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Genetic consequences of population decline in the Danish population of the little owl (*Athene noctua*)

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ABSTRACT

Background: Danish populations of the little owl (*Athene noctua*) have experienced dramatic declines in size over the past century. Before 1960 the little owl population was abundant in Denmark (estimated $N > 2000$), but between 1960 and 1980 the population declined rapidly, and since 1980 the little owl population has survived only in small and fragmented areas.

Question: Is the decline in population size associated with reduced genetic variation in these Danish populations of the little owl? Are the populations genetically fragmented?

Field site: Samples were collected from birds in Denmark (from 57°45′7″N to 54°33′35″N).

Methods: We extracted DNA from the feathers of museum specimens of Danish little owls collected between 1918 and 1980. We also extracted DNA from feathers collected between 1984 and 2010. We performed a genetic analysis of 15 microsatellites on these samples.

Conclusions: Older samples showed relatively little genetic variability, with more recent ones showing even less. In addition, pairwise F_{ST} values showed evidence for genetic substructuring with small but significant genetic differences between the extant population and the extinct owl populations on the Danish isle of Funen. The modest loss of genetic variability observed since the 1960s and 1970s may be associated with a diminished distributional range and population bottlenecks.

Keywords: conservation, microsatellites, old DNA, population bottlenecks, temporal and spatial variation.

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INTRODUCTION

Many species and populations are currently losing genetic variability, which may increase the risk of extinction (Avise and Hamrick, 1996; IUCN, 2011). Typically, they are species or populations previously abundant and widespread over large geographical areas and now confined to only a few, small, and fragmented populations (e.g. Willi *et al.*, 2007). It is expected that fragmentation and reduced population sizes will eventually lead to loss of genetic diversity and reduced fitness (Reed and Frankham, 2003; Allentoft and O'Brien, 2010). However, knowledge of the loss of variability that has taken place is often tempered by a lack of information on the genetic composition of the same populations prior to bottlenecks and fragmentations. Moreover, there is generally limited information about the genetic variation that has been lost when populations have gone entirely extinct. Some recent studies have addressed this problem by extracting 'ancient' DNA and analysing molecular markers from museum specimens or other types of historical samples and comparing the results with data from present populations (Pertoldi *et al.*, 2001, 2005; Shapiro *et al.*, 2004; Wandeler *et al.*, 2007).

The little owl (*Athene noctua*) is a red-listed bird species in Denmark and in other European countries (Birdlife Denmark, 2011). Until the 1970s, it was widespread and common in Denmark (Laursen, 2006). More than 1000 pairs were estimated in 457 25-km^2 atlas squares in the 1970s (Fig. 1), 13% of which were occupied in the 1990s (Thorup *et al.*, 2010). The species went extinct on the isle of Funen around 2003 and at that time approximately 100 pairs remained in Northern and Western Jutland. Since 2005 this last remaining population appears to have entered an extinction vortex with annual growth rates lower than 93% because of collapsed breeding success (Thorup *et al.*, 2010). In 2010, the total Danish breeding population amounted to 40–46 known breeding pairs (Eskildsen and Vikstrøm, 2011). Even though there is strong evidence that reduced fitness in the present Danish population is due to poor habitat quality and birds experiencing starvation during the breeding season (Thorup *et al.*, 2010), it is possible that inbreeding and genetic drift, as well as interactions between genetic and ecological factors have contributed to the dramatic reduction in size of the little owl population in Denmark, as suggested by, for example, Liao and Reed (2009).

In the present study, we focused on the genetic consequences of the decline in population size of the little owl in Denmark observed during the last 40–50 years, both on a temporal and a geographical scale. Our aim was to assess if the dramatic decrease in population size during the last decades could be observed at the genetic level. Furthermore, we wished to assess the genetic relationship between the surviving owl population in Northern and Western Jutland and the (now extinct) population on the isle of Funen. Our data can help to determine whether the remaining population was part of one large population that had declined, or if genetically distinct population segments had previously been present. These issues were addressed using data obtained by analysis of microsatellite DNA from museum samples of owls from Jutland and the isle of Funen, covering the period from 1918 to 2000, and from recent samples collected from the present owl population (2001–2010). The Danish little owl population serves as a good example for analysing genetic patterns of a declining and decomposing population. First, little owls are territorial and year-round residents as adults (Laursen, 2006; Sunde *et al.*, 2009), with mean natal dispersal distances of 22 km and thus very limited exchange of individuals between geographical regions (Bønløkke *et al.*, 2006). Second, with an adult annual survival rate of 60–68% (K. Thorup, D. Pedersen, P. Sunde, L.B. Jacobsen and C. Rahbek, unpublished) and a mean adult generation time of 2.5–3.1 years, our analysis

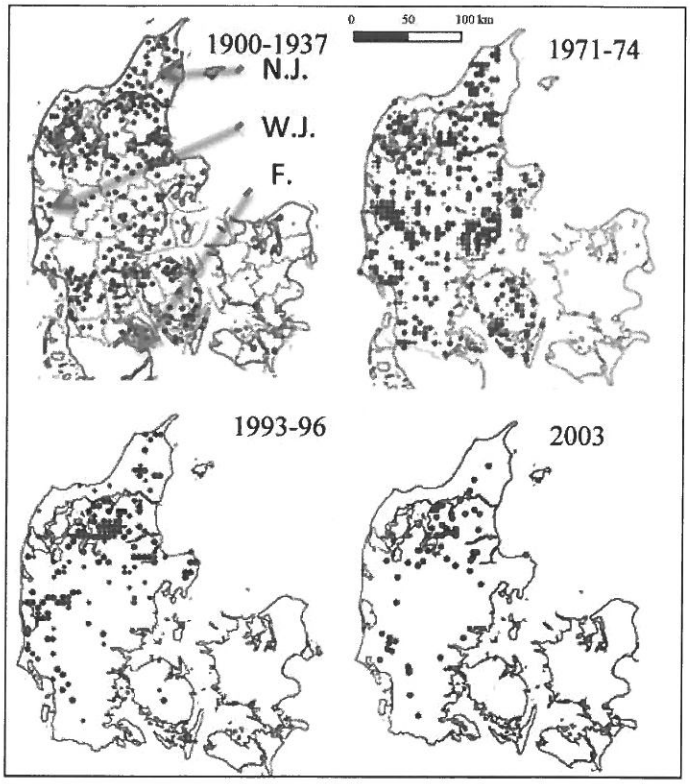

Fig. 1. Distributional range of the little owl in Denmark on Funen (F.), Western Jutland (W.J.), and Northern Jutland (N.J.) registered as reports of occurrence 1900–1937 (Jespersen, 1937), as presence in areas of 5×5 km^2 (size of dots indicate the certainty of breeding) in the Danish bird Atlas surveys in 1971–73 (Dybbro, 1976) and 1993–96 (Grell, 1998), and as all reports of presence (pairs or individual birds) in 2003 (data from Birdlife Denmark, 2011).

covers >20 generations (82 years) with negligible potential overlap of individuals between the three historic intervals considered in the study (see below).

MATERIALS AND METHODS

Samples

Our samples consisted of: (1) feathers from 123 stuffed specimens kept in schools ('old' samples) throughout Jutland and the isle of Funen; (2) feathers collected from nests during the period 1984–2010 ('modern' samples); and (3) one muscle sample obtained from a single owl found dead during the winter of 2009. In total, 124 samples were analysed. The samples were grouped according to geography: Jutland (JUT) and the isle of Funen (FUN). The Jutland and Funen samples were further subdivided based on period of collection: before 1960 (Period 1), between 1965 and 1980 (Period 2), and after 1984 (Period 3 – the 'modern period'). The number of specimens analysed in the five groups were as follows: JUT1 ($n = 32$), JUT2 ($n = 27$), JUT3 ($n = 43$), FUN1 ($n = 16$), and FUN2 ($n = 6$). The reasons for

these groupings are that before 1960 the little owl population was abundant in Denmark (estimated $N > 2000$), between 1960 and 1980 it declined significantly (mainly due to dramatic changes in agricultural practices in Denmark), and since 1980 the little owl population has survived in small and fragmented areas in Jutland, whereas on Funen the population had already become extinct by the second period (1960–1980).

Molecular analyses

DNA was extracted from 1 to 1.5 cm of the calamus tip of large feathers, including pulp cells inside the feather shaft if available, and from the entire shaft for small feathers. DNA was extracted from muscle tissue from the one bird found dead. All extractions were performed using the Qiagen DNeasy tissue kit (QIAGEN, Valencia, California) (Hogan *et al.*, 2008). The DNA obtained from old samples is often of low quantity and degraded. Amplification by polymerase chain reactions of such material may produce random non-amplification of some alleles (allelic drop out, or ADO). To detect the presence of ADO, we performed the following steps: (1) the extracted DNA was run on 1% agarose gel to verify the amount and the integrity of total DNA; and (2) analyses at six loci on 15 samples collected between 1920 and 1950 were independently replicated with the aim of determining the percentage of positive PCR and the occurrence of ADO. Allelic drop out and positive PCR were calculated using GIMLET v.1.3.3 (Valière, 2002).

Amplification of all samples was performed in a Pre-PCR area provided with standard equipment (micropipette, UV hoods, thermocyclers, laboratory coat and gloves). Amplifications were carried out under the flow hood after UV decontamination. Surfaces were cleaned with a blench solution at the beginning and at the end of operations. Amplified DNA was managed in a properly equipped Post-PCR area. Negative controls were used in each step to check for contamination.

Samples were genotyped for the following 15 microsatellite loci: Atn1, Atn2, Atn3, Atn4, Atn5, Atn7, Atn8, Atn9, Atn11, and Atn12 previously isolated in little owls (G. Segelbacher, J. Hurst and W. Fiedler, unpublished), together with 15A6 (Thode *et al.*, 2002), Oe053 (Hsu *et al.*, 2003), Oe085 (Hsu *et al.*, 2006), Ta212 and Ta216 (Burri *et al.*, 2008) isolated in other species from the order Strigiformes.

We used the following touchdown PCR protocol: pre-denaturation at 94°C for 5 min, followed by 32 cycles with denaturation at 94°C for 45 s. Annealing temperatures ranged from 60°C to 55°C (for 'Ath' primers) and from 55°C to 50°C (for 15A6, Oe053, Oe085, Ta212, and Ta216) for 45 s, followed by extension at 72°C for 45 s. The amplified *loci* were analysed on an ABI3130XL automatic sequencer. Genotypes were assigned using GENEMAPPER v.4.0 (Applied Biosystems).

Population genetic structure

The effective number of alleles per locus (A_E), unbiased expected heterozygosity (UH_e), and the number of loci successfully amplified were estimated using the software GENALEX v.6.41 (Peakall and Smouse, 2006; http://www.anu.edu.au/BoZo/GenAlEx/).

The software GENEPOP v.4.1.1 (Raymond and Rousset, 1995; http://kimura.univ-montp2.fr/~rousset/Genepop.htm) was used to estimate linkage disequilibrium, and for testing for deviations from Hardy-Weinberg equilibrium (HWE) by exact tests (Guo and Thompson, 1992). The tests were performed for each locus in each spatial and temporal group.

Genetic differentiation between populations was characterized by exact tests (Raymond and Rousset, 1995) using GENEPOP, by estimating pairwise F_{ST} (Weir and Cockerham, 1984) in FSTAT v.2.9.3.2 (Goudet, 1995), and by estimating Nei's unbiased genetic distance (D) with GENALEX (Nei, 1978). We adjusted the statistical significance for multiple tests using the sequential Bonferroni method (Rice, 1989).

Inferring population bottlenecks

Recent population bottlenecks were inferred using Wilcoxon's test (for heterozygosity excess) implemented in BOTTLENECK v.1.2 (Cornuet and Luikart, 1996), assuming an infinite allele model (IAM), a stepwise mutation model (SMM), or a two-phase model of mutation (TPM, with 95% SMMs). These three models were used to produce the distribution of heterozygosity expected from the observed number of alleles given the sample size of each population under the assumption of mutation–drift equilibrium. This enables the calculation of the average expected heterozygosity, and computation of the P-value for the difference between observed and expected heterozygosity at each locus. To determine whether a population exhibits a significant number of loci with heterozygosity excess, we performed a Wilcoxon sign-rank test (Luikart *et al.*, 1998). The software can detect only severe and recent declines, which occurred within $0.2N_e$ to $0.4N_e$ generations, where N_e is effective population size. The Wilcoxon sign-rank test was not performed for FUN2, as the sample size was too small ($n = 6$).

The mean and median of A_E and UH_e for each locality and each period were estimated. Tests of differences in mean and median values were performed with a paired t-test and a Wilcoxon's test, respectively, using the software PAST version v.2.12 (Hammer *et al.*, 2001; http://folk.uio.no/ohammer/past). The following comparisons were made: JUT1 vs. JUT2, JUT1 vs. JUT3, JUT2 vs. JUT3, JUT1 vs. FUN1, FUN1 vs. FUN2, and JUT2 vs. FUN2.

RESULTS

DNA quantity and quality

The amount and quality of DNA tested on a 1% agarose gel was heterogeneous. Samples with no or very small amounts of DNA were not included in the analyses.

Additional amplification of 15 old samples at six loci identified a very low allelic drop out (ADO) value (0.036) and a very high percentage of positive amplification (mean = 0.99). The observed low ADO value indicates that allelic drop out cannot influence the variability of the investigated population. Furthermore, the observed ADO values observed in the old samples are comparable with values observed in fresh tissue.

Genetic variability

Twenty-seven of 1860 (124 samples × 15 loci) scores could not be genotyped, suggesting a sufficient level of successful amplifications. The number of alleles per locus varied from 2 (loci Oe053 and Ta212) to 10 (locus ATn5) with a mean ± standard error of 6 ± 0.58 and a median of 5 (see Table 1). The mean number of alleles in the Danish populations ranged from 3.27 for FUN2 to 5.07 in JUT3. All pairs of loci showed no significant genotypic linkage disequilibrium after applying a sequential Bonferroni correction.

Table 1. Summary of the mean number of loci successfully amplified (NLSA), effective number of alleles per microsatellite locus (A_e), observed heterozygosity (H_o), unbiased expected heterozygosity (UH_e), and associated standard errors (S.E.)

Pop.		NLSA	A_E	H_o	UH_e
JUT1	Mean	31.4	3.04	0.52	0.61
	S.E.	0.35	0.32	0.06	0.05
JUT2	Mean	26.73	2.82	0.52	0.60
	S.E.	0.15	0.28	0.04	0.04
JUT3	Mean	42.93	2.79	0.55	0.60
	S.E.	0.07	0.24	0.04	0.05
FUN1	Mean	15.6	3.04	0.58	0.63
	S.E.	0.19	0.29	0.07	0.05
FUN2	Mean	5.53	2.45	0.38	0.57
	S.E.	0.16	0.29	0.06	0.06

Note: Deviations from expected Hardy-Weinberg proportions (HW test) were never significant. See Materials and Methods for explanation of sample abbreviations. JUT1 and FUN1 represent 'old' individuals collected before 1960 (Period 1) in Jutland and Funen respectively, JUT2 and FUN2 represent 'old' individuals collected between 1965 and 1980 (Period 2), and JUT3 represent individuals from the 'modern' population collected after 1984 (Period 3). The number of samples analysed from each of the five groups was as follows: JUT1 ($n = 32$), JUT2 ($n = 27$), JUT3 ($n = 43$), FUN1 ($n = 16$), and FUN2 ($n = 6$).

The range of the mean estimated UH_e ranged from 0.57 to 0.63. The tests for HWE using GENEPOP identified 12 significant deviations ($P < 0.05$) out of 74 tests. However, after Bonferroni correction ($K = 74$), no significant deviations from HWE were observed. Locus Ta216 was found to be monomorphic in FUN2 (Table 2).

Population genetic structure

After Bonferroni correction ($K = 10$), the exact test for differences in allele frequencies between samples showed significant differentiation between JUT1 and JUT3, between JUT2 and FUN1, and between JUT3 and FUN1. However, the F_{ST} and D values were both quite low (below 0.03 and 0.043, respectively) (Table 2). Differentiation was also observed between JUT1 and JUT2, JUT2 and JUT3, and JUT1 and FUN1 when Bonferroni correction was not performed (see Table 2).

Inferring population bottlenecks

Results obtained using Cornuet and Luikart's (1996) procedure showed that, in the four groups for which tests were conducted, the Wilcoxon's tests revealed significant probabilities for the IAM in all populations (JUT1, $P < 0.01$; JUT2, $P < 0.0001$; JUT 3, $P < 0.01$; FUN1, $P < 0.001$). This is strong evidence for bottlenecks. However, the significance of the results was strictly dependent on the assumed mutation model. Under the SMM or TPM, none of the tests revealed significant results ($P > 0.05$).

Table 2. Pairwise F_{ST} values and Nei's unbiased genetic distances (D; in **bold** in parentheses) (below the diagonal), and exact tests for homogeneity of allele frequencies with probabilities combined over loci using Fisher's method (above diagonal)

	JUT1	JUT2	JUT3	FUN1	FUN2
JUT1		(*)	*	(*)	N.S.
	0.0025				
JUT2	**(0.008)**		(*)	*	N.S.
	0.0173	−0.0003			
JUT3	**(0.029)**	**(0.002)**		*	N.S.
	0.0037	0.0139	0.0255		
FUN1	**(0.010)**	**(0.027)**	**(0.043)**		N.S.
	0.0037	0.0056	0.0252	−0.0163	
FUN2	**(0.027)**	**(0.028)**	**(0.049)**	**(0.000)**	

Note: The asterisks in parentheses (*) denote that significant differentiation did not remain so after Bonferroni correction ($K = 10$).

The tests for differences in mean and median A_E and H_e revealed significant differences in a few cases: FUN1 showed significantly higher mean (paired t-test; $P < 0.01$) and median (Wilcoxon's test; $P < 0.05$) A_E compared with FUN2.

DISCUSSION

Genetic variability

The ranges of the mean estimated H_o and H_e for the Danish population of little owls (H_o: 0.38–0.58; H_e: 0.57–0.63) were lower than those for a south-west German population investigated with an overlapping set of markers [H_o and H_e ranges of 0.30–0.84 and 0.58–0.8, respectively (G. Segelbacher, J. Hurst and W. Fiedler, unpublished)]. The number of alleles in the five Danish populations (Table 1) exhibited lower values than the German population. Thus, it can be concluded that the Danish owl populations exhibit relatively low genetic variability. The low level of genetic variation observed in the Danish owls versus the German ones may have several causes: (1) recent population decline resulting in genetic bottlenecks (for which we found some evidence); (2) a historical low effective population size because the Danish population has probably always been geographically more isolated than Central European populations because it is a peninsular population; (3) little owls fluctuate in size and can, for example, experience a dramatic reduction in numbers during severe winters (as in the 1940s, when the species disappeared from the Danish isle of Lolland and in Sweden); thus bottlenecks could have been frequently experienced by the Danish population. However, most European populations of little owls experience reductions in population size (van Nieuwenhuyse *et al.*, 2008). The Danish population may have lower levels of genetic variation compared with the German ones and potentially other European populations because it is more isolated and extremely small. We argue that the observed trend towards a temporal decline in genetic variation observed in the Danish little owl population is likely to be seen also in other European little owl populations that experience a decline in population size.

When interpreting the results, it should be noted that evidence for population bottlenecks was found also in the two oldest populations (JUT1 and FUN1). This suggests that even before 1960, when the number of birds in Denmark was more than 2000, there was evidence of population bottlenecks. This could be related to founder events (e.g. during postglacial re-colonization) or to metapopulation dynamics involving frequent extinctions and re-colonizations or periodic decimations (e.g. during cold winters) followed by recoveries that shaped the genetic structure and variability of the Danish little owl population before our samples were collected. The evidence for population bottlenecks was, however, weak. With the SMM or TPM, no significant signs of population bottlenecks were observed. This phenomenon may be partly explained by the fact that alleles are lost randomly and independently of population size when a population is subjected to strong demographic fluctuations and/or undergoes a metapopulation dynamics. Therefore, the loss of alleles will result in an allelic pattern that resembles the distribution of alleles in a population governed by an IAM (Cornuet and Luikart, 1996). Another reason for the dependence of the results on the mutation model used is that most of the samples are small and collected over sampling areas of different size. Therefore, the results obtained from these tests should be interpreted with caution (see Cornuet and Luikart, 1996).

Population genetic structure

The tests for genetic differences between samples clearly showed that the present JUT3 population is genetically divergent from the historical JUT1, which can be explained by genetic drift (Table 2). Drift has acted strongly as a consequence of the low effective population size of the owl population and this phenomenon has probably accelerated in the last few decades with the marked reduction in the little owl population in Denmark (Thorup *et al.*, 2010). Restricted gene flow and genetic drift have also been observed to cause population differentiation in other bird species (Méndez *et al.*, 2011).

The genetic differentiation between the recent JUT3 population and the FUN2 population ($F_{ST} = 0.0252$, $D = 0.049$) was considerably higher than that between the two oldest populations (JUT1 and FUN1; $F_{ST} = 0.0037$, $D = 0.010$). This suggests the occurrence of gene flow between the JUT1 and the FUN1 populations, which is to be expected given the short distance between Southern Jutland and Funen (less than 1 km across the sea 'Lillebælt'). The rate of divergence between two populations is inversely proportional to the effective population size. Furthermore, if the geographic distance between populations is increased, the divergence will increase further. The concomitant reduction in population size on the isle of Funen and on the Jutland peninsula could have reduced the range of the owl populations through breakdown of the initial meta-population structure. This phenomenon may have increased the geographic distance and thereby reduced gene flow between the populations (see also Schaub *et al.*, 2006). The estimated F_{ST} values in this study were all below 0.03. These values are low but comparable to those of boreal owl (*Aegolius funereus*) subpopulations throughout North America ($F_{ST} = 0.04$) (Koopman *et al.*, 2007) and to *those reported* for the greater sage-grouse (*Centrocercus urophasianus*) ($F_{ST} = 0.027$) (Oyler-McCance *et al.*, 1999). However, lower values are also reported in the literature. For example in the yellow warbler (*Dendroica petechia*) and the burrowing owl (*Athene cunicularia*), F_{ST} values < 0.014 have been reported (Gibbs *et al.*, 2000; Korfanta *et al.*, 2001). Given these estimates and the fact that our study was conducted over a much smaller spatial scale compared with most other studies, the relatively low F_{ST} values are to be expected.

Inferring population bottlenecks

The evidence for recent population bottlenecks within $0.2N_e$ to $0.4N_e$ generations was quite strong with the IAM. Under the IAM, results from the Wilcoxon sign-rank test revealed a significant excess of heterozygotes, which is a common sign of a recent bottleneck. This is because rare alleles are most likely to be removed by drift and consequently UH_e is only affected to a limited extent (Cornuet and Luikart, 1996). However, as previously mentioned, these results should be interpreted with caution as no evidence for bottleneck was found for the more realistic TPM model. The evidence for bottlenecks is, however, supported also by the tests for differences in the mean and median of A_E, which showed a reduction of genetic variability over time in the populations from the isle of Funen. However, the small sample size of FUN2 weakens the conclusions that can be drawn from the results of the tests. In theory, the effects of repeated bottlenecks will greatly reduce the expected level of heterozygosity of the population (Motro and Thomson, 1982), and in our study levels of H_e were low, supporting the hypothesis that the Danish little owl population has undergone several bottlenecks.

Ice sheets that covered northern and Central Europe until the last glacial maximum (~18,000 years ago) could be responsible for the bottlenecks detected in the two oldest populations (JUT1 and FUN1). In most parts of Europe, cold periods were followed by two extremely dry climatic cycles 13,000 to 10,000 years ago [the Older and the Younger Dryas (Starkel, 1991)]. During those periods, permafrost and dry steppe habitat conditions could have been critical for the little owl populations throughout Northern and Central Europe. Post-glacial founder events and re-colonization of Northern Europe after the last glacial maximum, or more recent population declines during the early Holocene in Central Europe, might have led to population and genetic bottlenecks. The last glacial maximum has also been suggested to have shaped the genetic variability and structure of the Eurasian otter (*Lutra lutra*) (Pertoldi *et al.*, 2001).

Genetic diversification and conservation strategies for the little owl in Denmark

This study provides a first description of the genetic structure of little owl populations in Denmark. The data presented here indicate that the little owl populations do suffer from a loss of genetic variability. However, historical population declines of different orders of magnitude could have occurred on a time-scale of thousands of years, probably in relation to late glacial or post-glacial habitat changes. It remains to be determined whether the moderately low level of genetic variability described in this study can partly explain the low reproductive fitness observed in contemporary populations of little owls in Denmark.

Based on the fragmented subpopulations present in Jutland, our data reveal relatively low F_{ST} values; this, together with the rather homogenous habitats in Denmark suggest that a transfer of genetic material between remaining populations in Jutland or a reintroduction to Funen is unproblematic from an outbreeding depression perspective. Corridors enabling birds from Germany to make contact with the Danish population would also help to limit further loss of genetic variation. The establishment of a captive breeding programme and release of birds bred in captivity might also be a viable option to increase the size and thereby the long-term survival of the Danish population of little owls (L.H. Andersen, V. Loeschcke

and C. Pertoldi, unpublished). For such strategies to be successful, an important prerequisite is the availability of and connectivity between patches of suitable habitat. Current suitable habitats for the little owl in Denmark are limited in both number and size.

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