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Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline

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Most of the white-tailed eagle (*Haliaeetus albicilla*) populations in Europe experienced dramatic declines during the twentieth century. However, owing to intense conservation actions and the ban of DDT and other persistent pollutants, populations are currently recovering. We show that despite passing through demographic bottlenecks, white-tailed eagle populations have retained significant levels of genetic diversity. Both genetic and ringing data indicate that migration between populations has not been a major factor for the maintenance of genetic variability. We argue that the long generation time of eagles has acted as an intrinsic buffer against loss of genetic diversity, leading to a shorter effective time of the experienced bottleneck. Notably, conservation actions taken in several small sub-populations have ensured the preservation of a larger proportion of the total genetic diversity than if conservation had focused on the population stronghold in Norway. For conservation programmes targeting other endangered, long-lived species, our results highlight the possibility for local retention of high genetic diversity in isolated remnant populations.

Keywords: bottleneck; conservation genetics; generation time; microsatellites; mitochondrial DNA; single large or several small

1. INTRODUCTION

The white-tailed eagle (*Haliaeetus albicilla*) is used as a major flagship and umbrella species for conservation work throughout large parts of Europe. The species was once widely distributed across the continent (Helander & Stjernberg 2003), but direct persecution and low reproductive success as a result of environmental pollutants led to dramatic declines during the twentieth century (Bijleveld 1974; Helander *et al.* 2002). By the 1970s, many local populations in Europe had gone extinct and most surviving sub-populations barely reached one or a few tens of breeding pairs (figure 1). However, a stronghold remained along the Norwegian Atlantic coast with at least 700–800 breeding pairs (Folkestad 2003), corresponding to more than 70% of the northern and central European population (Helander *et al.* 2003). The population along the Norwegian coast utilized relatively uncontaminated food resources from the northern Atlantic and was thus largely saved from the detrimental effects of harmful pollutants (Helander *et al.* 1982). On the other hand, around the heavily polluted Baltic Sea, white-tailed eagles suffered strongly reduced breeding success (Helander *et al.* 1982).

With the ban in the 1970s of DDT, PCB and other detrimental chemicals, European white-tailed eagle populations soon started to recover, a process that is still ongoing (figure 1). For instance, the number of territorial pairs in southern and central Sweden increased from about 40 in 1974 to 250 in 2000 (Helander 2003*a*), and in Estonia from 10–15 between 1965 and 1975 to 116 in 2002 (Volke & Randla 2003). This population expansion appears to have been based mainly on local recruitment, since almost no inter-population exchange of breeders has been recorded from ringing data. In particular, the large Norwegian population does not seem to have acted as a major founding source for recovering populations; none of the more than 3000 eagles colour-ringed in Norway has so far been found breeding outside Norway (Folkestad unpublished data). Further, among more than 150 ringed breeding birds in Sweden, all but a few individuals from the nearby Finnish coast were offspring of resident pairs (Helander 2003*b*). Similarly, most of the few recorded emigrants from the German eagle population settled to breed nearby in Poland (Köppen 2003). Given this apparent trend of local recruitment, current populations might have been depleted of genetic diversity by the recent demographic declines.

Loss of genetic diversity can reduce both short-term viability (e.g. Saccheri *et al.* 1998) and long-term adaptability (Frankham 2005). To assess the genetic impact of population declines during the twentieth century, we therefore studied the genetic variability at mitochondrial DNA (mtDNA) control region sequences and autosomal microsatellite markers in north and central European white-tailed eagle populations.

2. MATERIAL AND METHODS

Several populations from northern and central Europe were sampled, yielding a total number of 218 presumably unrelated individuals: Estonia (EST, $n=13$ samples), Germany (GER, $n=18$), Swedish Lapland (LAP, $n=24$), southern and central Sweden (SWE, $n=117$), Kola peninsula, Russia (KOLA, $n=10$) and Norway (NOR, $n=36$). Most of the samples consisted of blood, but nine samples were moulted feathers that had been collected in the field.

Polymerase chain reaction (PCR) of 26 autosomal microsatellite loci was performed (details are given in Hailer *et al.* (2005) and the electronic supplementary material). In order to directly compare

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the amount of genetic variation (expected and observed heterozygosity, H_E and H_O, and the mean number of alleles per locus, N_A) between population samples, we standardized for sample size by employing a bootstrapping procedure (see electronic supplementary material). We tested for deviation from Hardy–Weinberg equilibrium (HWE) and genotypic differentiation using GENEPOP 3.4 (Raymond & Rousset 1995). GENETIX 4.05.2 (Belkhir *et al.* 2004) was used to calculate F_{ST} and its 95% confidence interval. Assignment tests were carried out using GENECLASS 2.0.d (Piry *et al.* 2004) and demographic simulations were performed in BOTTLESIM (Kuo & Janzen 2004). For the latter, population size was held constant at 300 actively reproducing pairs for 10 years, followed by a bottleneck of 30 reproducing pairs for 40 years (see electronic supplementary material for details). The program BOTTLENECK (Cornuet & Luikart 1996) was used to detect possible traces of population bottlenecks on the allele frequency spectrum, applying the recommended settings and tests for microsatellites (two-phased model: 90% one-step mutations and 10% multi-step changes; evaluation with standardized differences and Wilcoxon tests).

A part of the mtDNA control region was amplified and sequenced using the primers *HalHVR1F* (5′-CCCCCCCTATG-TATTATTGT-3′) and *HalHVR1R* (5′-TCTCAGTGAAGAGC-GAGAGA-3′) developed for *H. albicilla*, yielding a total of 500 bp for analysis. Similar to the microsatellite analyses, we used a bootstrapping procedure to correct diversity estimates for sample size (see the electronic supplementary material). DnaSP 4.10 (Rozas *et al.* 2003) was used to determine Tajima's D (Tajima 1989) and G_{ST}, an estimator of genetic differentiation among populations, using haplotype identity as the underlying statistic. Sequences obtained have been placed in the EMBL database (accession numbers AM156933–AM156934 and AM156936–AM156943).

3. RESULTS

A total of 139 alleles were observed across the 26 microsatellite markers. Significant heterozygote deficit was detected in the SWE, LAP and NOR populations. However, the signal disappeared when the locus *Hal 10* was excluded; we take this as an indication that this locus exhibits a null allele with restricted geographical distribution and that the populations otherwise do not deviate significantly from HWE.

Observed and expected heterozygosities estimated from data corrected for inter-population variation in sample size were in the range of 0.41–0.51 for all populations (table 1). Similarly, the mean number of alleles per locus did not vary much among populations, being in the range of 2.9–3.5. Importantly, there was no evidence for the large Norwegian population to show higher levels of genetic diversity ($H_E=0.48$, $H_O=0.49$, $N_A=3.1$), as compared to the other populations that had experienced a significant demographic bottleneck. Further, no population showed significant allele frequency shifts ($p>0.05$) which are expected after strong demographic bottlenecks.

One possible explanation for the homogeneous levels of genetic diversity among the populations could be that gene flow had passed unnoticed in the field surveys. Consistent with this idea, overall genetic differentiation was low ($F_{ST}=0.036$). However, F_{ST} was significantly different from zero (95% confidence intervals 0.029–0.044) and the test of genotypic differentiation found that all populations were significantly differentiated from each other ($p<0.01$ in all pairwise comparisons). Furthermore, an assignment test also indicated that populations were distinct units: despite the low overall F_{ST} value, about 80% of the analysed nestlings assigned to their natal population. Thirty-seven alleles found in other European populations were not observed in Norway, while only three of the Norwegian alleles were not found in the rest of the Europe.

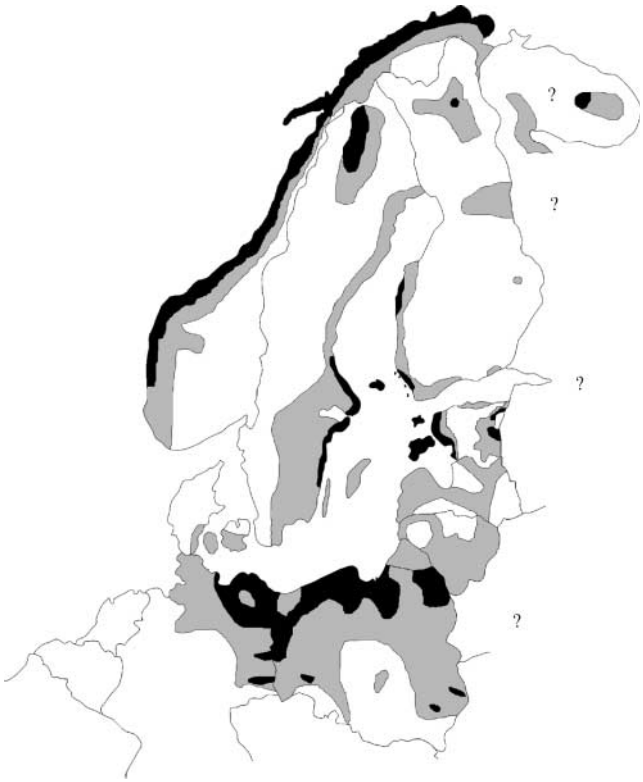

Figure 1. Distribution of the white-tailed eagle in north–central Europe. Black shading indicates regions where the species survived the mid-twentieth century population crash, light grey shading refers to regions re-colonized in the phase of population recovery since 1980.

Ten different mtDNA haplotypes were observed. Tajima's D was 1.522, but differed non-significantly from zero ($p>0.05$), compatible with neutral evolution of the DNA sequences. Haplotype frequencies differed significantly among populations, reflected by an overall G_{ST} of 0.228. The stronger population differentiation in mtDNA than nuclear markers probably reflects differences in effective population size between the marker types. Higher dispersal rates in males than in females are uncommon in birds (Greenwood 1980), and not supported by empirical data in white-tailed eagles (Helander 2003*b*).

The corrected number of haplotypes per population varied between 1.3 (Norway) and 7 (Kola peninsula; table 2), again indicating that bottlenecked populations do not contain lower levels of genetic diversity as compared to the Norwegian population. Further, our results confirm that the European populations could not have been derived largely from the coastal Norwegian population: only two mtDNA haplotypes were observed in Norway, one haplotype being very frequent (97%).

4. DISCUSSION

In summary, our data suggest that effective dispersal between populations has not been common during the past decades. We conclude that sustained genetic

Table 1. Genetic diversity at microsatellite loci. Number of samples analysed, unbiased expected (H_E) and observed (H_O) heterozygosity, mean number of alleles per locus (N_A) and their inter-locus standard deviations. For comparison between populations, standardized diversity values are also given.

population	sample size	H_E (s.d.)	corrected H_E	H_O (s.d.)	corrected H_O	N_A (s.d.)	corrected N_A
Norway	36	0.49 (0.05)	0.48	0.49 (0.02)	0.49	3.9 (2.3)	3.1
Estonia	13	0.49 (0.05)	0.48	0.50 (0.03)	0.50	3.5 (1.7)	3.1
Germany	18	0.43 (0.05)	0.42	0.42 (0.02)	0.41	3.4 (1.7)	2.9
Kola peninsula[a]	10	0.52 (0.05)	—	0.51 (0.03)	—	3.8 (2.0)	—
Lapland	24	0.51 (0.05)	0.49	0.49 (0.02)	0.49	4.2 (2.3)	3.4
mid and southern Sweden	117	0.51 (0.05)	0.51	0.50 (0.01)	0.51	4.8 (2.2)	3.5

[a]diversity statistics of the remaining populations were standardized to sample size of $n=10$, corresponding to the number for Kola peninsula.

Table 2. Genetic diversity at mitochondrial control region sequences. The number of analysed eagle territories (maternal lineages) is denoted by n, and N denotes the number of unique haplotypes found in each population. To correct for unequal sample sizes among populations, the mean number of alleles encountered in 100 bootstrap samples of 10 individuals each, and the standard deviation of these values, is presented.

population	n	N	corrected N (s.d.)
Norway	33	2	1.3 (0.5)
Estonia	12	5	4.0 (0.7)
Germany	18	3	2.6 (0.5)
Kola peninsula[a]	10	7	—
Lapland	22	6	3.7 (1.0)
mid and southern Sweden	44	4	2.9 (0.6)

[a]diversity statistics of the remaining populations were standardized to sample size of $n=10$, corresponding to the number for Kola peninsula.

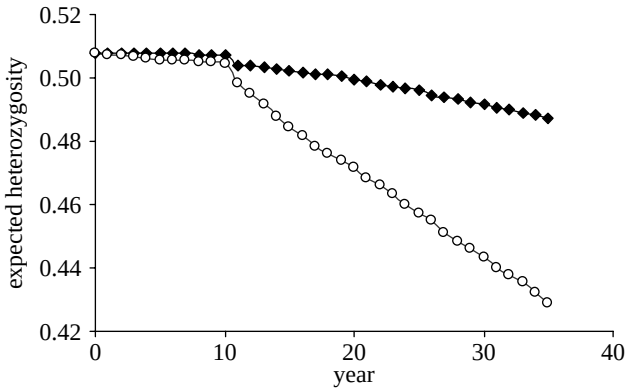

Figure 2. Loss of genetic diversity (as measured by expected heterozygosity) in a bottleneck of 30 reproducing pairs and overlapping generations. Filled diamonds and open circles consider an individual lifespan of 17 years and 1 year, respectively. The simulation was started at a population size corresponding to 300 reproducing pairs (years 0–10).

diversity in extant north–central European white-tailed eagle populations has a predominantly local origin, although some immigration may have contributed to local gene pools. We suggest that the long generation time of white-tailed eagles (average life span is around 17 years; Helander 2003*b*; Struwe-Juhl 2003) has minimized genetic drift and acted as an intrinsic buffer against rapid loss of genetic diversity. When a randomly mating population passes through a bottleneck, reduction of genetic diversity depends on population size and time according to $H_t=H_0(1-1/(2N_e))^t$; H_0 and H_t being the past and present heterozygosity, respectively, N_e the effective population size and t the time measured in number of generations. A bottleneck lasting about 20–30 years is equivalent to about two white-tailed eagle generations. Simulation of a bottleneck comprising 30 reproductive pairs (which corresponds to the situation in several European populations during the 1970s; see §1) shows that only about 4% of the original heterozygosity is expected to be lost in an eagle population during that time (figure 2). By contrast, for an organism with a generation time of 1 year, the corresponding loss of genetic diversity is considerably higher (16%; figure 2). Our results are compatible with the relatively high levels of genetic diversity seen in remnant and bottlenecked populations of other long-lived species, e.g. black rhinoceros (Swart *et al.* 1994) and greater one-horned rhinoceros (Dinerstein & McCracken 1990).

Starting in the late 1960s, several countries in Europe developed conservation programmes to protect eagles (Helander 1990). Nest site protection enabled fertile pairs to raise fledging young. Carcasses free of contaminants were laid out in winter and, as a result, juvenile survival was increased to 80% (Helander 2003*b*). Our results, therefore, indicate that these conservation efforts have led to the preservation of ample genetic diversity in local eagle populations. Moreover, conservation of eagles at local scales has resulted in the preservation of more total genetic diversity than if all conservation efforts had been centred on the coastal Norwegian population.

There has been an intense debate among conservation biologists about reserve design (the SLOSS debate—single large or several small; reviewed in Kingsland 2002). This debate was directly linked to the relative importance of local conservation versus a focus on population strongholds. Our results illustrate the high conservation value of local populations for species with a long generation time. A number of other species of conservation concern also have long generation times (e.g. raptors, turtles, large mammals and orchids) and for many of these significant genetic diversity may still exist in local populations. For the white-tailed eagle, protection of the local populations did not arrive too late.

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Accompanying information to the article by Hailer *et al.* (2006) in *Biology Letters*

"Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline"

Material and methods

Sample collection and DNA extraction

Blood samples were taken in the field from the brachial vein, immediately buffered in EDTA/SSC buffer and kept frozen until treatment in the lab. DNA extraction followed a standard protocol involving digestion with proteinase K and extraction with phenol-chloroform (Sambrook *et al.* 1989). Additionally, some naturally shed feathers were collected in the field. DNA from those was extracted using the DNeasy Tissue Kit (Qiagen) following the recommendations of Horváth *et al.* (2005). Our sampling within populations aimed at maximising the number of different territories, but never picking more than one sample per territory. We thus obtained a data set of presumably unrelated individuals, at least in the current generation. Spatially, our sampling in Estonia covers both the eastern and western distribution range, in Germany both northern and southern regions (Mecklenburg-Vorpommern and Brandenburg), in Sweden basically the entire distribution range, and the Norwegian samples stem from four different regions along the Atlantic coast (Møre-Romsdal, Sør-Trøndelag, Nord-Trøndelag and Troms).

PCR amplification and analysis of microsatellite markers

Fourteen loci cloned from the white-tailed eagle (*Hal 01* to *Hal 10* and *Hal 12* to *Hal 15*) were genotyped as described in Hailer *et al.* (2005). Additionally, 12 microsatellite markers developed for other raptor species were analysed in five multiplex reactions: *Aa35* (Martinez-Cruz *et al.* 2003), *Hle0B06*, *Hle0B10*, *Hle6A09*, *Hle6H10*, *Hle0E05*, *Hle0E12*, *Hle6F02*, *Hvo59* (Tingay *et al., in prep.)*, *IEAAAG04*, *IEAAAG05*, *IEAAAG14* (Busch *et al.* 2005). PCR reactions of the latter markers were performed in reaction volumes of 10 µL containing 10 ng of genomic DNA, 0.2 mM of each dNTP, 0.125-0.8 µM (see table S1) of each forward and reverse primer (one of them fluorescently labelled), 0.4 units of HotStarTaq DNA polymerase (Qiagen) and 1 µl of 10x HotStarTaq (Qiagen) reaction buffer containing Tris-Cl, KCl, $(NH_4)_2SO_4$ and a final concentration of 1.5 mM $MgCl_2$. We used the following PCR programme on a PTC-225 machine (MJ Research): 35 cycles with 95 °C for 30 sec., a locus-specific annealing temperature (see Table S1) for 30 sec., and 72 °C for 30 sec. Before the first

cycle, a prolonged denaturation step (95 °C for 15 min.) was included and the last cycle was followed by an additional annealing step at the corresponding annealing temperature for one minute and a final extension step for 8 min. at 72 °C.

PCR products were run on a MegaBACE 1000 capillary sequencer (Amersham Biosciences) and analyzed using the software GENETIC PROFILER 2.0. The MICROSATELLITE TOOLKIT for Excel (Park 2001) was used to calculate Nei's unbiased estimate of expected heterozygosity (Nei 1978), observed heterozygosity and mean number of alleles per locus. Deviation from Hardy-Weinberg equilibrium (HWE) was tested globally and separately for each locus in each population using the exact test implemented in GENEPOP 3.4 (Raymond and Rousset 1995). Theta, an estimator of F_{ST} (Weir & Cockerham 1984) was calculated using GENETIX (Belkhir *et al.* 2004) and its 95% confidence intervals were estimated by bootstrapping across loci 1000 times. Assignment tests were carried out using GENECLASS 2.0.d (Piry *et al*. 2004), employing the frequency-based method described in Rannala and Mountain (1997). As input data for the starting point of the demographic simulations in BOTTLESIM, we used all 26 loci from the Swedish (SWE) population. Simulations were performed assuming a life span of 17 years, sexual maturity at 5 years of age and fully overlapping generations (Helander 2003; Struwe-Juhl 2003). In BOTTLESIM, fertility and survival rates are held constant across each individual's life span.

Amplification and analysis of mtDNA control region sequences

We designed primers flanking the mitochondrial DNA control region using the published sequence of the common buzzard (*Buteo buteo*, GenBank accession number NC 003128). Primers *Bbu14834F* (5'-GGTCTTGTAAACCAAAAACTGAAGGC-3') and *Bbu16634R* (5'-CGGTTTAGGGGAGTCAGAGAGTAGTTTAA-3') were initially used to amplify the complete mtDNA control region in a few individuals of different geographic origin. Next, an especially variable 544 bp region was targeted by designing interior primers specific to *H. albicilla*: *HalHVR1F* (5'-CCCCCCCTATGTATTATTGT-3') and *HalHVR1R* (5'-TCTCAGTGAAGAGCGAGAGA-3'). PCR reactions were carried out in 10 µl volumes containing approximately 15 ng of genomic DNA, 0.3 µM of each primer, 0.2 mM of each dNTP, 0.25 units of HotStarTaq DNA polymerase (Qiagen) and 1 µl of 10x HotStarTaq (Qiagen) reaction buffer containing Tris-Cl, KCl, $(NH_4)_2SO_4$ and a final concentration of 1.5 mM $MgCl_2$. PCR was performed in a PTC-225 instrument with the following treatment: 15 min. at 95 ºC prior to 36 cycles of 30 sec. at 56 ºC, 30 sec. at 72 ºC and 30 sec. at 95 ºC. Finally, a 1 min. step at 56 ºC and an extension step of 10 min. at 72 ºC was performed. PCR

products were cleaned using the ExoSAP enzyme kit (Amersham Biosciences) and DNA sequencing was performed on both strands using the original PCR primers and the DYEnamic ET Terminator kit (Amersham Biosciences). Sequencing reactions were cleaned using AutoSeq plates (Amersham Biosciences) and run on a MegaBACE 1000 capillary instrument according to the manufacturer's recommendations. Electropherograms were checked manually and assembled in Sequencher 4.1.4 (Gene Codes). After removal of primer sequences and some additional bases close to the primers, this yielded a 500 bp fragment for analysis.

Bootstrap resampling to standardize estimates of genetic diversity for sample size

For each population, we used the Excel macro POPTOOLS (Hood 2005) to randomly resample individuals with replacement, creating 100 synthetic populations of equal size: 10 individuals for both the microsatellite and mtDNA analysis, corresponding to the number of samples in the smallest population sample (Kola peninsula). Then, for the microsatellite data, the macro MICROSATELLITE TOOLKIT for Excel (Park 2001) was used to calculate the unbiased expected heterozygosity, observed heterozygosity and mean number of alleles per locus for each of the synthetic populations. The average of these 100 values is given in Table 1. In order to calculate the corrected number of mitochondrial DNA (mtDNA) haplotypes, basically the same procedure was used. After bootstrap resampling of individuals, each haplotype was coded as a number and the data was then analyzed as haploid genotype data using MICROSATELLITE TOOLKIT. From that output, we determined the number of haplotypes per synthetic population by counting all haplotypes with a frequency larger than zero. The average number of haplotypes among the 100 replicates is given in Table 1.

Distribution map

Information regarding the distribution of white-tailed eagles was obtained from Folkestad *(unpublished data)* and Ganusevich *(unpublished data)*, Hauff (1998), Hauff *(unpublished data)*, Helander *et al.* (2003), Mizera (2002), Randla (1976) and Stjernberg *(personal communication)*.

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Table S1: Multiplex assays for 12 microsatellites cross-species amplified in the white-tailed eagle.

Multiplex	annealing temperature (°C)	marker	concentration (μM)
1	52	*Aa35*(F)	0.35
		Aa35(R)	0.35
		Hle0B06(F)	0.30
		Hle0B06(R)	0.30
		Hle0B10(F)	0.55
		Hle0B10(R)	0.55
2	52	*Hle6A09*(R)	0.35
		Hle6A09(F)	0.35
		Hle6H10(F)	0.45
		Hle6H10(R)	0.45
3	52	*Hle0E12*(F)	0.65
		Hle0E12(R)	0.65
		HleE05(F)	0.80
		HleE05(R)	0.80
4	54	*Hvo59*(F)	0.50
		Hvo59(R)	0.50
		Hle6F02(F)	0.50
		Hle6F02(R)	0.50
5	56	IEAAA*G04*(F)	0.125
		IEAAA*G04*(R)	0.125
		IEAAA*G05*(F)	0.125
		IEAAA*G05*(R)	0.125
		IEAAA*G14*(F)	0.50
		IEAAA*G14*(R)	0.50