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RESEARCH ARTICLE

The genetic structure of the European breeding populations of a declining farmland bird, the ortolan bunting (*Emberiza hortulana*), reveals conservation priorities

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Abstract

Anthropogenic activities, such as agricultural intensification, caused large declines in biodiversity, including farmland birds. In addition to demographic consequences, anthropogenic activities can result in loss of genetic diversity, reduction of gene flow and altered genetic structure. We investigated the distribution of the genetic variation of a declining farmland and long-distance migratory bird, the ortolan bunting *Emberiza hortulana*, across its European breeding range to assess the impact of human-driven population declines on genetic diversity and structure in order to advise conservation priorities. The large population declines observed have not resulted in dramatic loss of genetic diversity, which is moderate to high and constant across all sampled breeding sites. Extensive gene flow occurs across the breeding range, even across a migratory divide, which contributes little to genetic structuring. However, gene flow is asymmetric, with the large eastern populations acting as source populations for the smaller western ones. Furthermore, breeding populations that underwent the largest declines, in Fennoscandia and Baltic countries, appear to be recently isolated, with no gene exchange occurring with the eastern or the western populations. These are signs for concern as declines in the eastern populations could affect the strength of gene flow and in turn affect the western populations. The genetic, and demographic, isolation of the northern populations make them particularly sensitive to loss of genetic diversity and to extinction as no immigration is occurring to counter-act the drastic declines. In such a situation, conservation efforts are needed across the whole breeding range: in particular, protecting the eastern populations due to their key role in maintaining gene flow across the range, and focussing on the northern populations due to their recent isolation and endangered status.

Keywords Endangered species · Gene flow · Genetic diversity · Long-distance migrant

Introduction

Anthropogenic activities are a known cause for biodiversity decline. For instance, conversion of native land for food production leads to habitat loss and fragmentation while intensification of farming practices is responsible for large-scale declines of diverse taxa including insects (Benton et al. 2002; Potts et al. 2010) and birds (Donald et al. 2001, 2006; Gregory et al. 2005). However, the diversity and heterogeneity of land cover dedicated to agriculture is not similar across Europe (Eurostat 2016). Agricultural ecosystem quality is not uniform and the impact of agriculture on biodiversity is spatially heterogeneous (Reidsma et al. 2006), even within one geographical region (Norris 2008). For instance, the steepest farmland bird declines were observed in Western Europe due to agricultural intensification, while the fall of communism in Eastern Europe resulted in land abandonment and reduced agricultural intensity which allowed the short-term recovery of farmland birds (Eif 2013). Large-scale and long-term bird monitoring data revealed a steep 57% decline of European farmland birds during the period

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1980–2014 (Pan-European Common Bird Monitoring Scheme [PECBMS] 2016).

While the footprint of human activities on biodiversity is mostly measured at the species or population level, genetic impacts are equally important even if harder to determine and thus less frequently assessed. Indeed, intra-specific genetic variation is critical for population dynamics, community structure and ecosystem function (Mimura et al. 2017). It is largely accepted that populations poorly adapted to new conditions are more at risk of decline and extinction caused by rapid environmental changes (e.g. Thomas et al. 2004). Among and within population genetic diversity may reduce these risks through the portfolio effect (Schindler et al. 2015), i.e. large genotypic diversity may produce a wide range of responses to environmental conditions and thus increases population stability in the face of change. Furthermore, genetic variation, maintained by higher effective population size, reduces inbreeding depression, which can be responsible for lower fitness (Hoffman et al. 2014). It also contributes to the evolutionary potential of a population by providing genotypes that may allow adaptation to new conditions (Bijlsma and Loeschcke 2012), so that the conservation of genetic diversity in the face of environmental change should be an essential precautionary principle (Sarrazin and Lecomte 2016). Unfortunately, human activities strongly influence intraspecific variation. For instance, land-use change often leads to habitat loss and fragmentation and consequently to population declines and loss in connectivity. Genetic consequences may include increased inbreeding and loss of genetic diversity that are exacerbated by reduced gene flow, which cannot buffer diversity loss in the absence of connectivity (Frankham et al. 2004). Erosion of genetic diversity further impedes the adaptation potential of a population to environmental changes (Bijlsma and Loeschcke 2012). It is therefore essential to understand and monitor intraspecific genetic variation in the face of anthropogenic global change and at a scale large enough to integrate spatial heterogeneity since environmental changes can vary drastically across a species' range (Mimura et al. 2017).

In this study, we investigated the genetic diversity and structure of a widely distributed farmland bird, the ortolan bunting *Emberiza hortulana*. This passerine bird breeds from Spain to Finland and east to Mongolia, and is largely associated with agricultural landscapes, especially with mosaic habitats including areas of bare grounds for foraging (Vepsäläinen et al. 2007; Menz et al. 2009; Menz and Arlettaz 2011; Elts et al. 2015). While it is not listed as an endangered species under IUCN criteria due to its large range and moderate recent decline, the ortolan bunting underwent severe long-lasting or recent local population declines in Europe, leading to extinctions in several countries and to recent listing as Endangered to Critically Endangered on several national Red Lists (Jiguet et al. 2016a). Indeed, average decline since 1980 is reported to reach 88%, although last decadal trend is not as steep (− 14% decline) (PECBMS 1980–2014). Northern breeding populations are especially affected, in particular Fennoscandian countries, where some populations have become totally isolated with dramatic consequences for local demography, as high female dispersal precludes an equilibrated sex-ratio in absence of immigration (Dale 2001). Declines are not as drastic in southern breeding populations, and increasing trends are even reported in some Mediterranean countries (Jiguet et al. 2016a). Drivers for such trends probably involve multiple factors, including decreasing habitat quantity and quality on breeding grounds, altered populations dynamics in small fragmented populations, environmental changes on wintering grounds and hunting during migration (Dale 2001; Vepsäläinen et al. 2005; Menz et al. 2009; Menz and Arlettaz 2011). This species is a long-distance migrant (Selstam et al. 2015) with restricted wintering areas in sub-Saharan Africa and displays relatively strong migratory connectivity. A migratory divide occurs from Finland to Italy: birds west of the divide migrate across France along an Atlantic or Mediterranean flyway to overwinter in Guinea and neighbouring countries; birds east of the divide use an oriental flyway via the Middle East towards Ethiopia (Jiguet et al. 2016b). The ortolan bunting is thus a long-distance migrant with an identified migration divide, with a large but locally fragmented breeding range, and with declining population sizes since decades at least in central and northern Europe.

In this context, population genetics could provide further understanding of the ortolan bunting's conservation biology, and especially in revealing genetic connectivity at large scale and in identifying vulnerable populations that may be differentiated, prompting targeted conservation actions to maintain or restore connectivity and to define conservation units. We hypothesised that the large recent population declines should not have affected the genetic diversity of the breeding populations of ortolan bunting, as these populations are still large, with an estimated 3,319,000–7,057,000 pairs breeding in Europe for the 2012–2014 period (Jiguet et al. 2016a). We also expected a low genetic structure across the species' range due to its wide distribution and still relatively large numbers. However, the presence of well-defined migratory flyways may suggest some degree of structuring across the migratory divide. Finally, stronger fragmentation and population declines were recorded in north, central and western Europe while eastern populations remained larger and connected (Jiguet et al. 2016a). This led us to suspect some subtle genetic structuring, namely the recent isolation of some populations, likely in north Europe, and to asymmetric gene flow from the core eastern populations towards the western populations.

Materials and methods

Field work

We undertook a broad sampling strategy across most of the breeding range of the ortolan bunting, spanning from Spain to Russia, from Greece to Norway (Fig. 1; Table 1), thanks to international collaboration with local researchers and ornithologists. All samples apart from those from Sweden (n = 17) and Norway (n = 3) were collected in late spring 2013, 2014 and 2015 (late May and June) by capturing buntings in mist-nets around singing posts and in some cases playing territorial male songs to elicit visits. Geolocator analyses indicated that birds come back from migration early to mid-May and we also observed nesting birds and egg clutches when collecting samples. While we cannot exclude that we sampled a few migrants, birds that were sampled were either singing males (establishing their territory) or females that were answering their call. As such, we argue that the vast majority of our samples were from breeding individuals and not migratory birds on a stop-over. A tail feather was collected for DNA extraction. The Swedish and Norwegian blood and muscle tissue samples were similarly collected during breeding in 2003, 2006, 2008, 2012 and 2014, after the largest population declines and obtained from the Oslo museum of natural history. They are therefore samples from contemporary breeding populations.

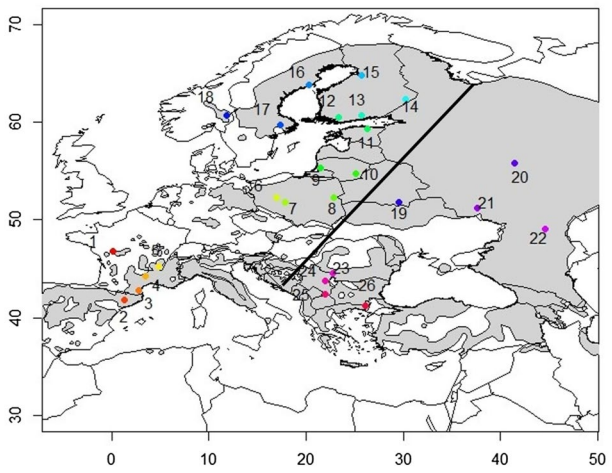

Fig. 1 Sampling locations of breeding ortolan buntings indicated by identifying numbers. Grey area indicates *E. hortulana* European distribution (BirdLife International 2015) and the black line indicate the position of the migratory divide revealed by light-level geolocators (Jiguet et al. 2016b)

Laboratory procedure

We used a shotgun sequencing approach on the Ion PGM platform (Life Technologies) to develop 24 microsatellite loci from muscle tissue. The microsatellites were combined into five multiplex panels. Details of the procedure are included in Supplementary Material.

DNA was extracted from tail feathers after overnight lysis at 56 °C in 180 µl lysis buffer (Macherey–Nagel, Düren, Germany), 25 µl proteinase K (Macherey–Nagel) and 20 µl Dithiothreitol. Lysates were processed through NucleoSpin PCR Clean-up kits (Macherey–Nagel). Blood samples were processed using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the kit protocol. DNA extracts from blood were diluted by factor two in 1× Tris–EDTA (TE) before being plated along the feather samples.

We prepared a 10× primer mix in 1× TE for each multiplex set (see Table S1 for primer concentrations). Multiplex reactions consisted of 5 µl Type-it PCR mix (Quiagen, Hilden, Germany), 1 µl primer mix, 1 µl bovine serum albumin (1 mg/ml), 2 µl PCR-grade water and 1 µl DNA. Cycling conditions were: 95 °C /5 min denaturation, followed by ten cycles of 95 °C/30 s, 65 °C/90 s and decreasing by 1 °C per cycle, 72 °C/60 s, followed by 25 cycles of 95 °C/30 s, 55 °C/90 s, 72 °C/60 s, and a final elongation step of 72 °C for 40 min. PCR products were diluted 1/225 in water and formamide and a size standard was added GeneScan 500LIZ (ThermoFisher Scientific, Waltham, MA, USA) before processing on a 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA).

Data preparation

A total of 1127 samples, including duplicates, were genotyped for the 24 microsatellite loci. Genotypes were examined in Genemapper v5 (Applied Biosystems) to determine peak alleles and raw allele sizes were exported to AutoBin (Salin 2013). This Excel macro examines the size difference between contiguous alleles and detects gaps to infer allele binning. Binning was carefully inspected and manually corrected when necessary. DNA amplification success was calculated for each sample and each locus. We re-amplified and genotyped 113 individuals to estimate genotyping error. We also used CERVUS (Kalinowski et al. 2007) to identify 30 further replicated genotypes that corresponded to recaptures in successive years and confirmed the birds' identity by checking the ringing database. The 143 replicated genotypes thus represent a 12.69% sub-sample of the dataset.

Microsatellite characteristics, genetic diversity and relatedness

Breeding birds were mostly captured within an approximately 5 km radius from a core study site. Where breeding

Table 1 Sample size (N), mean number of alleles (A), mean allelic richness (AR), observed (HO) and expected (HE) heterozygosity, fixation indexes (FST and FIS) (Weir and Cockerham 1984) over 21 microsatellite loci and relatedness as the percentage of related pairwise individual relationships

Breeding site	N	A	A_R	H_O	H_E	F_{IS}	F_{ST}	Relatedness
West								
France 1	8	7.0	6.2	0.769	0.778	0.080	–	21.43
Spain 2	28	11.6	6.5	0.708	0.830	0.165*	–	6.61
France 3	3	–	–	–	–	–	–	–
France 4	12	9.0	6.6	0.740	0.812	0.133*	–	9.09
France 5 (Drôme)	12	7.2	5.6	0.733	0.756	0.073	–	43.94
Poland 6	8	7.0	6.2	0.685	0.781	0.190	–	7.14
Poland 7	25	11.2	6.4	0.727	0.812	0.126*	–	6.67
Poland 8	15	9.4	6.4	0.713	0.807	0.151*	–	8.57
Lithuania 9	3	–	–	–	–	–	–	–
All western sites excluding France 5	**102**	**9.2**	**6.4**	**0.724**	**0.803**	**0.144***	**0.006***	**9.92**
All western sites including France 5	**114**	**8.9**	**6.3**	**0.725**	**0.797**	**0.144***	**0.006***	**14.78**
North								
Lithuania 10	34	11.2	6.2	0.731	0.816	0.120*	–	8.38
Estonia 11	21	10.0	6.1	0.700	0.790	0.138*	–	7.62
Finland 12	47	12.4	6.2	0.758	0.818	0.085	–	9.62
Finland 13	61	12.7	6.2	0.713	0.814	0.133*	–	8.03
Finland 14	10	7.4	6.0	0.760	0.764	0.061	–	6.67
Finland 15	14	8.9	6.2	0.722	0.793	0.128*	–	7.69
Sweden 16	2	–	–	–	–	–	–	–
Sweden 17	15	9.0	6.1	0.719	0.797	0.132*	–	9.52
Norway 18	3	–	–	–	–	–	–	
All north sites	**207**	**10.2**	**6.2**	**0.729**	**0.799**	**0.116***	**0.007***	**8.22**
East								
Belarus 19	42	12.9	6.5	0.753	0.833	0.108*	–	7.32
Russia 20	20	10.5	6.3	0.726	0.809	0.127*	–	5.26
Russia 21	99	16.0	6.6	0.747	0.844	0.120*	–	6.56
Russia 22	76	15.3	6.5	0.742	0.834	0.118*	–	5.23
Serbia 23	3	–	–	–	–	–	–	–
Serbia 24	8	7.4	6.4	0.732	0.771	0.117	–	17.86
Serbia 25	2	–	–	–	–	–	–	–
Serbia 26	3	–	–	–	–	–	–	–
All east sites	**253**	**12.4**	**6.5**	**0.740**	**0.818**	**0.118***	**0.005***	**8.44**
All sites excluding France 5	**563**	**10.5**	**6.3**	**0.730**	**0.806**	**0.122***	**0.010***	**8.85**
All sites including France 5	**575**	**10.3**	**6.3**	**0.730**	**0.803**	**0.121***	**0.011***	**10.70**

Diversity indices were averaged over sites within each population

*Indicates significantly different from 0 at $p < 0.05$ after 1000 bootstraps, in bold if concerning F_{ST}. Indices were not evaluated for breeding sites which sampling size was under eight individuals

densities were low, such as in Finland, individuals were sampled over larger geographical scales (up to 108 km distance between sampling sites) and grouped together as breeding sites. Sites with less than eight individuals were excluded from population-level analyses. The resulting breeding dataset consisted of 555 individuals sampled at 19 breeding sites for population level analyses and 575 individuals from 26 sites for individual level analyses (Fig. 1; Table 1).

GENEPOP 4.0 (Rousset 2008) was used to test loci in departure from linkage equilibrium at each site (Markov chain parameters: 10,000 dememorisation, 100 batches, 5000 iterations) and sequential Bonferroni correction for multiple tests was applied (Rice 1989). We tested the correlation between the number of homozygotes and of missing data across samples and loci to determine whether any putative allelic dropout was due to low DNA quantity or poor DNA quality (correlation across samples) or to locus specific factors including null alleles. Tests were carried out in MICRODROP (Wang et al. 2012). The presence of scoring errors or null alleles was determined for each locus and each site using MICRO-CHECKER (Van Oosterhout et al. 2004). Finally, we conducted 456 exact tests for deviation from

Hardy–Weinberg equilibrium (HWE) for each locus (24) and each site (19) with 1000 Monte Carlo replicates using the PEGAS package (Paradis 2010) in R 3.3.1 (R Core Team 2016). Bonferroni correction for multiple tests was applied to the nominal 5% p value ($p = 0.00011$ for 456 tests).

Further analyses were carried out without three loci that displayed high frequency of null alleles and deviated from HWE. Genetic diversity indices were obtained for each sampling site in GENETIX 4.05.2 (Belkhir et al. 2004) and FSTAT (Goudet 1995, 2001). Estimated F_{ST} averaged over loci (Weir and Cockerham 1984) and the 95% confidence intervals (CIs) after 1000 bootstraps over loci were calculated in GENETIX. A randomised G-test was performed with 1000 replicates to test for genetic differentiation among sites in R (Goudet 2005). The relationship between individuals within a breeding site was estimated with ML-RELATE (Kalinowski et al. 2006) that estimates the log likelihood for four types of relationship: unrelated, half sibs, full sibs and parent/offspring. We calculated the proportion of unrelated and related relationships (pooling half and full sibs and parent/offspring together).

Population structure

Two methods were used to uncover genetic population structure. First, the Bayesian clustering program STRUCTURE (Pritchard et al. 2000) allowed the assignment of the 575 breeding individuals to K populations by minimizing deviations from HWE. Since the differentiation index F_{ST} was very low, indicating weak structure and likely high admixture, we used a correlated allele frequency and admixture model. We ran the program for 1–6 clusters using a burn-in of 5×10^5 iterations followed by 10^6 Markov Chain Monte Carlo iterations. Each K value was run five times with and without population information (sampling sites as prior). The optimal number of clusters K was obtained from ΔK, based on the rate of change in the log probability of data in successive K values (Evanno et al. 2005) as implemented on STRUCTURE HARVESTER (Earl and VonHoldt 2012). We used CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) to merge results from replications of each K before plotting results.

Secondly, a multivariate method, discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was applied to the breeding dataset. DAPC is free from population genetic assumptions and inferences are made on allelic similarity. It summarises genetic variability of individuals within groups while optimizing group discrimination. Breeding sites were used as the grouping variable. The first 115 principal components (PC) were retained in the data transformation step, corresponding to 84.2% of genetic variance, and three discriminant functions were saved. Analyses were carried out using the ADEGENET 2.0.1 package (Jombart 2008) in R.

The exploratory methods revealed two well defined clusters consisting of the northern populations and the rest of Europe, the latter being also more subtly sub-structured. This confirmed our suspicion of the isolation of the northern populations due to declines and fragmentation in Central Europe. We tested the partition of the genetic variance within and among these two clusters by assigning sampling sites to one of the clusters and performing an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using the POPPR 2.2.0 package (Kamvar et al. 2014) in R. Sampling sites with less than eight individuals were excluded from this analysis. Log-likelihood G tests for differentiation were performed between and within populations using 10,000 permutations (Goudet et al. 1996).

We also tested the influence of the east–west migratory divide on the partition of genetic variance in an AMOVA by assigning sampling sites to an eastern or a western cluster according to the flyway used during migration as revealed by light-level geolocator analysis (Jiguet et al. 2016b).

Finally, based on these prior results and knowledge, we tested for a consensus structure of three clusters to account for the influence of the migratory divide and the isolation of the northern populations from the south–western populations due to fragmentation in central Europe.

Validation and characterization of the structure

We performed a cross-validation of these three consensus clusters. The full breeding dataset excluding highly related breeding sites (Drôme and Corbières) was randomly split into a training and a validation dataset by assigning 70% of individuals from each sampling site to the training set (394 individuals) and the remaining 30% (166 individuals) to the validation set. The training set defined the genetic makeup of the clustering to be tested, and individuals from the validation set were assigned one of these populations by the program GENECLASS 2 (Piry et al. 2004) using the Bayesian method described by Rannala and Mountain (1997). The process was repeated ten times.

Pairwise F_{ST} between the clusters were calculated by the HIERFSTAT (Goudet 2005) package in R to assess the level of connectivity.

Isolation by distance was tested between sampling sites with over eight individuals across the whole range, and within each defined population. Mantel tests were performed between matrices of linearized pairwise F_{ST} (Rousset 1997) and log-transformed geographical distances using the HIERFSTAT package in R setting the number of repetitions to 1000.

Contemporary gene flow

The magnitude and direction of contemporary gene flow occurring between the consensus clusters were estimated using the program BAYESASS 3.0.1 (Wilson and Rannala 2003). The simulation was run with 2×10^7 iterations, with the first 10^6 iterations discarded as burn-in. Samples were collected every 1000 iterations. We tested a combination of mixing parameters to ensure that the acceptance rates were between 0.2 and 0.6. Eventually, allelic frequencies coefficient was set at 1, inbreeding coefficient at 0.9, and migration rate at 0.06. The trace file was examined in TRACER 1.5 to ensure mixing and convergence of the chains (Rambaut and Drummond 2009).

Results

Data preparation

813 samples out of 1127 could be amplified at all loci, giving a 72.14% success rate after only one PCR. Most samples that failed to amplify did so for only one locus (190 samples), and only 3.02% of samples failed to amplify at ten or more loci. Thirteen samples were removed from the dataset because of amplification failure for more than eleven loci. No locus showed excessive amplification failure rate (mean ± SD = 3.25 ± 1.71%, range 1.69–9.49%).

Ninety-one replicates had identical genotypes, corresponding to a 63.64% correct typing rate. Of the 53 samples that displayed typing errors, 80.95% were mistyped at only one locus, mostly as homozygote rather than heterozygote due to weak amplification of the second allele, and no individual was mistyped at more than four loci (occurred in only one sample). No locus showed excessive typing error (mean ± SD = 2.04 ± 1.65%, range 0–6.38%).

The final dataset consisted of 575 unique individuals typed for a minimum of 14 loci.

Microsatellite characteristics, genetic diversity and relatedness

Only ten tests for linkage disequilibrium out of 5244 (24 loci, 19 sites) were significant. No significant correlation between the number of homozygotes and of missing data was found at the sample level ($r = -0.14$, $p = 0.998$), indicating that any allelic dropout was not likely due to DNA quality or quantity. In contrast, significant correlation was detected at the locus level ($r = 0.45$, $p = 0.021$), indicating that allelic dropout could be due to null alleles. Four microsatellites displayed moderate to high null allele frequencies (> 0.2) in over four sites, and in particular Embhort12 (Table S1) had high frequencies of null alleles in most sites. Fifty-three tests for deviation from HWE were significant after Bonferroni correction and due to deficiency in heterozygosity. Three loci were out of HWE in nearly 50% of populations, likely due to the presence of null alleles and were thus removed from further analyses (Embhort05, Embhort08 and Embhort12; Table S2).

Mean allelic richness was high and similar for all sites (mean ± SD = 6.28 ± 0.24). Observed heterozygosity was moderate and similar across all sites (mean ± SD = 0.730 ± 0.022), and was significantly lower than expected heterozygosity (mean ± SD = 0.803 ± 0.025, $p < 0.01$). Inbreeding indices F_{IS} were low to moderate for all sites (mean ± SD = 0.101 ± 0.048) and significantly different from zero after 1000 bootstraps for 14 out of 19 populations. Estimated F_{ST} over loci was low at 0.011 but significant (95% CI 0.010–0.014) and the G-test was significant ($p < 0.001$), indicating that breeding sites were not part of a panmictic population.

Most individuals within a breeding site were unrelated (mean proportion of unrelated relationships ± SD = 89.30 ± 9.01%, range 56.06–94.77%), however three sites displayed proportion of related individuals larger than 15%: France 1, France 5 and Serbia 24 (21.43, 43.94 and 17.86% respectively; Table S2). France 5, located in the Drôme area, has an exceptionally high proportion of related individuals which could affect inference of population structure, and was thus removed from subsequent population-level analyses.

Population structure

Bayesian modelling

Genetic structure was consistent with sampling geography. The prior and non-prior Bayesian models indicated similar patterns although the non-prior model displayed large levels of admixture (Fig. 2). At K = 2, breeding sites from northern Europe (Fennoscandia, Estonia and western Lithuania: sites 10–18) formed one cluster, while western, central, eastern and southern Europe formed a second one. At K = 3, the north–south clustering remains but the Drôme site from France (site 5) stood out as an independent cluster. At K = 4, the French samples from Corbières (site 3) distinguished themselves from the other clusters (these three samples are an adult and its two nestlings). At K = 5 and 6, the Belarus breeding site (site 19) started to separate from the eastern cluster and so did the western and central sites which displayed large levels of admixture. The standardised second-order rate of change ΔK indicated that the most likely number of clusters describing the data were two for both prior and non-prior models. As this method only distinguished higher structuring level, each cluster was then run separately with the same settings. No obvious geographical

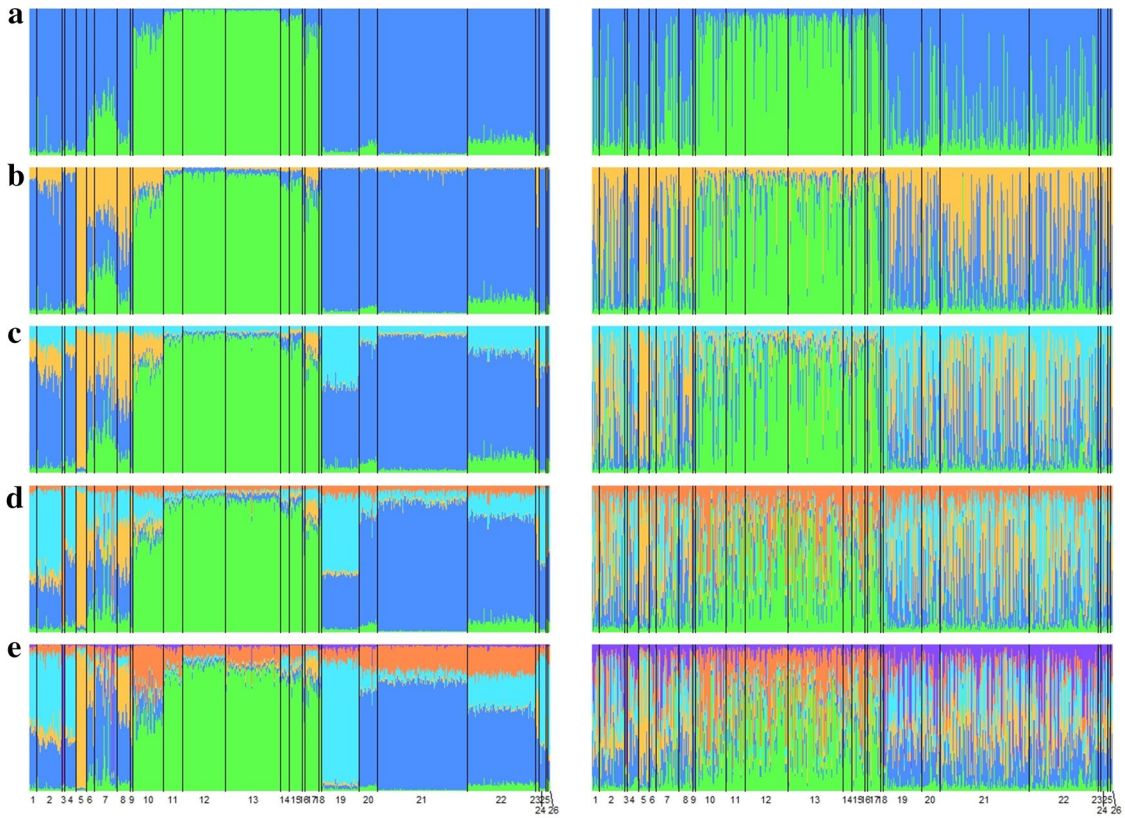

Fig. 2 Bayesian posterior probability of membership to one of K populations obtained by STRUCTURE, using breeding sites as prior for the left column and no prior on the right column. Each vertical line represents an individual. Solid lines separate sites the individuals were sampled from. The sites are ordered according to their geographical location and using the same identifying numbers as in Fig. 1. **a** K = 2, **b** K = 3, **c** K = 4, **d** K = 5, **e** K = 6

substructure was supported for northern Europe at the exception of larger admixture in Lithuania, possibly indicating a contact zone (the eastern Lithuania site was assigned to the southern cluster). In contrast, the optimal number of clusters for the southern group was four (prior model), consisting of the Drôme site, the Corbières site, the Russian sites, and an admixed group gathering western, central and southern Europe as well as Belarus (Figs. 2, 3).

Multivariate analysis

The discriminant analysis on sampling sites indicated a similar pattern of clustering. Most of the discrimination occurred on the first axis and distinguished northern Europe (Fennoscandia) from the rest of Europe. The second axis isolated the Drôme samples from the southern cluster, and to a lesser degree, Eastern Europe from Western Europe. Within these clusters, admixture seems high, and individuals cannot be reliably assigned to the breeding site they were captured from (41.57% of correct assignment after leave-one-out cross-validation) (Fig. 3).

AMOVA and genetic differentiation among clusters

Based on these results, we considered two clusters: northern populations (Fennoscandia, western Lithuania and Estonia: sites 10–18), and southern populations (western, central, eastern and southern Europe: sites 1–4, 6–9 and 19–26). AMOVA indicated that most genetic variation occurred within breeding sites (97.89%, $\Phi_{ST} = 0.021$, $p < 0.001$), but still very marginally supported the clustering, with larger genetic variation between populations than between sites within populations (1.07% $\Phi_{CT} = 0.011$, $p < 0.001$ vs. 1.04% $\Phi_{SC} = 0.011$, $p < 0.001$) (Table 2).

We also tested the effect of the migratory divide (western flyway: France, Spain, Norway, Sweden, Poland, Lithuania, Estonia, Finland; eastern flyway: Russia, Belarus, Serbia, Greece) on genetic partitioning. It was found to be a weak driver for genetic structuring (larger genetic variation between sites within flyways than between flyways (0.57% $\Phi_{CT} = 0.006$, $p < 0.01$ vs. 1.28% $\Phi_{SC} = 0.013$, $p < 0.001$)) (Table 2).

Finally, we tested the hypothesis that the European ortolan population is subtly structured on an eastern–western

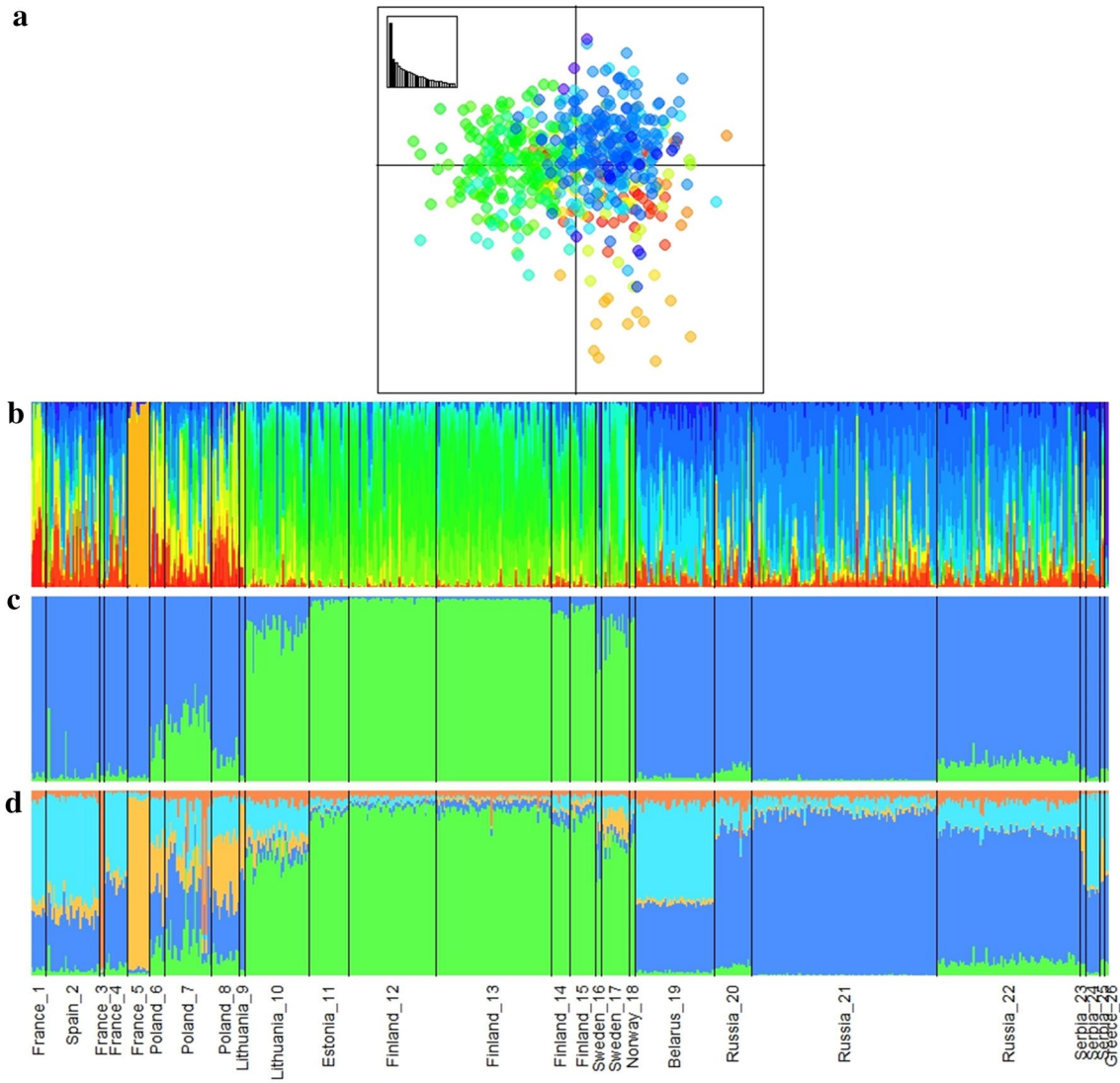

Fig. 3 Individual-based analyses on microsatellite data using multivariate (**a**, **b**) and Bayesian (**c**, **d**) assignment methods. **a** Scatterplot of individual principal components on the first two axes. Each site is colour coded as Fig. 1. **b** Posterior probability of membership to one of the sites after discriminant analysis and leave-one out cross-validation. Each vertical line represents an individual. Solid lines separate sites the individuals were sampled from. The sites are ordered according to their geographical location and colour coded as in **a**. **c** Bayesian posterior probability of membership to one of two populations obtained by STRUCTURE. Individuals and sites are displayed in the same order as in **b**. **d** Bayesian posterior probability of membership to one of five populations obtained by STRUCTURE. (Color figure online)

axis influenced by the migratory divide, and that recent declines and population fragmentation in central Europe (Jiguet et al. 2016a) isolated northern populations from the south–western ones, resulting in three clusters: the northern population, a western one (France, Spain, Poland and eastern Lithuania), and an eastern one (Russia, Belarus, Serbia, and Greece). The AMOVA marginally supported this clustering with larger genetic variation between populations than between sites within populations (0.97% $\Phi_{CT} = 0.010$, $p < 0.001$ vs. 0.93% $\Phi_{SC} = 0.009$, $p < 0.001$) (Table 2).

Pairwise F_{ST} demonstrated weak differentiation among the three consensus sub-populations with the highest F_{ST} of 0.006 occurring between the northern population and the western and eastern ones (Table 3).

Cross-validation

We performed a cross-validation on the three populations that supported the strength of the northern and the eastern clusters with an average of 84.68 and 79.48% of individuals

Table 2 Analysis of molecular variance for three clustering: two populations (consensus from STRUCTURE and DAPC analyses), three populations (consensus from STRUCTURE and DAPC analysis and prior knowledge of migratory flyways), two populations (test for the two migratory flyways)

Source of variation	Sum of squares	Variance components	Percentage variation	Phi-statistics
Two populations (north, south)				
Between populations	67.827	67.827	1.074	0.011***
Between sites within populations	328.752	20.547	1.039	0.011***
Between individuals	8326.625	15.860	97.887	0.021***
Three populations (north, west, east)				
Between populations	97.259	48.629	0.970	0.010***
Between sites within populations	299.321	19.955	0.925	0.009***
Between individuals	8326.625	15.860	98.105	0.020***
Two flyways (western, eastern)				
Between flyways	52.261	52.261	0.576	0.006**
Between sites within flyways	344.319	21.520	1.282	0.013***
Between individuals	8326.625	15.860	98.142	0.019***

**Significance at 0.01 level

***Significance at 0.001 level

Table 3 Pairwise F_{ST} among the three populations

	West	North
North	0.006	–
East	0.003	0.006

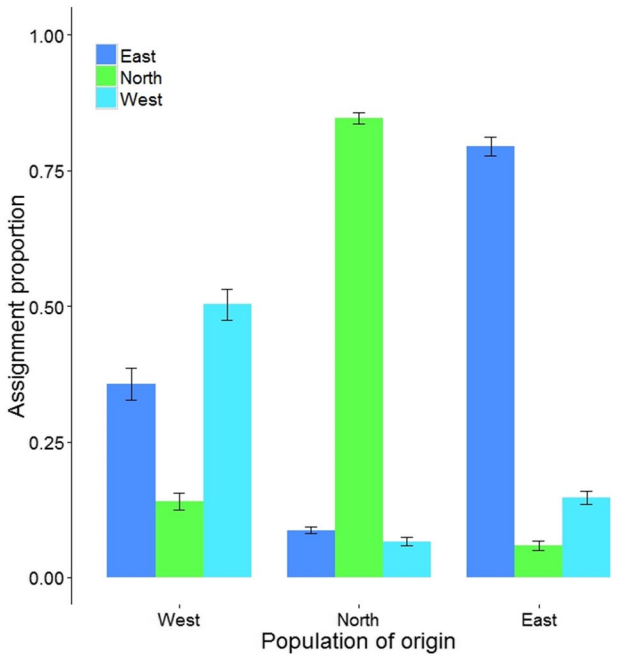

Fig. 4 Mean and standard errors assignment proportions to one of the three described sub-populations obtained by cross-validation. The dataset was split into a training set (70% of individuals) and a validation set (30% of individuals) and the cross-validation process was repeated ten times

Table 4 Contemporary gene flow among populations as percentage of genetic migrants per generation (±95% confidence interval) as revealed by the BAYESASS analysis

	From		
	West	North	East
To			
West/central	66.98 (±0.61)	2.88 (±2.67)	30.13 (±2.72)
North	0.16 (±0.31)	97.59 (±2.25)	2.25 (±2.23)
East	0.13 (±0.25)	0.56 (±0.82)	99.32 (±0.86)

correctly assigned (Fig. 4). Moderate correct assignment to the western populations (50.33%) with a large contribution of the eastern populations (35.67%) confirmed low differentiation between these groups and the low influence of the migratory divide in structuring populations.

Isolation by distance

No isolation by distance was detected on the full dataset and within the southern and northern populations. When splitting the southern cluster into western and eastern populations, no correlation between genetic and geographic distances were found within the west/central cluster. In contrast, marginally significant isolation by distance was detected in the eastern population ($r^2 = 0.38$, $p = 0.045$).

Contemporary gene flow

Recent migration rates were estimated as the fraction of individuals in a population that are migrants derived from another population, per generation (Table 4). We predicted that most gene flow would occur from the eastern cluster

towards the western cluster since it contains the largest breeding populations in Europe, while the northern populations would essentially be isolated from both the western and the eastern clusters due to population fragmentation in central Europe. The disequilibrium-based program inferred a nearly complete lack of contemporary gene flow towards the eastern populations and towards the northern populations. Emigration rates from the northern populations were very low (0.56–2.88%) indicating low contemporary dispersal from these populations. In contrast, the emigration rate from the eastern populations towards the western one was especially high (30.13%). This analysis confirmed the eastern populations as a source population for Western Europe while northern Europe now appears genetically disconnected from the rest of Europe.

Discussion

We examined patterns of genetic variation and genetic diversity across the European breeding range of a long distance migrant bird, the ortolan bunting, in order to investigate any potential effect of recent population declines and fragmentation, and to infer structure that could identify conservation priorities for this species. As predicted, our results suggest that, given the still quite large breeding populations, the population declines observed have not resulted in dramatic loss of genetic diversity, despite being a concern from a demographic point of view. The low overall F_{ST} indicated weak differentiation among breeding sites, pattern reported for species with high mobility potential, such as birds (Crochet 2000) and bats (Moussy et al. 2013). The migratory divide only subtly shapes genetic structure with a weak east–west partitioning of genetic variation, but considerable asymmetric gene flow from the large eastern breeding populations towards the western ones is eroding this historic signal. In contrast, the population declines in central Europe leading to local extinctions and fragmentation (Jiguet et al. 2016a) lead to interrupted contemporary gene flow between the Fennoscandia and Baltic populations = and the southern populations, driving its genetic differentiation and triggering conservation concerns.

Genetic diversity indices, allelic richness and observed heterozygosity, indicated moderate to high level of diversity within all breeding sites, consistent with many other widely distributed migratory species such as blackcap *Sylvia atricapilla* (Mettler et al. 2013) or reed warbler *Acrocephalus scirpaceus* (Prochazka et al. 2011), and with other another *Emberiza* species, reed bunting *E. schoeniclus* (Kvist et al. 2011). Breeding populations underwent large declines since at least 1980 and continue to decrease (Jiguet et al. 2016a). In particular, northern populations are declining more rapidly. Large population declines and fragmentation can drive loss of genetic diversity through genetic drift, and especially in peripheral populations following the core-marginal theoretical framework (Eckert et al. 2008). This has been recorded for a subspecies of the reed bunting, *E. schoeniclus lusitania* in the Iberian Peninsula (Kvist et al. 2011) for instance. However, despite the worrying demographic trends, the breeding populations of ortolan bunting still have an effective population large enough to maintain reasonably high genetic diversity. Similarly, large declines in breeding populations of corncrake *Crex crex* were also reported with no apparent effect on genetic diversity and differentiation (Fourcade et al. 2016). However, gene flow is now severely limited towards the northern populations and their isolation and large reported declines could make them particularly sensitive to genetic drift and associated loss of genetic diversity in the future. This potential impact on genetic diversity might not be detectable early enough due to the lag between current processes and genetic consequences (Epps and Keyghobadi 2015).

The East–West migratory divide running from Belarus to Serbia and uncovered by a geolocator study (Jiguet et al. 2016b) is highly permeable and therefore contributes only little to genetic structuring. Indeed, cross-validation of the west/central cluster indicated that over 35% of breeders from these populations could be assigned to the eastern cluster. It could be the result of the re-colonisation process from the last ice age, from refugia located in the Iberian Peninsula for the SW route, and in the Balkans or Central Asia for the SE route. Migratory divides can indeed contribute to creating or maintaining genetic structure as found in the European bee-eater *Merops apiaster* (Ramos et al. 2016). In contrast, migratory divides do not appear to act as barriers to gene flow in many other bird species. For instance, low differentiation was found across the migratory divide in the Eurasian reed warbler (Prochazka et al. 2011), the willow warbler *Phylloscopus trochilus* (Bensch et al. 1999) or for the traditional SW–SE divide in blackcaps (Rolshausen et al. 2009; Mettler et al. 2013). High historic and ongoing gene flow could gradually erase the historic signature, especially when using high mutation rate microsatellites as we did (Wan et al. 2004). Most of the gene flow we reported is asymmetric, with high emigration from the eastern populations. Those populations are the largest in Europe (Jiguet et al. 2016a) and dispersal from the core to the margin is congruent with a source-sink dynamic which homogenises populations (Eckert et al. 2008). The Fennoscandia and Baltic populations use the same SW migratory route as the western populations and we would have expected them to belong to the same cluster However, recent extinctions attested in Belgium, the Netherlands or Denmark and quasi extinction in Switzerland (Jiguet et al. 2016a) due, at least partly, to human activity leading to habitat loss and disruption of population connectivity (Menz and Arlettaz 2011),

seem to be driving the recent differentiation of the northern population. Contemporary gene flow is indeed reported very low towards this population, indicating that this is an ongoing process. The ortolan bunting is highly associated with agricultural land, at least outside the Mediterranean region (Menz and Arlettaz 2011), and intensification practices resulted in habitat fragmentation and loss of habitat heterogeneity, highly detrimental to farmland birds (Donald et al. 2001, 2006). Northern populations, located at the range margin, could have thus been particularly affected, as the large population declines reported seem to indicate (Jiguet et al. 2016a). Lower effective population sizes and lower rates of gene flow due to limited long-distance dispersal could accelerate genetic drift and genetic divergence. If such a scenario is true, genetic differentiation is expected to increase and genetic diversity to decrease with time (Epps and Keyghobadi 2015). For instance, fragmentation of two subspecies of the reed bunting *E. schoeniclus* in the Iberian peninsula is reported to be responsible for the loss of genetic variation and small effective population size in the *lusitanica* subspecies, and of population differentiations in the *witherbyi* subspecies, prompting strong conservation concerns (Kvist et al. 2011). We did not find evidence of isolation by distance within the northern or the western clusters, indicating that local gene flow occurs irrespectively of distance. However, weak isolation by distance was found in the eastern cluster, probably because of the broader geographic scale compared to other groups of populations. Dispersal in the ortolan bunting occurs through female-biased natal dispersal (Dale 2001), as reported in most bird species (Greenwood 1980; Paradis et al. 1998), but also through adult movement. Breeding dispersal was indeed reported in males that failed to attract a female in their first territory and occurred over longer distances than natal dispersal (Dale et al. 2005). Adult male movement could therefore contribute strongly to gene flow. Nonetheless, site fidelity of both sexes may occur in this species and can lead to a high likelihood of siblings settling close together if no breeding dispersal occurs later (Dale 2010). This was particularly noticeable in the breeding population located in the Drôme area of France which stood out in the individual-based analyses. The 12 territorial males sampled over 2 years were more related than in any other populations and indicated extreme philopatry.

Disentangling historical and contemporary processes that shape genetic structure and diversity is a continued issue in population genetics and while we could infer some scenarios to explain the patterns we observed, the use of other molecular markers could help clarify the drivers of genetic variation. Mitochondrial DNA is maternally inherited and has shorter coalescent time than nuclear markers and is thus particularly adapted in retracing a species history and in inferring sex-biased dispersal compared to microsatellites (Wan et al. 2004). For instance, a spatial structuring of mitochondrial haplotype across the migratory divide could be expected if it results from re-colonisation from separate refugia and if female dispersal is not too strong. High haplotype diversity could be expected if the population has long been established with high levels of gene flow, while low haplotype diversity is the signature of a recent population expansion.

While the exact mechanisms shaping the distribution of genetic variation of the ortolan bunting are uncertain and we can only propose likely scenarios, the high population connectivity in the southern populations should prompt the implementation of large scale conservation plans to halt the large ongoing population declines, while the recent differentiation of the declining northern populations should drive targeted action to preserve what is left. With contemporary gene flow in the southern cluster being asymmetric and western breeding sites acting as population sinks, it is essential to protect the core eastern populations. Agricultural practices in post-communist era might have been beneficial to these populations while agricultural intensification in western and northern Europe lead to landscape homogenisation and loss of structural heterogeneity which were detrimental to numerous farmland birds (Donald et al. 2001, 2006). Renewed intensification of agriculture in eastern countries has however already impacted grassland birds in Eurasian steppes (Kamp et al. 2011) and could therefore also affect the ortolan bunting. Population declines in the eastern core have already been reported (Jiguet et al. 2016a) and could further affect the western populations relying on eastern immigration to be sustained. Intensified efforts in the western countries are also required to stabilize the populations in current sinks, by preserving suitable habitats, notably insect-rich habitats providing access to prey in the form of patches of bare ground, allowing the maintenance of western populations and the recruitment of eastern immigrants. Some particular efforts are also required in the Fennoscandia and Baltic countries as those populations suffered the largest declines and we brought evidence of a lack of contemporary gene flow resulting in what seems to be the beginning of divergence of this northern cluster from the southern populations. Improving connectivity and quality habitats within this cluster would be essential to maintain an effective population large enough to avoid loss of genetic diversity in the quasi-absence of immigration from the core populations. Identifying all effective causes of population decline are urgently needed to act and prevent the extinction of this northern cluster. Continued monitoring of sensitive populations would be needed to infer the impact of current demographical trends on genetic diversity and structure through longitudinal studies (Cousseau et al. 2016). In conclusion, this study highlighted the complex patterns and drivers of genetic variation in a widely distributed

long-distant migrant bird and helped inform the scale of conservation actions required to limit population declines and identify vulnerable populations where targeted effort are required to prevent future extinctions.

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