Genetic impoverishment of the last black grouse (*Tetrao tetrix*) population in the Netherlands: detectable only with a reference from the past

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Abstract

We have studied a small isolated population of black grouse (*Tetrao tetrix*) in the Netherlands to examine the impact of isolation and reduction in numbers on genetic diversity. We compared the genetic diversity in the last extant Dutch population with Dutch museum samples and three other black grouse populations (from England, Austria and Norway, respectively) representing isolated and continuous populations. We found significantly lower allelic richness, observed and expected heterozygosities in the present Dutch population compared to the continuous populations (Austria and Norway) and also to the historical Dutch population. However, using a bottleneck test on each population, signs of heterozygosity excess were only found in the likewise isolated English population despite that strong genetic drift was evident in the present Dutch population in comparison to the reference populations, as assessed both in pairwise $F_{ST}$ and structure analyses. Simulating the effect of a population reduction on the Dutch population from 1948 onwards, using census data and with the Dutch museum samples as a model for the genetic diversity in the initial population, revealed that the loss in number of alleles and observed heterozygosity was according to genetic drift expectations and within the standard error range of the present Dutch population. Thus, the effect of the strong decline in the number of grouse on genetic diversity was only detectable when using a reference from the past. The lack of evidence for a population reduction in the present Dutch population by using the program **BOTTLENECK** was attributed to a rapidly found new equilibrium as a consequence of a very small effective population size.

Keywords: black grouse, bottleneck, conservation biology, genetic variation, historical DNA, microsatellites, museum samples

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Introduction

Genetic diversity of populations is negatively affected by human-related activities such as habitat destruction and fragmentation that has caused previously continuous populations to become fragmented and reduced in size (Frankham *et al*. 2002). Small populations will lose genetic variation as a result of increased genetic drift (Gilpin & Soulé 1986; Soulé 1987). Not only genetic drift increases in fragmented populations but the frequency of consanguineous mating may also increase, leading to a loss of fitness and adaptive potential (Westemeier *et al*. 1998; Madsen *et al*. 1999). This in turn will lead to yet lowered population sizes and the population may enter an extinction vortex (Gilpin & Soulé 1986; Lande 1988; Vrijenhoek 1994; Frankham 1996; Hedrick & Kalinowski 2000; Srikwan & Woodruff 2000).

It has recently been suggested that the impacts on genetic diversity of a population size reduction can be...
estimated from data in extant populations by examining patterns of heterozygosity excess and observed allele frequencies without knowledge of the genetic variation in the past (Cornuet & Luikart 1996; Piry et al. 1999). By examining the difference between the expected heterozygosity under Hardy–Weinberg equilibrium ($H_E$) and the heterozygosity expected at mutation-drift equilibrium ($H_{eq}$), inferences about losses of genetic variation can be made. In populations that have not been reduced in numbers and that are near mutation-drift equilibrium, $H_{eq}$ will equal $H_E$ (Luikart & Cornuet 1998). Since alleles are lost more rapidly than heterozygosity during a population size reduction the effect will be a heterozygosity excess (higher $H_E$) in reduced populations (Piry et al. 1999). Threatened species are rarely assayed continuously during population size reductions for genetic diversity. Thus, the method implemented in BOTTLENECK has appeal since it allows detection of loss of genetic variation by only requiring on a single ‘snap-shot’ point estimate. However, although theory predicts that a new mutation-drift equilibrium may be set rapidly when effective population size becomes low (Watterson 1984), there are, to our knowledge, no empirical studies on under what circumstances the ‘snap-shot’ approach may fail to detect loss of genetic variation.

Black grouse (*Tetrao tetrix*) are widespread in their northern and eastern distribution, but there is a major conservation concern in Western and Central Europe. Small population size and isolation appear to significantly limit the chances for survival and recovery in temperate Europe (Storch 2000). In the Netherlands, a dramatic decline in black grouse numbers took place in the last 60 years (Fig. 1). From the original population of 5000–8000 displaying cocks before 1940, no more than 30 cocks divided among less than 10 populations were found around 1990. Currently, only the population on the Sallandse Heuvelrug exists, with numbers of displaying cocks fluctuating between nine and 32 over the last 20 years (Jansman et al. 2004). The average dispersal range for black grouse is 5 km and 30 km for cocks and hens, respectively (Caizergues & Ellison 2002). The nearest populations at the Hoge Venen (Belgium) and the Lüneburger Heide (Germany) are located more than 200 km from the Sallandse Heuvelrug, and out of the dispersal range of the species. The Dutch black grouse population can thus be considered as extremely small and isolated. Conservation measures have been taken to improve the habitat and to control predators. The population is now more or less stable at a low population size (average number of displaying cocks since 1996 is 17), but still not growing (Niewold et al. 2003). Some hypotheses for this lack of growth are disturbance by an increasing recreation pressure, effects of climate change (especially humid winters), and loss of genetic diversity and/or inbreeding depression. As a result of the small population size in combination with the isolated situation, it is expected that due to genetic drift, the genetic variability has been reduced.

The aim of this study was to evaluate the magnitude of genetic variation and the effect of genetic drift in selectively neutral genetic markers (microsatellites) in this small and up until recently declining population. We present data on microsatellite variation in the current Dutch black grouse population in relation to a number of reference populations. To estimate the genetic composition of the historically more or less continuous Dutch black grouse population in the Netherlands, samples from stuffed birds in a museum collection was used. As current reference populations with large and more or less thriving populations, samples from Scandinavia and the Alps were included. Also, an isolated population from England, representing the outermost western distribution of the black grouse, was included.

We focused on the following issues. (i) Is it possible to detect a reduction in genetic variation following a reduction in numbers in the Dutch population only using data from the present population as suggested by Cornuet & Luikart (1996) and as implemented in the software program BOTTLENECK? (ii) Is the Dutch population genetically depauperate as a result of the small population size in combination with isolation when compared with the continuous Dutch population in the last century? (iii) Is there genetic differentiation and differences in genetic diversity between the Dutch population and the Austrian, Norwegian and English population samples?

**Methods**

**Collection, DNA extraction, amplification and visualization**

Between 1991 and 2005, feathers, eggshells and tissue samples from 165 individuals were sampled as a source for
genomic DNA from the Netherlands (52°20’N, 6°24’E),
Austria (46°40’N, 14°0’E), Norway (58°2’N, 7°41’E) and
England (54°31’N, 2°4’E) (Table 1). The current wild Dutch
samples (collected 2003–2005) originate all from the
Sallandse Heuvelrug population; the last remaining
population in the Netherlands. The Austrian samples com-
prised a local alpine population in the Tauern mountains.
The English samples were all collected from a population
in the Northern Pennines. The Norwegian samples origin-
ated from southern Norway. Moulted feathers or feathers
from carcasses were collected, put individually in envelopes
or plastic bags and stored dry at room temperature for
1–20 months until analysis. Most samples were freshly
moulted feathers collected in the field during the summer
months; other feathers came from carcasses killed by
raptors and birds killed by hunters. Genomic DNA was
extracted from about a 1-cm segment at the root end of the
feathers using the DNeasy Tissue Kit (QIAGEN) as described
by Segelbacher (2002). Blood was stored in EDTA buffer
and muscles were stored deep-frozen and DNA was
extracted similarly or with a standard phenol/chloroform
extraction. Thirty samples from stuffed black grouses,
collected between 1893 and 1941 throughout the Nether-
lands, stored in Naturalis (Dutch Natural History Museum
in Leiden), were taken as a reference for the historical
genetic make-up of the Dutch population. During this period
of time, the black grouse population of the Netherlands
was more or less continuous. From the stuffed birds, a toe
scrape of ~3 × 3 mm² was taken as a source of DNA and
treated similarly to the other samples.

Individual samples were genotyped at 10 tetranucle-
etide microsatellite loci (Tut1, Tut2, Tut3, Tut4, BG10,
BG15, BG16, BG18, BG19, BG20). Polymerase chain reaction
(PCR) amplifications were conducted as described in
PCR fragments were resolved by electrophoresis by
labelling the primers with fluorescent dye and running
the fragments on an ABI 377 genetic analyser or a LICOR
4200 DNA analyser. To detect whether contamination with
exogenous DNA or PCR products had occurred, tubes
without samples were included in the DNA extraction and
PCR amplification procedure as negative controls. Ampli-
fication of the cloned locus aided in size determination and
also served as a positive control. To avoid contamination,
DNA extractions, pre- and post-PCR pipetting were carried
out in different rooms and aerosol-resistant filter pipette
tips were used throughout.

Genetic analyses

To obtain standard estimates of genetic diversity within
and between sample sites, we used genotype and allele
frequencies of the microsatellite loci. Genetic variation in
each population was assessed by calculating the mean
number of alleles, observed heterozygosity \((H_o)\), expected heterozygosity \((H_e)\) and \(F_{IS}\), i.e. deviance from Hardy–Weinberg equilibrium, using the microsatellite toolkit 3.1 (Park 2001) and genetix 4.02 (Belkhir et al. 2000) software. The genotypic match function in the microsatellite toolkit was used to identify samples of putatively the same origin which were then considered as one sample in further analyses. The program micro-checker (van Oosterhout et al. 2004) was used to check for allelic dropouts and null alleles and genepop 3.4 (Raymond & Rousset 1995) was used to check for linkage disequilibrium and genetic differentiation \((F_{ST})\) between populations. The pairwise population differentiation estimates were bootstrapped 15 000 times over all loci to get a 95% confidence interval in fstat 2.93 (Goudet 2001) for pairwise \(F_{ST}\) tests (Weir & Cockerham 1984). Allelic richness, which corrects the number of alleles for sample size, was assessed using fstat 2.93 (Goudet 2001), and a principal coordinate analysis (PCA) on the codominant genetic distances between individuals as described by Smouse & Peakall (1999) was made in genalex (Peakall & Smouse 2005).

We obtained an estimate of the number of independent genetic clusters in our data set using the program structure 2.1 (Pritchard et al. 2000). This approach does not require a priori information about population structure, and thus, provides an estimate of genetic structure independent of the origin of samples. We used the no-admixture algorithm without prior population information and used 50 000 runs as burn-in and 500 000 runs for each of three Markov chains. For each simulation of \(k = 1–10\) (no. of clusters), we used 10 replicates. We chose the most likely number of clusters given the data by choosing the number of clusters where we observed the largest difference in log likelihoods (\(\Delta K\), Evanno et al. 2005).

To check for possible genetic footprints of a recent decline in the Dutch present population, we ran the program bottleneck (Piry et al. 1999). This software tests for heterozygosity excess based on the theoretical expectation of a more rapid loss of alleles than heterozygosity in declining populations (cf. Cornuet & Luikart 1996) which was examined under the infinite alleles model (IAM), stepwise mutation model (SMM) and two-phased model (TPM) of mutation, with TPM characteristics set as suggested by Piry et al. (1999) (SMM, 95% with a variation of 12).

To examine whether the loss of genetic variation could be explained by genetic drift and since we had access to historic samples, we simulated the changes over generations in allele numbers and \(H_o\) based on the variation in the Dutch museum samples, using the program bottlesim (Kuo & Janzen 2003). Census numbers of black grouse cocks in subpopulations in the Netherlands every 10th year from 1945 to 1975 and then every year until the year 2007 were used to estimate the number of breeding black grouse during 52 years (1948–2000). In the simulation, we assumed that in every subpopulation in the Netherlands, there would be a lek for every 10 black grouse cocks, and that on average, two males would breed on each lek (except when the census was one male) in order to get the effective male population size (Alatalo et al. 1992). The black grouse hen number was considered to be equal to the male census size. The simulation parameters were set to random mating and generation overlap with 1 year to maturity and an average lifespan of 3 years (Hjorth 1970). The random mating assumption is unrealistic in this lekking species; thus, we adjusted the effective male population size to fit the assumptions of two(one) males mating per lek. To tests for the robustness of the results of these simulations, we run additional simulations with varying assumptions of sex ratio (from all males mating to the above assumption) and generation time (2–5 years).

**Results**

**Present and past genetic variation**

Black grouse numbers in the Netherlands have been reduced drastically since the 1940s both when considering number of displaying males (cocks) and number of areas used (Fig. 1). Among the microsatellite loci used in the study, the loci Tut1 and BG10 were removed from further analyses, because of repeated evidence of null alleles. We detected no significant deviations from linkage equilibrium among the remaining loci within populations. The present Dutch population showed signs of genetic impoverishment in comparison with the Dutch museum samples (Table 1). Both observed and expected heterozygosities were significantly higher in the museum sample (paired \(t\)-tests, \(t = 2.84, d.f. = 7, P < 0.05\) and \(t = 3.36, d.f. = 7, P < 0.01\), respectively) as was allelic richness (paired \(t\)-test, \(t = 4.39, d.f. = 7, P < 0.005\)). The present Dutch population also deviated from the observed heterozygosities in the putatively larger populations in Norway and Austria (paired \(t\)-test, \(t = 3.93, d.f. = 6, P < 0.01\) and \(t = 6.86, d.f. = 7, P < 0.001\), respectively). There was no difference in genetic diversity \((H_e)\) (paired \(t\)-test, \(t = 0.24, d.f. = 7, P < 0.82\)) or allelic richness (paired \(t\)-test, \(t = 0.97, d.f. = 7, P < 0.365\)) between another isolated and presumably bottlenecked population, England, and the Dutch present population (Table 1). \(F_{IS}\), a putative sign of inbreeding, was however, significantly positive in the English population, but not in the present Dutch or any other population (Table 1).

**Population differentiation**

A principle coordinate analysis (PCA) indicated the existence of four more or less separated clusters (Dutch present, England, Norway and Austria/Dutch historical; Fig. 2). This was confirmed by the structure analysis. The
program STRUCTURE identified four distinct genetic clusters out of the five putative populations (Table 2). The pairwise $F_{ST}$ values showed that all the populations significantly differed from one another (at $P < 0.001$; Table 3). The Dutch museum population was most similar to the Austrian population as indicated by the STRUCTURE analyses and hinted by the pairwise $F_{ST}$ values, although the Austrian population was most similar to the Norwegian population (Table 3). England was the most genetically distant population compared to all others.

### Table 2 Proportion of individuals in each population assigned to one of four clusters detected by the program STRUCTURE

<table>
<thead>
<tr>
<th>Population</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
<th>Cluster 3</th>
<th>Cluster 4</th>
<th>Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch present</td>
<td>0.904</td>
<td>0.053</td>
<td>0.028</td>
<td>0.016</td>
<td>53</td>
</tr>
<tr>
<td>Dutch museum</td>
<td>0.055</td>
<td>0.755</td>
<td>0.169</td>
<td>0.02</td>
<td>30</td>
</tr>
<tr>
<td>Norway</td>
<td>0.016</td>
<td>0.174</td>
<td>0.793</td>
<td>0.017</td>
<td>29</td>
</tr>
<tr>
<td>Austria</td>
<td>0.02</td>
<td>0.331</td>
<td>0.603</td>
<td>0.045</td>
<td>25</td>
</tr>
<tr>
<td>England</td>
<td>0.019</td>
<td>0.054</td>
<td>0.038</td>
<td>0.889</td>
<td>28</td>
</tr>
</tbody>
</table>

**Genetic footprints of a bottleneck**

The tests carried out in BOTTLENECK did not reveal any evidence of lost genetic diversity for the present Dutch population except under the infinite alleles model ($P < 0.01$). Under the stepwise mutation model and the two-phased model, none of the loci were significantly different from expected under mutation-drift equilibrium, regardless whether the standardized differences test or a Wilcoxon test was used to test for a loss of alleles. Furthermore, there were no signs of mode shifts, since allele frequencies showed a normal L-shaped distribution. The Dutch museum samples showed significant excess of heterozygosity ($H_{O} > H_{E}$) under an infinite alleles model as did all the other analysed populations under the IAM model (Dutch museum $P < 0.02$, Norway $P < 0.01$, Tauern $P < 0.02$, England $P < 0.01$).

The locus Tut2 was almost monomorphic, and when using the program BOTTLENECK to obtain confidence limits around the estimates of the loss of genetic variation, we obtained very high bootstrapped variance among loci. This made it impossible to accept or reject a possible reduction of genetic diversity. To get rid of this artefact, we removed the locus Tut2 from this analysis. The new simulation revealed that the number of alleles shown in the present Dutch population was within the 95% confidence intervals of the simulation 14 years before the last census (Fig. 3). The results for $H_{O}$ showed the same pattern and the simulated observed heterozygosity was within the 95% confidence interval of the Dutch present observed heterozygosity beginning in 2001 (Fig. 4).

The results of the simulations were robust for varying assumptions (about generation time and sex ratio) when it comes to loss of alleles, since all our simulations fall within the confidence limits of the observed variation. As might be predicted, the results for heterozygosity were more sensitive. When we assumed a 50:50 sex ratio and random mating, or when we assumed generation time to be 5 years, we did not reach within the observed variation. However, we note that when using biologically more relevant assumptions (shorter generation times and a female biased sex ratio, i.e. not all males mating), we could indeed simulate also the loss of heterozygosity.

### Table 3 Pairwise $F_{ST}$ values between black grouse populations. Ninety-five per cent confidence intervals were determined by 15 000 bootstraps over all loci

<table>
<thead>
<tr>
<th></th>
<th>Dutch museum</th>
<th>Norway</th>
<th>Austria</th>
<th>England</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch present</td>
<td>0.111 (0.062–0.177)</td>
<td>0.16 (0.124–0.193)</td>
<td>0.152 (0.108–0.194)</td>
<td>0.208 (0.083–0.317)</td>
</tr>
<tr>
<td>Dutch museum</td>
<td>0.05 (0.011–0.109)</td>
<td>0.036 (0.006–0.078)</td>
<td>0.138 (0.066–0.228)</td>
<td>0.110 (0.068–0.144)</td>
</tr>
<tr>
<td>Norway</td>
<td>0.031 (0.013–0.05)</td>
<td>0.110 (0.068–0.144)</td>
<td>0.096 (0.060–0.122)</td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td></td>
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</table>
Discussion

The genetic variation of the Dutch present population is consistent with what is seen in other small and isolated populations (Höglund et al. 2007). The structure analysis indicates no contact between the present Dutch population and other European populations. A large part of the genetic structure of Dutch black grouse is thus probably due to increased genetic drift, because the Dutch historical population was almost indistinguishable from the Austrian population which is clearly different from the Dutch present population. The known decline of population size in the Netherlands has probably sped up this process with loss of allelic richness as another consequence. All together, this causes the current Dutch population to stand apart from the other European populations.

When we ran simulations of a potential bottleneck/population crash of the Dutch population using the museum samples as a reference population, the loss of alleles was as expected under strong genetic drift. As previously observed in prairie chicken (e.g. Bellinger et al. 2003), the effects of a bottleneck/population crash are less obvious when considering heterozygosities. This was also evident in Dutch black grouse. The population today appears not to deviate from mutation-drift equilibrium.

The tests in bottleneck revealed a heterozygosity excess only under an IAM, which is an unlikely mutational model for microsatellites (Piry et al. 1999), in both the museum sample, and in the present Dutch population. This is not strong evidence for loss of genetic variation since we also revealed the same pattern also for the populations from Norway and Austria. These two populations do not show any signs of being reduced in numbers and are putatively large and in connection with other black grouse populations. In any case and regardless of mutation model, we can not find any evidence of reduced genetic variation in the present Dutch population using the program bottleneck. It is recommended in order to be statistically conservative to use only the SMM when analysing microsatellite data to test for recent bottlenecks (Luikart & Cornuet 2000).

Fig. 3 The simulated decline in mean number of alleles over 52 years. Black circles, number of alleles simulated from the estimated number of breeding males from census with standard errors (see text). The grey line is the mean number of observed alleles in the present Dutch population with grey dotted lines above and below to indicate 95% confidence interval.

Fig. 4 The simulated observed heterozygosity decline over 52 years. Black circles, observed heterozygosity simulated from the estimated number of breeding males from census with standard errors (see text). The grey line is the mean number of observed heterozygosity in the present Dutch population with grey dotted lines above and below to indicate 95% confidence interval.
Furthermore, it is recommended that the number of polymorphic loci should be between 10 and 20 to achieve statistical power (Luikart & Cornuet 1998). However, most threatened bird or mammal populations are strongly reduced in numbers and are characterized by the presence of many monomorphic loci. However, we did detect loss of genetic variation using bottleneck in the English population, showing that a bottleneck could indeed be detected with these markers and that the failure of the bottleneck program in the case of the Dutch population cannot entirely be explained by a low power due to a low number of microsatellite loci. Furthermore, genetic variation was significantly higher in Norway and the Alps, which suggest that the current levels of genetic variation cannot be explained by Pleistocene bottlenecks in all the sampling localities.

The present Dutch population is on the brink of extinction, and yet bottleneck failed to uncover genetic footprints of a documented reduction in numbers. However, by modelling and by the observation of loss of allelic richness and heterozygosity with a reference in the past, we could document a loss of genetic variation in the Dutch population. After a population crash, the effects on heterozygosity will stay for 0.2–4 N_e generations before a new equilibrium is set (Luikart & Cornuet 1998). In the Dutch black grouse population, there was a decline in population numbers from 7500 to about 1000 individuals between the 1950s to the 1970s. This would lead to a measurable heterozygosity excess for the population for about 67–200 generations if the population would have remained constant at that size. However, with a census size of about 20 males the 5 years before the sampling of the extant population, one could estimate a conservative effective population size of about 13 (N_e = 4 N_m * N_m / (N_m + N_f), N_m = 20 and N_f = 4). It is then possible that a new equilibrium (N_E = N_m) has been set (0.2 * 13 = 2.6 generations), and that the only way to suspect that such population has been subjected to severe genetic drift by is by comparison to other continuous populations or to access samples from prior to the population crash.

In summary, almost all extant Dutch birds were sampled for this study, which allowed valuable insights of the genetic variation of a small, isolated, population which has recently decreased rapidly in numbers. The use of museum samples allowed us to assess the historical genetic variation of the population. With bottlemim and census numbers, it was possible to show that loss of genetic variation has indeed occurred and yet was undetectable by the bottleneck approach. This suggests that measures may be warranted to restore the genetic variation of the last extant Dutch black grouse population to reduce any possible detrimental effects of low genetic variation such as inbreeding depression and lost adaptive potential. When effective population size becomes very small (in the order of 10^1) and when generation times are short, the program bottleneck and similar approaches will fail to detect loss genetic variation. Under such circumstances only knowledge of, or estimates of the genetic diversity prior to the bottleneck, will reveal loss of genetic variation.

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References


