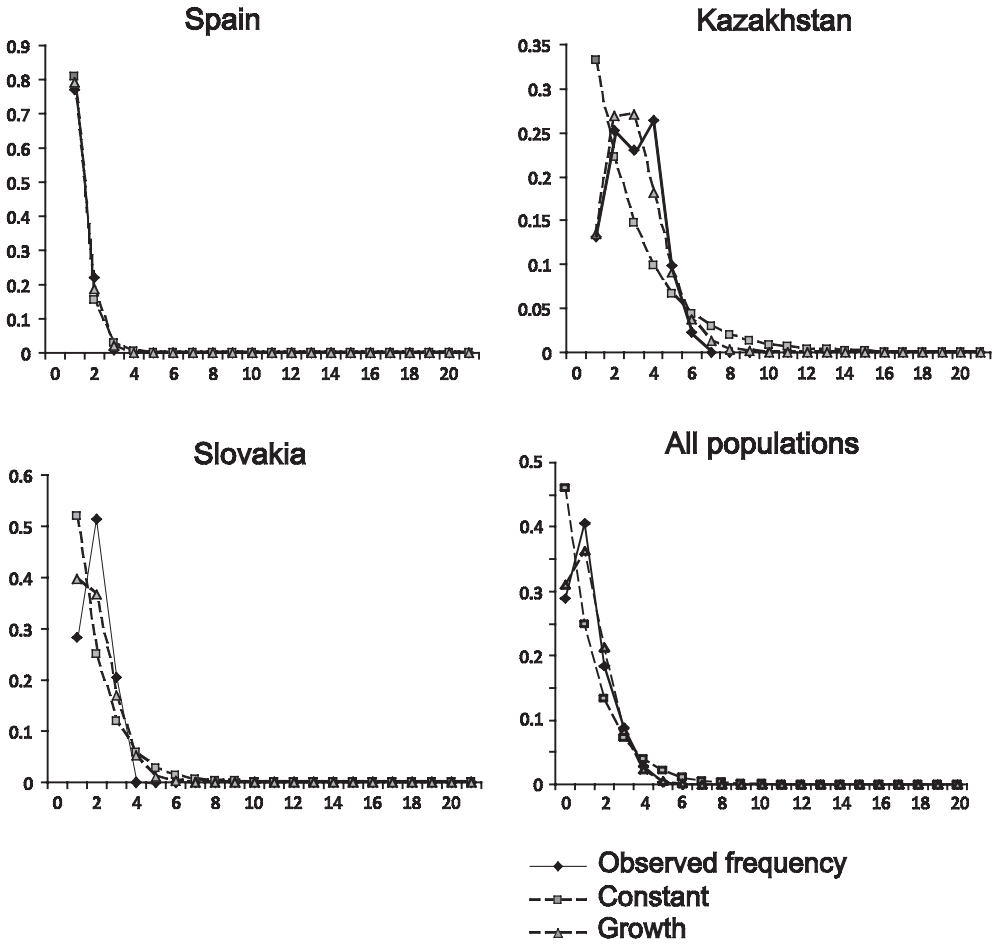

Figure 3. Mismatch distributions with the observed distributions as well as the expected distributions for constant or growing populations are depicted.

Hungarian population and also showed quite low heterozygosity in the other populations (range 0.063–0.375, the Georgian population being the exception, Table 4). Heterozygosity was also low in most populations for loci *STG4* and *Pocc6* (range 0.063–0.750). Linkage disequilibrium was found between *LS2* and *Ppi2* (in the Spanish population, $P < 0.05$) and *LS4* and *STG4* (in Slovakia, $P < 0.01$), but the significance disappeared after Bonferroni correction. Allelic richness (Table 4) was highest in the Hungarian and Slovak populations (4.633 and 4.518, respectively), and the lowest in Spain (3.352), but the difference was not significant (ANOVA: $F = 0.673$, df = 4, $P = 0.615$). In the Spanish population, the mean heterozygosity estimated across loci was also lower (expected heterozygosity 0.489, Table 4) than in the other populations (range 0.531–0.643) but, again, not significantly (ANOVA, observed heterozygosity: $F = 1.057$, df = 4, $P = 0.392$, expected heterozygosity: $F = 0.397$, df = 4, $P = 0.809$, Spanish populations combined), whereas F_{IS} was positive ($P > 0.05$, Table 4). Estimated effective population size was only 3.9 individuals (95% CI 3.2–4.9) in the Spanish population. Other populations, with adequate sample sizes, provided estimates of 35.5 for Slovakia and 28.2 for Kazakhstan (95% CIs were 9.7–118.2 and 16.8–68.11, respectively). The bottleneck test failed to find any evidence of population bottlenecks in any population. This indicates that the present bottleneck has not (yet) reduced the number of alleles relative to heterozygosity in the Spanish population. There was also no shift in the mode of the allele frequency distribution. However, the M-ratio for the Spanish population was only 0.65, suggesting that the number of

Table 4. Expected heterozygosity (H_{exp}), observed heterozygosity (H_{obs}) and allele richness (R) per locus and population. Mean F_{IS} is estimated over all loci for each population. Allelic richness is estimated as the weighted mean for each locus (R).

	Spain			Slovakia			Hungary			Kazakhstan			Georgia			
Locus	H_{exp}	H_{obs}	R	H_{exp}	H_{obs}	R	H_{exp}	H_{obs}	R	H_{exp}	H_{obs}	R	H_{exp}	H_{obs}	R	Mean R
S1	0.772	0.857	4.967	0.710	0.733	5.416	0.810	0.889	6.654	0.841	0.938	6.347	0.833	1	6.000	6.641
LS2	0.644	0.444	3.403	0.811	0.800	6.117	0.784	0.556	6.654	0.802	0.813	5.695	0.767	1	4.000	5.649
LS3	0.401	0.375	2.497	0.513	0.600	3.322	0.314	0.333	3.667	0.688	0.938	3.498	0.725	0.750	5.000	3.641
LS4	0.063	0.063	1.500	0.186	0.200	1.910	0	0	1.000	0.339	0.375	3.387	0.542	0.750	3.000	2.515
STG4	0.191	0.200	2.324	0.503	0.600	4.421	0.307	0.333	2.882	0.460	0.313	3.491	0.542	0.750	3.000	3.574
Ppi2	0.738	0.786	4.394	0.786	0.733	6.185	0.784	0.667	5.667	0.809	0.800	6.126	0.817	0.875	5.000	5.700
Lox1	0.671	0.556	4.749	0.834	0.667	6.859	0.856	1	7.549	0.643	0.688	4.328	0.675	0.500	4.000	5.412
pocc6	0.383	0.389	3.102	0.186	0.200	1.910	0.392	0.444	2.987	0.063	0.063	1.500	0.242	0.250	3.000	2.667
Mean	0.489	0.459	3.352	0.566	0.567	4.518	0.531	0.528	4.633	0.581	0.616	4.282	0.643	0.734	4.125	
F_{IS}	0.051			−0.001			0.007			−0.063			−0.154			

alleles has declined in relation to allelic range. M-ratios for the other populations were 0.71 for Slovakia, 0.87 for Hungary, 0.78 for Kazakhstan and 0.78 for Georgia. According to Garza and Williamson (2001), an M-ratio below 0.68 indicates a reduction in population size when analysed for more than seven loci, i.e. the index suggests a recent bottleneck in the Spanish population.

The program STRUCTURE did not reveal any evidence of genetic structuring between the sampling sites, and the highest log-likelihood obtained was for $k = 1$, i.e. a single panmictic population. Nevertheless, population structure was suggested by the factorial correspondence analysis, in which all the populations cluster separately (Fig. 4).

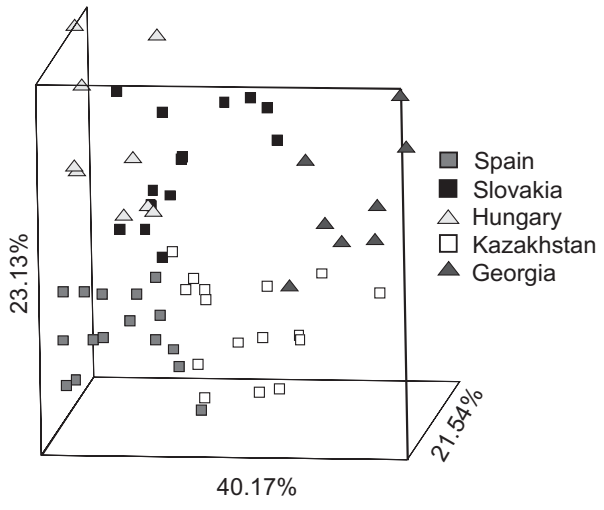

Figure 4. Factorial correspondence analysis with the first three factors presented.

Some structuring was also detected by AMOVA, with overall $F_{ST} = 0.0381$ ($P < 0.001$). It is possible that STRUCTURE failed to detect any differences between the populations due to the small sample size and the number of loci used. The largest pairwise values were found in comparison with the Georgian population (0.056–0.124), whereas the smallest were found between the European populations (0.010–0.012, Table 2). The Spanish population did not differ significantly from other European populations, but the Asian and European populations did, and the two Asian populations differed from each other. Total F_{ST} increased to 0.0508 ($P < 0.001$) when populations were grouped according to subspecies *minor* and *turanicus* and to 0.0525 when three groups, i.e. Europe, Kazakhstan and Georgia, were formed. As with the mitochondrial data, the highest F_{ST} (0.0806, $P < 0.001$) was obtained when all the populations except Georgia were grouped into one, and this grouping arrangement was supported by SAMOVA. This increased the among-group variation to 6.08%, and also the among-population within-group variation to 1.99%. The correlation between gene flow ($F_{ST}/1 - F_{ST}$) and the natural log of geographical distance was calculated using a Mantel test, and was 0.3842 ($P = 0.062$), which is slightly greater than the value obtained with the mitochondrial sequence data. Although the differences between the European populations were small, most of the individuals were correctly assigned to their population of origin (all the Spanish birds, 13 of 15 birds from Slovakia, eight of nine birds from Hungary, 15 of 16 birds from Kazakhstan and all the Georgian birds).

DISCUSSION

Population structure

The endangered Spanish population differed significantly from all others in mitochondrial markers, but only from Caucasian and Asian populations in nuclear markers, even though the distance to the next sampled European breeding population was considerable. This might indicate exchange of breeders among European populations, but a more likely possibility is that the decline and isolation of the Spanish population is still so recent that genetic drift has only had time significantly to affect mtDNA. As mtDNA has a quarter of the effective population size of nuclear markers (assuming equal variance in male and female reproductive success), the former should show the effects of drift four times faster, irrespective of the fact that the mutation rate in microsatellites is generally higher than in mtDNA (see Zink & Barrowclough 2008). Failure to find signs of a genetic bottleneck in microsatellite markers in two of the three bottleneck tests used might also indicate that the observed population declines and resulting isolation are too recent to be detected using genetic data.

AMOVA suggested genetic differentiation between populations within the assumed subspecies *L. m. turanicus*: birds from Georgia and Kazakhstan differed significantly and even more so from each other than from some European populations (Table 2). Therefore, the existence of two subspecies, one from Europe (*L. m. minor*) and one from Asia (*L. m. turanicus*), may need re-evaluation. It is worth highlighting that the Georgian population, which was the most divergent from all others, is surrounded by the Caucasus Mountains, the Black Sea and the Caspian Sea, suggesting stronger physical barriers for this population. On the migration route from Africa to breeding sites, Lesser Grey Shrikes diverge in migration direction from the Arabian Peninsula onwards, heading northwest through Turkey to Europe, north through Georgia to Caucasia and southern Russia, or northeast through Iran to Kazakhstan (Dowsett 1971, Lefranc & Worfolk 1997). This spatial segregation coupled with the high site-fidelity reported at least for adults (Hantge 1957, Kristin *et al.* 2007) could restrict gene flow and therefore explain the relatively greater differentiation between European, Georgian and Kazakh populations. Additional sampling of populations and individuals is necessary to test this conclusion and to reject the alternative possibility that the genetic structure described here has arisen solely through isolation by distance.

Finally, mitochondrial data suggest a historical expansion that may have occurred close to the last glacial period, perhaps beginning during one of the warm interstadial periods. The most common haplotype W1 was found in all populations and lies at the centre of the haplotype network, suggesting that it is an ancestral haplotype already present at the time of the expansion and that most or all other haplotypes evolved subsequently. Similar expansions have been detected for many species whose populations were confined to southern European refugia when glaciers spread across northern latitudes (Newton 2003). Furthermore, current migration routes may retrace ancestral routes of expansion during past colonization (Ruegg & Smith 2002). Thus, it is possible that Lesser Grey Shrikes migrate along flyways that evolved from historical colonization routes after an expansion from a refuge in the Middle East.

Within-population diversity and breeding range fragmentation

Genetic variation is lower in the Spanish population of the Lesser Grey Shrike than in any of the others we sampled. Given that the Spanish population is extremely small, geographically isolated and distant from the continuous breeding range, this is not a surprise. Thus, it seems that the recent reduction in population size coupled with peripheral isolation has resulted in loss of genetic diversity through genetic drift and/or reduced gene flow. Since the beginning of the 20th century, one-third of the previous geographical range of the Lesser Grey Shrike has been lost, almost all in the westernmost parts of its range (Lefranc 1995, 1999, Lefranc & Worfolk 1997). This has resulted in the current distribution of small, isolated and still declining populations in Italy, France and Spain (Lefranc & Worfolk 1997, Rufray & Rousseau 2004, Giralt & Valera 2007).

An alternative explanation for the low genetic diversity of the Spanish population could be a founder effect associated with recent colonization. Wallace and Sage (1969) suggested a recent expansion to northern Spain from France along the

Mediterranean coast in the 1960s. However, the oldest historical record in the Iberian Peninsula is from the 19th century (Vayreda 1883) and additional observations were reported in the 1940s (Heymer 1964). Therefore, it is likely that the species was abundant in France during the 19th century and that the range in Europe contracted mostly during the 20th century. Thus, the low genetic variation of the Spanish population is more probably related to recent fragmentation and population decline than to a recent colonization event. Analysis of historical museum samples (e.g. Goldstein & Desalle 2003, Munõz-Fuentes *et al.* 2005) would enable a more definitive test of these alternatives.

Conservation implications

A census of the Spanish population during 2004 and 2005 recorded 24 and 20 pairs, respectively. In 2006 and 2007, the population size declined to 13 and nine pairs, respectively. The effective population size estimated here is extremely small (3.9), genetic variation is low and F_{IS} is positive (though not significantly), suggesting a substantial loss of genetic diversity in this population. At the same time, the population is facing long-term habitat loss (Giralt & Bota 2003, Giralt *et al.* 2008a). When small, isolated populations face environmental, demographic and/or genetic degradation, the population may end in an extinction vortex: a downward spiral in which the three factors mutually enhance each other, ultimately resulting in extinction (Gilpin & Soulé 1986). The present Spanish population is facing all three threats: habitat loss and instability, population decrease and loss of genetic variability. Moreover, the most likely source of immigrant birds for this population, the nearest French population, is also in serious decline (Rufray & Rousseau 2004), making it likely that the Spanish population will soon become extinct.

Even though the Spanish population (or any of the other studied populations) is not reciprocally monophyletic for mitochondrial markers, as would be required for treating the population as an ESU (*sensu* Moritz 1994), we found significant divergence in mitochondrial haplotype frequencies from all other populations and in microsatellite allele frequencies from the two easternmost populations. This implies that the population should be considered an MU. The eastern populations (Georgia and Kazakhstan) could each form their own MUs, whereas the Slovakian and Hungarian populations could be combined into one. According to Crandall *et al.* (2000), management decisions should be based on ecological and genetic exchangeability between populations at present and in the past. Present genetic exchangeability is clearly rejected in our case and there is support also for rejection of ecological exchangeability between the Georgian, Kazakh and European populations (e.g. due to differences in migration routes). However, there are indications, such as spatial synchronization of western populations (Giralt & Valera 2007), that the Spanish population belongs to the same MU as other European populations (ecological exchangeability). In this case, the present genetic differentiation is simply a result of recent population fragmentation through anthropogenic effects. Thus, our results suggest at least three MUs, given the likelihood of ecological exchangeability between European populations at present and in the past.

Urgently needed conservation measures include large-scale habitat management (Giralt & Valera 2007) to provide breeding sites and allow for gene flow. Habitat management has been tested at a small scale, but its effectiveness has been difficult to estimate reliably (Giralt *et al.* 2008b). Other apparently more effective conservation actions include provisioning of supplementary food and controlling Eurasian Magpies to increase shrike nesting success (Giralt *et al.* 2008b). However, the situation may already be so desperate that even if reproductive output were improved, the population would not recover.

Another possibility could be initiation of a re-introduction or a reinforcement programme to increase genetic variation and population size. Our results imply that if desperate measures are needed to maintain the Spanish population, the stocks used for reinforcement should be drawn from other European populations, preferably the closest ones in France and Italy. It was unfortunate that we were not able to obtain samples from these populations for this study because the genetic variation and structure of these populations should be investigated prior to any reintroduction or reinforcement project. In addition, the origin of founders must be considered in the context of migration behavior, given that directional preferences at least differ among European Lesser Grey Shrike populations (Lefranc & Worfolk 1997).

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REFERENCES

Amos, W. & Harwood, J. 1998. Factors affecting levels of genetic diversity in natural populations. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **353**: 177–186.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. 2004. *GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171*. Montpellier: University of Montpellier II.

Bensch, S., Price, T. & Kohn, J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Mol. Ecol.* **6**: 91–92.

BirdLife International 2004. *Birds in Europe: Population Estimates, Trends and Conservation Status.* BirdLife Conservation Series no. 12. Cambridge: BirdLife International. .

Clancey, P.A. 1980. On the Lesser Grey Shrike *Lanius minor* in southern Africa. *Durban Mus. Novit.* **12**: 161–165.

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

Cornuet, J.M. & Luikart, G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**: 2001–2014.

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.

Dowsett, R.J. 1971. The Lesser Grey Shrike *Lanius minor* in Africa. *Ostrich* **42**: 259–270.

Dupanloup, I., Schneider, S. & Excoffier, L. 2002. A simulated annealing approach to define the genetic structure of populations. *Mol. Ecol.* **11**: 2571–2581.

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: Cambridge University Press.

Garza, J.C. & Williamson, E.G. 2001. Detection of reduction in population size using data from microsatellite loci. *Mol. Ecol.* **10**: 305–318.

Gilpin, M.E. & Soulé, M.E. 1986. Minimum viable populations: the processes of species extinctions. In Soulé, M.E. (ed.) *Conservation Biology: the Science of Scarcity and Diversity*: 13–34. Sunderland, MA: Sinauer Associates.

Giralt, D. & Bota, G. 2003. Alcaudón chico, *Lanius minor*. In Martí, R. & Del Moral, J.C. (eds) *Atlas de las Aves Reproductoras de España*: 532–533. Madrid: Dirección General de Conservación de la Naturaleza-Sociedad Española de Ornitología.

Giralt, D. & Valera, F. 2007. Population trends and spatial synchrony in peripheral populations of the endangered Lesser Grey Shrike in response to environmental change. *Biodivers. Conserv.* **16**: 841–856.

Giralt, D., Brotons, L.L., Valera, F. & Kristin, A. 2008a. The role of natural habitats in agricultural systems for bird conservation: the case of the threatened Lesser Grey Shrike. *Biodivers. Conserv.* **17**: 1997–2012.

Giralt, D., Rivas, J.L. & Albero, J.C. 2008b. Situación límite del Alcaudón chico en España. *Quercus* **270**: 14–21.

Goldstein, P.Z. & Desalle, R. 2003. Calibrating phylogenetic species formation in a threatened insect using DNA from historical specimens. *Mol. Ecol.* **12**: 1993–1998.

Goudet, J. 2001. *FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3)*. Available from http://www.unil.ch/izea/softwares/fstat.html (accessed 5 February 2002).

Hantge, E. 1957. Zur Brutbiologie des Schwarzstirnwürgers (*Lanius minor*). *Vogelwelt* **78**: 137–146.

Herremans, M. 1998. Monitoring the world population of the Lesser Grey Shrike (*Lanius minor*) on the non-breeding grounds in southern Africa. *J. Ornithol.* **139**: 485–493.

Heymer, A. 1964. Repartition de la Pie-Grièche à Poitrine Rose *Lanius minor* dans le sud-ouest de l'Europe. *Oiseau Rev. Fr. Ornithol.* **34**: 25–29.

Hoffmann, A.A. & Blows, M.W. 1994. Species borders: ecological and evolutionary perspectives. *Trends Ecol. Evol.* **9**: 223–227.

del Hoyo, J., Elliott, A. & Sargatal, J. (eds). 2008. *Handbook of the Birds of the World*, Vol. 13. Barcelona: Lynx Edicions.

Keane, T.M., Creevey, C.J., Pentony, M.M., Naughton, T.J. & Mclnerney, J.O. 2006. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. *BMC Evol. Biol.* **6**: 29.

Kristin, A., Hoi, H., Valera, F. & Hoi, C. 2007. Philopatry, dispersal patterns and nest-site reuse in Lesser Grey Shrikes (*Lanius minor*). *Biodivers. Conserv.* **16**: 987–995.

Kuhner, M.K. 2006. LAMARC 2.0: maximum likelihood and Bayesian estimation of population parameters. *Bioinformatics* **22**: 768–770.

Kvist, L., Martens, J., Higuchi, H., Nazarenko, A.A., Valchuk, O.P. & Orell, M. 2003. Evolution and genetic structure of the Great Tit (*Parus major*) complex. *Proc. R. Soc. Lond. B* **270**: 1447–1454.

Kvist, L., Broggi, J., Illera, J.C. & Koivula, K. 2005. Colonisation and diversification of the Blue Tits (*Parus caeruleus teneriffae*-group) in the Canary Islands. *Mol. Phylogenet. Evol.* **34**: 501–511.

Kvist, L., Arbabi, T., Päckert, M., Orell, M. & Martens, J. 2007. Population differentiation in the marginal populations of the Great Tit (Paridae: *Parus major*). *Biol. J. Linn. Soc.* **90**: 201–210.

Lefranc, N. 1995. Decline and current status of the Lesser Grey Shrike (*Lanius minor*) in Western Europe. In Reuven, R. & Lohrer, F.E. (eds) *Shrikes (Lanius) of the World: Biology and Conservation. Proc. West. Found. Vertebr. Zool.* **6**: 93–97.

Lefranc, N. 1999. Shrikes and the farmed landscape in France. In Pain, D.J. & Pienkowsli, M.W. (eds) *Farming and Birds in Europe*: 236–268. London: Academic Press.

Lefranc, N. & Worfolk, T. 1997. *Shrikes. A Guide to the Shrikes of the World*. Robertsbridge, East Sussex: Pica Press.

Lesica, P. & Allendorf, F.W. 1995. When are peripheral populations valuable for conservation? *Conserv. Biol.* **9**: 753–760.

Martínez, J.G., Soler, J.J., Soler, M., Møller, A.P. & Burke, T. 1999. Comparative population structure and gene flow of a brood parasite, the Great Spotted Cuckoo (*Clamator glandarius*), and its primary host, the Magpie (*Pica pica*). *Evolution* **53**: 269–278.

Moritz, C. 1994. Defining evolutionary significant units for conservation. *Trends Ecol. Evol.* **9**: 373–375.

Mundy, N.I. & Woodruff, D.S. 1996. Polymorphic microsatellite markers in the Loggerhead Shrike (*Lanius ludovicianus*) isolated from a library enriched for CA repeats. *Mol. Ecol.* **5**: 811–813.

Munõz-Fuentes, V., Green, A.J., Negro, J.J. & Sorenson, M.D. 2005. Population structure and loss of diversity in the endangered White-headed Duck, *Oxyura leucocephala*. *Conserv. Genet.* **6**: 999–1015.

Newton, I. 1995. Relationship between breeding and wintering ranges in Palaearctic-African migrants. *Ibis* **137**: 241–249.

Newton, I. 2003. *Speciation and Biogeography of Birds*. London: Academic Press.

Peel, D., Ovenden, P.R. & Peel, S.L. 2004. *NeEstimator: Software for Estimating Effective Population Size*, Version 1.3. Brisbane: Queensland Government, Department of Primary Industries and Fisheries.

Piertney, S., Marquiss, M. & Summers, R. 1998. Characterization of tetranucleotide microsatellite markers in the Scottish Crossbill (*Loxia scotica*). *Mol. Ecol.* **7**: 1261–1263.

Pritchard, J.K., Stephens, M. & Donelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Raymond, M. & Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**: 248–249.

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. & Rozas, R. 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**: 174–175.

Ruegg, K.C. & Smith, T.B. 2002. Not as the crow flies: a historical explanation for circuitous migration in Swainson's Thrush (*Catharus ustulatus*). *Proc. R. Soc. Lond. B* **269**: 1375–1381.

Rufray, X. & Rousseau, E. 2004. La Pie-Grièche à poitrine rose (*Lanius minor*): une fin annoncée. *Ornithos* **11**: 36–38.

Sinclair, R.E., Fryxell, J.M. & Caughley, G. 2006. *Wildlife Ecology, Conservation and Management*. Malden, MA: Blackwell Science.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P. & Shipley, P. 2004. Program note: micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**: 535–538.

Vaurie, C. 1955. Systematic notes on Palearctic birds, No 17. Laniidae. *Am. Mus. Novit.* **1752**: 1–19.

Vayreda, E. 1883. *Fauna ornitológica de la provincia de Gerona*. Gerona: Paciano Torres.

Wallace, D. & Sage, B. 1969. Las aves de la Costa Brava (Cataluña). *Ardeola* **14**: 143–157.

Zink, R.M. & Barrowclough, G.F. 2008. Mitochondrial DNA under siege in avian phylogeography. *Mol. Ecol.* **17**: 2107–2121.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Microsatellite allele frequencies for each sampled population.

Table S2. Microsatellite allele scores for each individual.

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