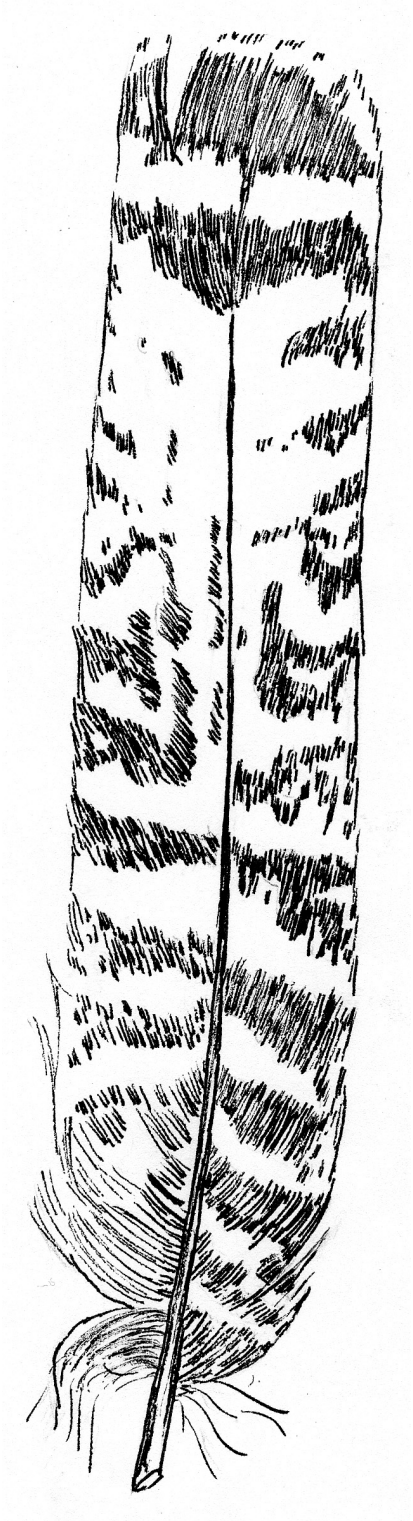

Genetic structure of capercaillie populations: a non-invasive approach at multiple spatial scales

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Vollständiger Abdruck der von der
Fakultät Wissenschaftszentrum Weihenstephan
für Ernährung, Landnutzung und Umwelt
der Technischen Universität München

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr.forest. Gerhard Müller-Starck
Prüfer der Dissertation:	1. Univ.-Prof. Dr.forest. Dr.rer.silv.habil. Wolfgang Schröder
	2. apl.Prof. Dr.agr. Dr.agr.habil. Oswald Rottmann

Die Dissertation wurde am 29.01.2002 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 25.04.2002 angenommen.

Sebastién Sachot

We only preserve what we love,
we only love what we understand,
we only understand what we study.

Tibetan Saying

Results! Why, man, I have gotten a lot of results.
I know several thousand things that don´t work.

Thomas A. Edison

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0 Preface

The general framework of this thesis is given by the introduction (chapter 1). General methods are described in chapter 2. I subdivide this thesis into six papers, which address different aspects of analytical advances with non-invasive genetic sampling in birds (chapter 3) and population genetics of capercaillie (chapter 4).

In chapter 3.1 I describe the development of microsatellites for capercaillie and their use for this study. Thereafter, I describe the methodology of using bird faeces samples for genetic studies (chapter 3.2). Subsequently, I stress the reliability of feather samples for population genetic studies (chapter 3.3). This sets the frame for the following papers, treating the genetic variation of capercaillie at multiple spatial scales (chapter 4).

In Chapter 4.1 I analyse the genetic diversity of capercaillie across Europe and demonstrate the importance of connectivity among populations. I hypothesise that capercaillie populations in the south-west, i.e. western and central Europe, will reveal a more structured genetic signature than birds from different sites within the contiguous and relatively undisturbed northern parts of the range, i.e. Fennoscandia and Russia. I assume genetic diversity to be lower in the Pyrenees and in small isolated populations in central Europe than in more contiguous populations in the Alps and northern Europe. The following chapter 4.2 deals with the metapopulation structure in the Alps. I analyse the spatial pattern of genetic variation in the Alps, testing the metapopulation hypothesis for those populations. I compare the genetic structure of populations along the northern edge of the Alpine distributional range with populations from other parts of the Alps. In the final chapter 4.3 I focus on fine-scale patterns of gene flow and genetic diversity. I attempt to assess how habitat availability might influence genetic population structure and demonstrate source-sink dynamics in a metapopulation framework. For each of these chapters I provide an abstract and introduction, describe methods and present and discuss results.

The Conclusion (chapter 5) summarises the major findings of the previous chapters and points out consequences of these findings for capercaillie conservation and management.

The main chapters of this thesis have been published or submitted for publication to scientific journals with the following authorships and titles:

Segelbacher G, Paxton R, Steinbrueck G, Trontelj P, Storch I (2000) Characterisation of microsatellites in capercaillie (*Tetrao urogallus*) (AVES). *Molecular Ecology*, **9,**1934-1935. (Chapter 3.1)[1]

Segelbacher G, Steinbrück G (2001) Bird faeces for sex identification and microsatellite analysis*. Vogelwarte*, **41**, 139-142. (Chapter 3.2)

Segelbacher G (in press) Non-invasive genetic analysis in birds: testing reliability. *Molecular Ecology Notes.* (Chapter 3.3)[1]

Segelbacher G, Höglund J, Storch I (submitted manuscript) Variation in genetic diversity across the European range of capercaillie (*Tetrao urogallus*). (Chapter 4.1)

Segelbacher G, Storch I (submitted manuscript) Metapopulation structure of capercaillie (*Tetrao urogallus)* in the Alps. (Chapter 4.2)

Segelbacher G, Tomiuk J, Storch I (submitted manuscript) Source and sink populations in Alpine capercaillie: evidence from microsatellites. (Chapter 4.3)

[1] Papers are reprinted with the permission of the publisher.

Parts of this thesis have also been presented in the following papers:

Segelbacher G, Storch I (1999) Genetic markers for studying spatial structure of grouse populations. *Grouse News* (*Newsletter of the IUCN Grouse Specialist Group*) **18**, 4-8.

Segelbacher G, Storch I (1999) Comparison of geographic and genetic distance in grouse (abstract). 2nd International Wildlife Management Congress, Gödöllö, Hungary.

Segelbacher G, Storch I (1999) Geographic and genetic distance in European grouse (abstract). 8th International Grouse Symposium, Rovaniemi, Finland.

Segelbacher G, Höglund J (2000) Conservation of black grouse – a study using microsatellites. *Cahiers d'Ethologie,* **20**, 411-420.

Segelbacher G, Storch I (2000) Welche Möglichkeiten bieten genetische Marker im Naturschutz ? Zusammenhang zwischen geographischer und genetischer Distanz bei Rauhfußhühnern (abstract). 150. Jahrestagung der DO-G in Leipzig. *Journal für Ornithologie,* **142**, 215.

Segelbacher G, Storch I (2000) Spatial distribution and genetic differentiation of black grouse populations in the Alps: preliminary results from a study with microsatellite markers. Workshop on the fate of black grouse (*Tetrao tetrix*) in European moors and heathlands, (abstract). Liège, Belgium.

Storch I, Segelbacher G (2000) Genetic correlates of spatial population structure in central European capercaillie *Tetrao urogallus* and black grouse *Tetrao tetrix*: a project in progress. *Wildlife Biology,* **6,** 305-310

Segelbacher G, Storch I (2001) Möglichkeiten molekularer Methoden im Naturschutz – am Beispiel des Auerhuhns (abstract). Workshop der LWF.

Segelbacher G, Storch I (2001) Spurensuche im Gebirge - was wir mit molekularen Methoden über Rauhfußhühner erfahren (abstract). 134. Jahresversammlung DO-G, Schwyz, Schweiz.

Segelbacher G, Storch I (2001) Genetic differentiation of capercaillie in Europe – a non-invasive approach (abstract). 8th Congress of the European Society of Evolutionary Biology, Aarhus, Denmark.

Segelbacher G, Storch I (2001) Population genetics of capercaillie and black grouse in the Alps (abstract). Conservation genetics meeting. Lausanne, Switzerland.

1 Introduction

The landscape of central Europe is dominated by farmland. Forests are distributed as isolated fragments within a matrix of open land; small woodlots no more than a few hectares in size prevail. Contiguous forests covering several hundred km² are restricted to mountainous regions. These are the areas where most of the remaining capercaillie (*Tetrao urogallus*) populations are found. Capercaillie are coniferous forest obligates. They prefer extensive areas of forest with moderate canopy cover and rich ground vegetation, dominated by ericaceous shrubs such as bilberry (*Vaccinium myrtillus*). In their boreal distribution range, capercaillie live in contiguous forest landscapes. In central Europe, however, grouse habitats, and thus populations, are spatially structured at two hierarchical levels of scale: forest-dominated uplands versus farmland-dominated lowlands at the continental scale (i.e. central Europe), and forested mountain slopes versus open habitats at the regional scale (e.g. in the Alps) (Klaus & Bergmann 1994). As a consequence, distribution ranges of grouse are separated by up to 100 and more km at the continental scale (see Klaus & Bergmann 1994), and local populations by up to 10 and more kilometre at the regional scale (I. Storch unpubl. data). From this distribution pattern, metapopulation structure has been hypothesised for central European capercaillie populations (see Rolstadt 1991, Storch 1993, 95, 97). The distribution of capercaillie has always been fragmented in central Europe after the last Ice Age due to the naturally patchy distribution of suitable habitats. However, the degree of fragmentation and the properties of both habitat patches and surrounding matrix have been altered by human land use practices. In general, habitat conditions are assumed to have deteriorated for capercaillie in the course of the 20th century (Storch 2001), and grouse populations may have been shifting towards decreasing connectivity along the gradient from spatially structured over metapopulations to isolated populations (Storch & Segelbacher 2000).

I want to use this metapopulation framework for analysing the threat of fragmented capercaillie populations. A metapopulation is a system of geographically or ecologically isolated populations within which there is sufficient migration among populations to have a significant impact on either the demography or genetic structure of each component population (Stacey *et al*. 1997). Each subpopulation has its own local dynamics (birth and death rates) but is also connected through immigration and emigration to other subpopulations (Hanski 1997, 1998). Extinction and recolonisation probabilities thus depend on both local (births, deaths, population size) and general processes (dispersal probabilities). When distances between subpopulations increase (due to local extinction and fragmentation), the probabilities of dispersal (and thus gene flow) between subpopulations decrease. At the extreme dispersal ceases completely and given time, the populations might diverge and become genetically isolated or even go extinct (Whitlock &

Barton 1997). Additionally isolation and small population size may also have detrimental genetic consequences (Lynch 1996).

The degree of genetic differentiation among subpopulations is determined by the interplay among the forces of natural selection, random genetic drift, extinction, colonisation and migration (Wade 2001). The combined effects of random genetic drift diversifying populations on the one hand and migration homogenising them on the other hand result in a balanced, or equilibrium, level of genetic differentiation among subpopulations in a metapopulation (Wade & McCauley 1984). Habitat availability influences dispersal success and thereby secondarily influences population subdivision (Gibbs 2001). This supports that drift and gene flow processes associated with patch extinction dynamics (Lacy & Lindenmayer 1995, Whitlock & Barton 1997) are important as determinants of patterns of genetic diversity and divergence within wild populations.

The genetic structure of a metapopulation determines both the rate and the direction of evolutionary change. The greater the genetic differentiation among local populations, the more important are the effects of metapopulation structure on evolution (Wade 2001). Wade & Goodnight (1991) could show in an experimental study large, heritable differences among demes of the flour beetle *Triboluem castaneum* in a metapopulation even when population size and migration among demes was high. However, to fully understand the range of effects that metapopulation structure has on evolution and ecology additional studies on natural populations are necessary (Hanski 1999, Wade 2001).

Dispersal distances in capercaillie are roughly known from marked birds. Summarising published results, one can expect average seasonal movements of 1 to 2 km for adults and median dispersal distances of less than 10 km for juvenile birds (Table 1). The longest dispersal distances recorded are 75 km (Myberget 1978). Comparing the spatial distribution of habitats and recorded dispersal distances in capercaillie, one can assume genetic and demographic isolation at a continental scale. At a regional scale, it is likely that juveniles disperse between local populations so that metapopulation structure can be assumed (Storch 1993, 1995, 1997).

In the past, dispersal studies on birds had to rely on classical methods such as radio-tracking or banding. However, these methods are labour-intensive, provide limited datasets and cause a relatively high level of disturbance to the birds, which is particularly problematic when dealing with endangered species. To our knowledge, there is no published example of a study on grouse in central and western Europe that achieved more than anecdotal data on dispersal rates and distances. Advances in molecular biology now allow to address questions of dispersal and gene-flow more effectively. Genetic information on individuals and populations can be gained by analysing DNA from feathers or faeces (see Taberlet *et al*. 1999). These techniques may provide large datasets with relatively little effort and disturbance to the study species.

Based on capercaillie feather samples from different parts of Europe, and from different mountain ranges across the Alps, I expect to gain insight into the effects of geographic distance and habitat fragmentation on genetic differentiation at multiple spatial scales.

Knowledge of the spatial structure of populations and metapopulations (Hastings & Harrison 1994, Harrison & Hastings 1996) will help us to model spatial (meta-) population dynamics, and last but not least, to improve capercaillie conservation and management approaches in central Europe.

Table 1 Juvenile dispersal distances and adult seasonal movement distances of capercaillie. All data stemmed from radio-marked or wing-tagged birds. Not all studies distinguished sexes; some reported mean, other median distances. Maximum distances (Max) were rounded to full kilometres (from Storch & Segelbacher 2000).

	Sex	Mean	SE	Median	N	Max	Location	Author
Juvenile dispersal	F				?	24	Fennoscandia	Koivisto 1963*
	M				?	4	Fennoscandia	Koivisto 1963*
				<2	39	75	Scandinavia	Myrberget 1978
		6.7	1.1	3.0	55	38	Fennoscandia	Swenson 1991
	F	>5.0			1		German Alps	Storch 1993
	M	0.6			1		German Alps	Storch 1993
Adult movements				3-5	8	11-20	Scandinavia	Myrberget 1978
	F	1.9			15	7.6	Norway	Rolstad *et al*. 1988
	M	1.5			44	10.0	Norway	Rolstad *et al*. 1988
				<1	11	8	Pyrenees	Menoni 1991
	F	0.8	0.3		7	6.9	German Alps	Storch 1995
	M	1.4	0.4		19	9.0	German Alps	Storch 1995

* cit. in Klaus *et al*. 1989

2 General Methods

Sampling

We collected feather samples from capercaillie at different locations across the European range of the species (see maps (Figure 3, Figure 7, Figure 11) in the relevant chapters) in 1997-2001. Sampling was conducted on a continental scale (distribution areas: Alps, Black Forest, Vosges, Pyrenees, Northern Russia) in Europe and on a regional scale (mountain ranges) in the Alps. Most samples obtained were moulted feathers collected in the field during the summer months; others came from carcasses and birds killed by hunters. A total of 949 feather samples were collected, put individually in envelopes or plastic bags and stored dry until analysis (1-20 months). Most of the male and female feathers could be distinguished in the field and samples where sex could not identified clearly were additionally sexed with genetic methods (Segelbacher & Steinbrück 2002).

DNA extraction

Comparing different extraction methods, we found that a silica based column method gave the best yield of DNA and most reliable results in PCR compared to the Chelex method, magnetic beads or classical phenol-chloroform extraction. Therefore this method was chosen to extract all feather samples. We used a commercial kit (DNeasy Tissue Kit, Qiagen) according to the manufacturer's instructions.
The basal part of a feather was cut into small pieces with a sterile razor blade and digested overnight with 20 µl Proteinase K in 180 µl buffer ATL (as supplied by the manufacturer) in a shaking water bath at 55° C. The buffer volume was adjusted to the size of the feather tip (400-600 µl for large feathers, 200 µl for small ones). Further steps of the extraction followed the protocol of the manufacturer (Qiagen). DNA was finally recovered in 60-100 µl of elution buffer and stored at –20° C.

Genotyping

Two µl of template DNA was used in a 10 µl PCR reaction in an Eppendorf Gradient thermal cycler. Individual mixes contained approximately 10 ng template, 0.5 mM of each nucleotide, 10 pmoles of each primer, 2.5 mM (for BG primers)/ 1.5 mM (for TUT primers) $MgCl_2$, 0.25 units of Platinum Taq DNA polymerase (Invitrogen), 20 mM Tris-HCl (pH 8.4) and 50 mM KCl. PCR profiles consisted of 3 minutes denaturation at 94° C, 35 cycles of 30s denaturation at 94° C,

30s annealing at the specified temperature and 45s extension at 72° C with a final 5 minute 72° C step.

In a comparison of Taq DNA Polymerase from different suppliers, Platinum Taq DNA polymerase (Invitrogen) showed the best results and was therefore chosen for all analyses.

PCR fragments were resolved by electrophoresis on 6% (w/v) denaturing polyacrylamide gels and fragments visualised using silver staining. After a pre-run at 75 W, 48 PCR samples were loaded on a gel with standards of 100bp, 25bp, and 10bp. After approximately 30 minutes, another 48 samples were loaded with the same standards. Electrophoresis was conducted for approximately 1 1/2 hours depending on the fragment size. Gels were fixed for 25 min in 10% acetic acid. Fixation was followed by three washing steps (each 2 min) with distilled water. Staining was carried out in 1% $AgNO_3$ solution (800ml) with 1.5 ml formaldehyde (37%) for 30 min. After a brief washing step (10s) the gel was developed with 24 g sodium carbonate, 1.2 g sodium thiosulphate (2%) and 1.5 ml formaldehyde (37%) in 800ml ddH_20. Developing was stopped with 10% acetic acid then the gels were washed with distilled water and air dried.

Allele sizes were determined by reference to the ladder standards of 100bp, 25bp and 10bp. Two PCR products of the corresponding cloned locus aided as a positive control and a PCR mix without template DNA served as a negative control. For comparisons between gels, the same individual was run in three lanes on each gel to standardise scoring.

Guidelines for genotyping of feathers

Limitations of non-invasive sampling methods result from either low DNA quantity, low DNA quality (i.e. degraded DNA) or poor extract quality (i.e. the presence of PCR inhibitors). There is also a high risk of contamination during the extraction and amplification process. To avoid these problems I followed stringent guidelines to avoid contamination. To detect whether contamination of samples with exogenous DNA or PCR products had occurred, tubes without sample tissue were included in the DNA extraction and PCR amplification procedure as negative controls. To avoid contamination, DNA extractions, pre PCR and post PCR pipetting were carried out in different rooms and aerosol-resistant filter pipette tips were used throughout. Concentrated DNA extracts were not handled in the pre PCR room and pipettes dedicated only for PCR were used.

The following methods were used to minimise the impact of genotyping errors:

- Feathers were collected in the field and stored dry, separately and in bags. Feathers were dried carefully before storing if they were wet when collected.
- A silica method was used for DNA extraction, which is very effective for feathers.
- Non-target DNA amplification was reduced via a hot start polymerase reaction (Platinum Taq, Invitrogen). This means that a single target cell could be detected if the PCR conditions are optimised.
- PCR experiments were repeated several times.
- I used only tetranucleotide microsatellites instead of dinucleotide microsatellites to reduce the risk of obtaining false alleles and make scoring of the alleles easier.
- PCR primers were chosen that amplify short DNA fragments (≤ 200bp).

To test the reliability of genotyping, multiple extractions and multiple PCR reactions have been carried out.

Statistical analysis

Microsatellites have become the preferred marker in many studies because of their high levels of variability, ease and reliability of scoring, codominant inheritance, and short lengths, making them useful for studies of DNA from fossils and hair, faeces or feathers collected in the field. In parallel, many analytical and statistical tools (Rousset & Raymond 1997, Luikart & England 1999) have been developed in response to the large amount of genetic information provided by microsatellite data.

In this thesis, several of the most recent and innovative methods for retrieving information from microsatellites were used. Specific methods for the analysis of the microsatellite data will be presented in the particular chapters. Here I give a general overview of the methods and programmes used.

Classical population genetic statistics

The main question of interest to population geneticists is the determination of the forces responsible for the variation patterns revealed by polymorphic microsatellites (Hastings 1997). The amount of polymorphism at a locus results from the interaction of the following forces: selection, genetic drift, mutation and migration. When screening genetic markers, I attempt to estimate the strength of each of these forces. I therefore investigate allele frequencies and the genetic diversity of the markers, the proportion of each genotype and their expected proportion under a Hardy-Weinberg null model. To quantify the inbreeding effect of substructure, Wright (1921) defined what has come to be called the fixation index (F).

For examining the overall level of genetic divergence among subpopulations, F_{ST} is the informative statistic (Hartl & Clark 1997). The following qualitative guidelines for the interpretation of F_{ST} (from Hartl & Clark 1997) have been suggested: The range 0 to 0.05 may be considered as indicating little genetic differentiation. The range 0.05 to 0.15 indicates moderate genetic differentiation. The range 0.15 to 0.25 indicates great genetic differentiation. Values of F_{ST} above 0.25 indicate very great genetic differentiation. Genetic differentiation among subpopulations described by the hierarchical F statistics can be interpreted as a sort of inbreeding effect resulting from population substructure. The relationship between population substructure and inbreeding is a subtle one, but it has profound consequences in population genetics (Hartl & Clark 1997). F_{IS} is the inbreeding coefficient of a group of organisms relative to the subpopulation to which they belong.

These estimates and statistics have been calculated via FSTAT Version 2.9.3 (Goudet 2001).

Additionally calculations of these basic population genetic parameters have been conducted with GENEPOP Version 3.3 (Raymond & Rousset 1995b). An overview of the estimation methods used in GENEPOP can be found in Rousset (2001).

I used the program ARLEQUIN Version 2.000 for basic statistics and for analysing the genetic structure using an Analysis of MOlecular VAriance framework (AMOVA) (Weir & Cockerham 1984, Excoffier *et al*. 1992). By defining groups of populations, I specified a particular genetic structure to be tested. A hierarchical analysis of molecular variance partitions the total molecular variance into covariance components due to intra-individual, inter-individual and/or inter-population differences.

Genetic relationships between individuals

The application of molecular methods for non-invasive or ancient samples frequently generates data sets with a low quantity of data and a high proportion of missing information. Originally developed to assess relatedness among individuals found in historic human burial sites, we used BURIAL 1.0 (Schönfisch *et al*. 2001) to determine whether individual capercaillie are genetically related. Genetic similarity of individuals at a certain locus is defined as the sharing of at least one identical allele. BURIAL tests the null hypothesis that the observed grouping of individuals at a particular site reflects random placement of genotypes.

Another program for calculating genetic relatedness among and between demographically defined groups of individuals is RELATEDNESS 5.0 (Queller & Goodnight 1989). It uses a regression measure of relatedness. In a diploid organism, like birds, offspring are related half to their father and half to their mother. This will result in a relatedness value of r= 0.5 between offspring and parent; individuals not related to each other will show a value of r=0. I used both programs to test if males and females in populations were related to each other.

Assignment tests

Using the high information content of multiple hypervariable microsatellite loci, tests have been developed which assign an individual to the population where its genotype has the greatest probability of occurring (program GENECLASS, Cornuet *et al*. 1999; program STRUCTURE, Pritchard *et al*. 2000).

Assignment tests are increasingly gaining recognition as a novel means of studying dispersal (Favre *et al*. 1997, Waser & Strobeck 1998, Luikart & England 1999, Mossman & Waser 1999) and have multiple other applications in wildlife management, conservation genetics and forensics (Davies *et al*. 1999, Luikart & England 1999, Galbusera *et al*. 2000, Primmer *et al.* 2000, Eldridge *et al*. 2001). Recently dispersed individuals will be assigned to their natal population and not the population in which they were sampled, and this method therefore provides a unique direct estimate of dispersal which can readily be compared with indirect estimates (e.g. N*m* from F_{ST}) made through conventional analysis of the same microsatellite data. In addition, some assignment tests (e.g. Pritchard *et al*. 2000) are able to detect not only immigrants into a population but also their offspring.

The assignment process has recently been refined with the development of a simulation procedure which determines the probability of an individual originating from a series of potential source populations (Cornuet *et al*. 1999). This procedure results in a probability of exclusion, which can, in effect, be used as a probability of belonging. A further advance is the development of a Bayesian clustering method (Pritchard *et al*. 2000) which can utilise prior population information and estimate the posterior probability that each individual has originated from, or has recent ancestry in, a series of potential source populations. This methods can be used to demonstrate the presence of population structure, to assign individuals to populations and to identify migrants and admixed individuals.

Uni directional gene flow and effective population size

MIGRATE (Beerli & Felsenstein 1999) estimates numerous population parameters, effective population sizes and migration rates of populations, using genetic data. It estimates a maximum likelihood based on coalescens, and taking into account the history of mutations and uncertainty of genealogy. The main advantage of this approach is that uni directional gene flow rates can be estimated, giving more precise information on how migration among populations has occurred.

Population decline

Populations that have experienced a recent reduction in their effective population size will display a reduction in both allele number and gene diversity (heterozygosity), but allelic loss will occur more rapidly than loss of heterozygosity (Maruyama & Fuerst 1985). Based on this phenomenon, tests have been developed to detect excess heterozygosity in a population at mutation-drift equilibrium as an indicator of a recent population decline (program BOTTLENECK, Cornuet & Luikart 1996). In a population at mutation-drift equilibrium (i.e., the effective size which has remained constant in the recent past), there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit. The expected average heterozygosity (H_{EQ}) is calculated through simulations under various possible mutation models. For the infinite allele model (IAM), a single mutation is allocated to compute the resulting number of alleles. This is repeated until the resultant and observed numbers of alleles are equal. A Bayesian approach, as described in Cornuet & Luikart (1996), is used for the estimation under the stepwise-mutation model (SMM). The two phased model of mutation (TPM) is intermediate and consists of mostly one-step mutations and a small proportion (5-10%) of multistep changes (Luikart & Cornuet 1998). Populations without recent changes in size will be in mutation-drift equilibrium where the expected heterozygosity (H_{EQ}) (as estimated via IAM, TPM or SMM, based on the number of observed alleles) will equal the Hardy-Weinberg equilibrium heterozygosity.

To determine whether a population exhibits a significant number of loci with gene diversity excess, a Wilcoxon sign-rank test was used. The Wilcoxon test provides relatively high power and it can be used with only four polymorphic loci and any number of individuals (15-40 individuals and 10-15 polymorphic loci is recommend to achieve high power). It should be noted that this is not the same as tests for Hardy-Weinberg proportions (H_E). The tests of population decline compare the heterozygosity expected at mutation-drift equilibrium for a sample with the same size and number of alleles as the sample used to measure H_E. Populations or loci which are not in Hardy-Weinberg equilibrium may bias the results of this test (Cornuet & Luikart 1996, Luikart & Cornuet 1998).

3.1 Characterisation of microsatellites

Primmer *et al.* (1996) found that microsatellites could be cross-species amplified in a wide range of bird species. However, against expectation chicken primers proved to be not suitable in turkey *Meleagris gallopavo* (Liu *et al*. 1996), pheasant *Phasanius colchicus* (Baratti *et al*. 2001) and sage grouse *Centrocercus urophasianus* (Oyler-McCane *et al*. 1999). Even primers developed in red grouse *Lagopus lagopus scoticus* are of limited use in sage grouse and black grouse (Oyler-McCane *et al.* 1999, Höglund *et al.* 1999). Our own experience agrees with these results: We tested more then 30 microsatellite primer pairs developed for the chicken *Gallus gallus* (Cheng *et al*. 1995, Hanotte *et al.* 1997) and 16 primer pairs developed for the red grouse (Piertney & Dallas 1997, Piertney *et al*. 1998b) for their use in capercaillie. We found only one chicken primer pair (Lei0319) and 5 red grouse primer pairs (LLST1, LLSD2, LLSD3, LLSD4, LLSD8) showing clear scorable bands, of which only three (Lei0319, LLST1, LLSD3) amplified loci with ≥ 3 alleles in both species. Apparently, microsatellite primers developed in related species of the order galliformes are only of little use in tetraonids, especially if hypervariable loci are necessary for analysis. We therefore developed specific microsatellite primers for capercaillie (Segelbacher *et al.* 2000) and used a set of hypervariable microsatellites available for black grouse from other studies (Piertney & Höglund 2001). As the frequency of repeat sequences generally seems to be very low in birds (Primmer *et al.* 1997, Neff & Gross 2001) we used an enrichment protocol modified from Piertney *et al.* (1998a) to isolate highly variable microsatellites.

Screening of microsatellites

We developed microsatellites for the capercaillie using both non-enriched and enriched partial genomic libraries. DNA for cloning was extracted from tissue of an adult capercaillie female using standard proteinase K digestion and phenol-chloroform procedures (Sambrook *et al*. 2001). Appoximately 5 µg of DNA was digested to completion using *Sau*3AI restriction endonuclease. Fragments of 300-900bp were extracted using a the QIAquick Gel extraction kit (Quiagen) according to the manufacturer´s protocol.

For the non-enriched library the fragments were ligated into plasmid vector pUC18/ BamHI (Amersham/Pharmacia). Vector molecules were transformed into INVαF´One-Shot™ *Escherichia coli* (Invitrogen) which were then grown overnight on Luria-Bertani (LB) medium with ampicillin. Colony lifts were made from the plates onto Hybond N+ membranes (Amersham International Ltd.) and screened for the presence of microsatellites using digoxigenin (DIG)-end labelled $(GA)_{10}$ and $(CA)_{10}$ as described by Estoup & Turgeon (1996).

For the enriched library fragments were ligated to a SAU linker molecule, which was formed by ligation of equimolar amounts of the SAU-L-A oligonucleotide (5´-GCGGTACCCGGGAAGCTTGG-3´) and the phosphorylate SAU-L-B oligonucleotide (5´-GATCCCAAGCTTCCCGGGTACCGC-3´). Resultant fragments were denatured and hybridized to a 1 cm² piece of Hybond N+ membrane to which a synthetic polymer, either $(CA)_n$ or $(GATA)_n$, was bound. Hybridization took place at 60° C overnight in 2x SSC, 0.1% SDS for $(CA)_n$ and in 5x SSC, 0.1% SDS for $(GATA)_n$. After three low stringency washes with 2x SSC, 0.1% SDS for $(CA)_n$ / 5x SSC, 0.1% SDS for $(GATA)_n$ the enriched fraction was removed by heating to 95° for 5 min in sterile water. The enriched fraction was precipitated and complementary strands were reformed in a PCR reaction (30 cycles at 94° C denaturation, 55° C annealing and 72° C extension) using the SAU-L-A oligonucleotide as a primer. SAU linkers were removed by restriction with *Sau*3AI endonuclease and fragments were ligated into a phosphorylated, *Bam*H1-cut pUC18 vector (Pharmacia Ltd). The now enriched fragments were transformed into INVαF´One-Shot™ *Escherichia coli* (Invitrogen) which were then grown overnight on Luria-Bertani (LB) medium with ampicillin. Colony lifts were made from the plates onto Hybond N+ membranes and screened for the presence of microsatellites following Estoup & Turgeon (1996) in 5x SSC, 0.1 % SDS at 55° C.

Plasmid DNA was extracted from positive colonies using the QIAgen plasmid isolation kit (Qiagen) following the manufacturer´s protocol. Inserts were cycle-sequenced using Big Dye Terminator chemistry (Perkin Elmer) and fragments were resolved on an ABI Prism.373A automated sequencer.

Of the non-enriched library 2900 clones were screened and 41 positive clones sequenced. Only one primer pair could be designed and used. Of the enriched library 744 clones were screened for GA repeats and 550 clones for GATA repeats. Ten clones were sequenced for GA, 23 clones for GATA repeats resulting in 4 respectively 5 primer pairs amplifying polymorphic microsatellite loci. Primers were designed on sequences flanking repeat elements of >10 (CA) or >8 (GATA) repeats using the Primer 3.0 software. (Whitehead Institute).

High levels of variability could be detected in the analysed loci (Table 2), making them useful for paternity analysis and population genetic studies (see also Appendix).

Table 2 Description of microsatellite loci for *Tetrao urogallus;* sequences and repeat type, PCR fragment length (based on the clone sequence), number of resolved alleles (number of analysed birds are in parentheses), optimal annealing temperature, heterozygosity, and Genbank accession numbers of the clone sequences. Significant differences between the observed and expected heterozygosity are marked with an asterisk.

Locus	Primer sequences (5´-3´)	Repeat type	Size (bp)	No. of alleles	Annealing temp.	H_O	H_E	Accession number
TUD1	F: ATTTGCCAGGAAACTTGCTC R: AACTACCTGCTTGTTGCTTGG	$(CA)_{14}$	209	8 (19)	59	0.70	0.82	AF254644
TUD2	F: GTGACAACTCAGCCCCTGTC R: AATAAGGGTGCGCATACACC	$(CA)_{13}$	200	10 (19)	59	0.83	0.89	AF254645
TUD3	F: TCCAAGGGGAAAATATGTGTG R: TTCTTCCAGCCCTAGCTTTG	$(TG)_{12}$	192	11 (19)	60	0.66	0.82	AF254646
TUD4	F: TTAGCAACCGCAGTGATGTG R: GGGAGGACTGTGTAGGAGAGC	$(CA)_{21}$	167	11 (19)	60	0.61*	0.88	AF254647
TUD5	F: CCTTGCTGCACATTTTCTCC R: GGTGCTGAGCATGTACTAGGG	$(GT)_{23}$	193	12 (19)	57	0.72	0.88	AF254648
TUD6	F: GGTGAGCAAGCCACAAATAAC R: GAGGACTGCAGAACCCACTG	$(CA)_{21}$	210	13 (16)	58	0.69*	0.88	AF254649
TUD7	F: TGACACTGGGGTCATTAGGC R: AACATGGGCAGGAGGAGAC	$(CA)_{11}$	200	5 (19)	59	0.58	0.63	AF254650
TUD8	F: TGCAGCCTCCTCTAATTTCG R: CTGGACATCAGCAATCATGC	$(GT)_{15}$	187	11 (17)	59	0.70*	0.85	AF254651
TUT1	F: GGTCTACATTTGGCTCTGACC R: ATATGGCATCCCAGCTATGG	$(CTAT)_{12}$	217	8 (20)	60	0.60	0.83	AF254653
TUT2	F: CCGTGTCAAGTTCTCCAAAC R: TTCAAAGCTGTGTTTCATTAGTTG	$(GATA)_{12}$	160	9 (20)	60	0.70	0.76	AF254654
TUT3	F: CAGGAGGCCTCAACTAATCACC R: CGATGCTGGACAGAAGTGAC	$(TATC)_{11}$	154	8 (20)	60	0.50	0.80	AF254655
TUT4	F: GAGCATCTCCCAGAGTCAGC R: TGTGAACCAGCAATCTGAGC	$(TATC)_8$	179	7 (20)	60	0.80*	0.77	AF254656

3.2 Bird faeces for genetic analysis

It is often difficult to obtain blood or tissue samples for population genetic studies of birds without disturbing the animals. We therefore developed a method for extracting DNA from small amounts of bird faeces, and describe the use of this method for sexing birds and for microsatellite analysis. Results obtained from faecal samples were identical to those obtained from tissue or feather samples. With non-invasive sampling, it is possible to gain information about free ranging animals with a minimum of disturbance to them, which is especially important when studying endangered species.

Introduction

Blood or tissue samples for population genetic studies of birds are often difficult or impossible to obtain. Particularly in studies of threatened species, non-invasive sampling is highly desirable. In many bird species, faeces can be sampled easily and are an accessible source of DNA (e.g. Nota & Takenaka 1999), because PCR-based methods of genetic analysis require only minute amounts of template DNA from which to amplify additional copies. We describe a method of DNA extraction from small amounts of bird faeces sufficient for identification of the sex of an individual and its genotype at microsatellite loci. Sex determination using molecular methods and analysis of microsatellite variation have proven to be valuable tools in wildlife conservation and for studies of sex allocation and the behavioural ecology in birds (e.g. Lens *et al.* 1998, Sheldon 1998, Galbusera *et al.* 2000a, Lee *et al.* 2001).

Material and Methods

DNA was isolated from fresh (less than one day old) and old (more than one week old) excrement of white-rumped shamas (*Copsychus malabaricus*) (2 females, 2 males), thrush nightingales (*Luscinia luscinia*)(2 females, 2 males), house sparrows *Passer domesticus*)(2 females, 2 males) and capercaillies (*Tetrao urogallus*) (4 females, 5 males). Samples of white-rumped shamas and thrush nightingales were collected from the cage bottom of caged birds. Samples of house sparrow and capercaillie were sampled at natural roosting sites, both in southern Germany. Droppings were put into paper envelopes and stored dry until analysis.

Alternatively a tube containing desiccating silica gel beads was used for large and humid samples.

For sex identification, we used a method which exploits the difference in length between introns in the CHD-Z and CHD-W genes (Griffiths *et al.* 1998, Kahn *et al.* 1998) that are located on the avian sex chromosomes of all non-ratite birds. Males of non-ratite birds have two identical sex chromosomes (WW) and therefore two identical CHD-W genes. A PCR reaction using DNA of males and suitable "sex-primers" (Griffiths *et al.* 1998, Fridolfsson & Ellegren 1999) which bind to the W chromosomes gives a single PCR product which can be detected by gel electrophoresis. On the other hand, female birds have different sex chromosomes (WZ) and are therefore heterozygous with respect of the CHD gene (CHD-W and CHD-Z). Due to length differences of introns in the CHD-W and CHD-Z gene copies, a PCR reaction with "sex-primers" results in PCR products which give two bands on a gel. For our analysis, we used the "sex primers" P2 and P8 (Griffiths *et al.* 1998). For microsatellite analysis, we used the primer sets (TUT1, TUT4) specially developed for capercaillie (Segelbacher *et al.* 2000).

To test the reliability of faecal typing, we analysed DNA from freshly moulted or plucked feathers of each of the caged individuals from which we had collected faeces. For sex identification, we used samples of 2 females and 2 males of each species. For microsatellite analysis, we analysed droppings and feathers of 9 capercaillie (4 males, 5 females) with primers TUT1 and TUT4. As a positive control, we used DNA extracted from muscle tissue of one female and one male capercaillie. To avoid false genotyping of all samples due to low DNA content, three PCR reactions were amplified for each DNA sample (multiple-tube approach).

A silica-based column method was used for DNA extraction from faeces that was originally developed for the isolation of DNA from human faeces (QIAamp DNA stool kit, Qiagen). DNA from feathers and muscle tissue was extracted as described in Segelbacher *et al.* (2000). A sample of 100 to 300 mg of faeces was put into a 15 ml tube and gently washed with 1-3 ml buffer ASL (supplied by the manufacturer) or phospate-buffered saline buffer (PBS) pH 7.4. As bird faeces contains a high amount of uric acid which could interfere with the extraction procedure, only the dark parts of the droppings were chosen. Washing was performed for 20-60 minutes on a rotating wheel to release shed epithelial cells of the intestinal lining from the surface of the excrement. We avoided disintegration of the dropping to minimize the amount of PCR-inhibiting substances. This supernatant was then transferred to a 2 ml microcentrifuge tube and centrifuged briefly to pellet particles. Then 1.4 ml of the supernatant was transferred to a new 2 ml microcentrifuge tube and an provided InhibitEX tablet (Qiagen) added. Additional steps were performed according to the manufacturers protocol. The extracted DNA was then eluted in 50 µl of H_2O. PCR reactions were performed in 10 µl volumes with an Eppendorf Gradient Thermal Cycler. Individual mixes contained 1 µl of the eluted DNA, 0.2 mM dNTPs, 10pmoles of each primer, 2.5 mM $MgCl_2$, 200 mM $(NH_4)_2(SO)_4$, 750 mM Tris-HCl pH8.8, 1.5 mg/ml BSA, 100 mM beta-mercaptoethanol and 0.25 units of Platinum Taq DNA polymerase

(Invitrogen). PCR profiles consisted of 3 minutes denaturation at 94 °C, 35 cycles of 30s denaturation at 94 °C, 45s annealing at 60 °C for the microsatellite primers (TUT1, TUT4) and 48.5 °C for the sex primers (P2, P8), 45s extension at 72 °C, with a final extension step of 3 min at 72 °C. The products were run on a 6% denaturing polyacrylamide sequencing gel and visualised by silver staining (Promega). Scoring of the gel bands was done by a person who did not know the sex and origin of the samples. To avoid contamination, DNA extractions, pre PCR and post PCR pipetting were carried out in different rooms and areosol-resistant filter pipette tips were used throughout.

Results & Discussion

Identification of sex proved to be reliable in all tested bird species when using the multiple-tube approach, as proposed by Taberlet *et al.* (1999). In this approach each DNA amplification is repeated independently for each locus at least three times with the same DNA extract. Droppings of all species (n=21) could be sexed correctly, and faecal samples showed the same banding patterns as feather and tissue samples (Figure 1). In only one case a drop-out of one allele in the first PCR reaction was found; only the shorter allele of the CHD gene could be amplified in a female sample. However, two subsequent PCR´s of the same sample gave the correct pair of products. This finding supports the importance of a multiple-tube approach.

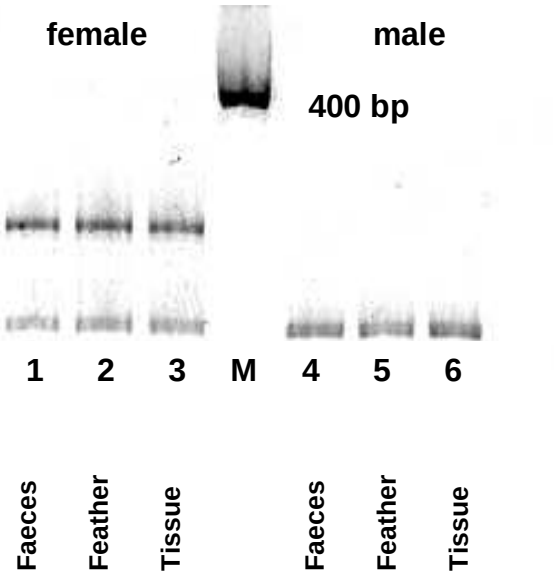

Figure 1 Sex identification using PCR with template DNA isolated from faeces, feathers or tissue. Lanes 1-3 show the PCR products obtained from a capercaillie female (two alleles amplified), 4-6 from a capercaillie male (one allele amplified). Lane M shows a 400 basepair molecular weight marker band.

Analyses of the 2 microsatellite loci amplified from capercaillie droppings of the four females and five males showed that genotypes were identical to those obtained when DNA was extracted from feathers. (Figure 2). Correct genotypes could be obtained from fresh and old droppings, indicating that even faeces, which were collected after some days in the field could be used for genetic analysis. However, amplification success of microsatellite markers was higher in fresh samples than in old samples (Segelbacher unpublished data). Effects of sampling and storage of faecal samples should therefore be taken into consideration to optimise genotyping from bird faeces.

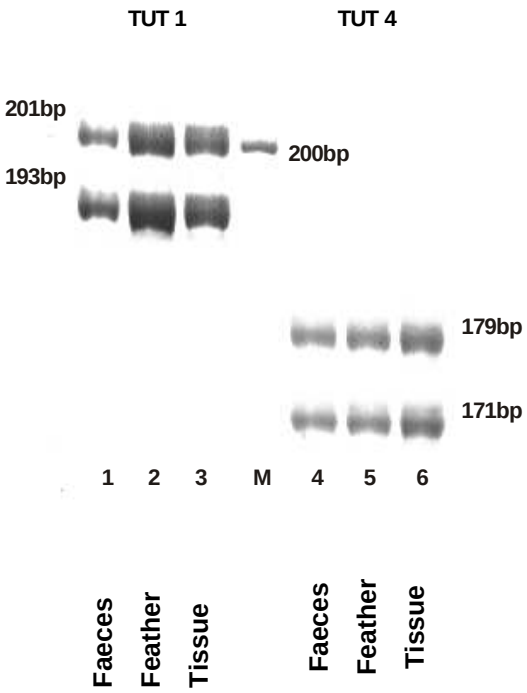

Figure 2 Banding patterns of two microsatellite loci amplified using DNA isolated from faeces, feather or tissue of capercaillie. Lanes 1-3 show the amplification product of locus TUT1 , lanes 4-6 locus TUT4. Lane M shows a 200 basepair molecular marker band. The microsatellite banding patterns obtained from the three different sources are identical.

As bird faeces contains a high amount of uric acid and PCR inhibiting substances, it is difficult to obtain DNA that is free of PCR inhibitors using previously published DNA extraction protocols (e.g. standard phenol-chloroform extraction or Chelex methods). We demonstrate that, using the described method, we can use minute amounts of bird faeces for reliable genotyping. Although DNA of droppings, which have been sampled in the field may be highly degraded, we could avoid typing errors that arise to the low amount of template DNA by using multiple, independent PCR reactions. We could also minimise errors in microsatellite genotyping using short (<200bp) microsatellite marker fragments.

This approach can be conducted in every molecular biology laboratory performing microsatellite genotyping or sexing of birds without any further need of equipment. Although the basic costs of this approach are similar to those when genotyping blood or tissue samples, one should be aware that multiple extractions and genotyping will be more expensive. As each study is unique and thus results cannot be transferred to another species or even other populations of the same species, a pilot study to assess the genotyping error rate and the laboratory effort and necessary costs is strongly recommended.

Several non-invasive sampling studies in mammals demonstrated their potential to identify individuals, to estimate relatedness among individuals, effective population size and level of genetic differentiation between populations (see review in Taberlet *et al.* 1999). In the case of small and endangered populations, species highly sensitive to disturbance, or in behavioural studies where capturing the individual of interest is not feasible, faeces or feathers might be the only accessible source of DNA. Our method offers the possibility to amplify microsatellites and sex markers from the same faecal sample. Therefore one can obtain information about the genotype and sex of the same individual. These non-invasive samples could also be very helpful to gain information on secretive species. As more and more microsatellite markers become available (e.g. Scribner & Pearce 2000, Galbusera *et al.* 2000b), many bird species may become identifiable by their faeces alone. Studies on European grouse species demonstrate the high potential of microsatellite markers to correctly assign any droppings to species (Segelbacher unpublished). This information could give additional data when mapping birds distribution and abundance. Analysing faecal samples could even allow us to track endangered populations, to gain information about the number of individuals or their home range size, as has been shown in brown bears (Tablerlet *et al.* 1997). Using bird faeces for microsatellite analysis and sex identification offers a new perspective in ecological and population studies of birds.

3.3 Non invasive studies on feather samples

Non-invasive samples are useful for molecular genetic analysis of free ranging animals. I tested if moulted feathers collected in the field are a reliable source of DNA for genotyping microsatellite loci. I pre-screened extracts for DNA quantity and, using only samples with higher amount of DNA, obtained reliable genotyping results. PCR amplification success was higher from extracts of plucked feathers compared to moulted feathers. DNA quantity of larger feathers was higher than that of smaller feathers. This study clearly demonstrates that moulted feathers could be used for genetic studies in birds.

Introduction

Non-invasive sampling of hair or faeces has become an important tool for molecular genetic studies in free-ranging animals. Although several studies demonstrate the use of this methods in mammals (e.g. Tablerlet *et al*. 1997), non-invasive large scale studies in birds using moulted feathers collected in the field have not been undertaken. Collecting feathers or even faeces could be an alternative genetic sampling strategy and is strongly recommended for endangered species. However, one problem in using feathers for genetic analysis is that the amount of DNA extracted is lower than when using blood or tissue samples. It has been demonstrated that it is easier to obtain PCR products than to obtain reliable genotyping results for conditions, where DNA content of samples is limited (Taberlet *et al.* 1996). Taberlet *et al.* (1999) therefore recommended that a multiple-tube approach should be used for studies involving samples with a low DNA content. Each DNA amplification should be repeated independently for every locus several times. However this method is extremely time consuming and expensive when applied to large sample sizes, and in practice, few investigators working with low concentration DNA follow the multiple-tube approach (Gagneux *et al.* 1997, Kohn *et al*. 1999).
Recently Morin *et al.* (2001) proposed a new assay in which they pre-screened sample extracts for DNA quantity and controlled for this by quantitative PCR. They reduced the total number of PCR´ s and still obtained high reliability. To study the population structure of capercaillie (*Tetrao urogallus*), I am using microsatellite polymorphisms of DNA extracted from feathers.

3.3 Non-invasive studies on feather samples

As genotyping of feathers collected in the field might lead to similar problems, as has been demonstrated for hair samples I focus in this paper on four questions:

(i) Can feather samples collected in the field be used for reliable genotyping ?
(ii) Is a single tube approach reliable for feather samples ?
(iii) Does feather type affect PCR amplification success ?
(iv) Is PCR amplification success different between moulted and plucked feathers ?

Methods

Sampling of the feathers was conducted at different locations across the European range of the species (Pyrenees; Alps, Black Forest, Vosges, Norway, Northern Russia) in 1997-2000. A total of 949 feather samples were collected, put individually in envelopes or plastic bags and stored dry until analysis (1-20 months). Most samples obtained were moulted feathers collected in the field during the summer months (n=804), others came from carcasses and birds killed by hunters (n=145). Feather samples consisted of 201 large feathers (remiges and rectrices) and 748 smaller feathers.

Genomic DNA was extracted from an ≈1cm segment at the root end of individual feathers using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions with the following modifications. Digestion was performed overnight in a shaking water bath at 55° C. The buffer volume was adjusted to the size of the feather tip (400-600 µl for large feathers, 200 µl for the small ones). DNA was finally recovered in 60-100 µl of elution buffer and stored at –20° C. Individual samples were genotyped at 10 tetranucleotide microsatellite loci (Tut1-4, Tut10, BG 4-6, BG 15, BG18). Primer sequences have been described elsewhere (Segelbacher *et al*. 2000, Piertney & Höglund 2001). 2 µl of template DNA was used in a 10 µl PCR reaction in an Eppendorf Gradient thermal cycler. Individual mixes contained 0.5 mM of each nucleotide, 10 pmoles of each primer, 2.5 mM (for BG primers)/ 1.5 mM (for TUT primers) $MgCl_2$, 0.25 units of Platinum Taq DNA polymerase (Gibco BRL), 200 mM $(NH_4)_2(SO)_4$, 750 mM Tris-HCl pH 8.8, 1.5 mg/ml BSA and 100 mM beta-mercaptoethanol. PCR profiles consisted of 3 minutes denaturation at 94° C, 35 cycles of 30s denaturation at 94° C, 30s annealing at the specified temperature and 45s extension at 72° C with a final 5 minutes 72° C step. PCR fragments were resolved by electrophoresis on 6% denaturing polyacrylamide gels and afterwards stained with silver.

To detect whether contamination of samples with exogenous DNA or PCR products had occurred, tubes without samples were included in the DNA extraction and PCR amplification procedure as negative control. To avoid contamination, DNA extractions, pre PCR and post

PCR pipetting were carried out in different rooms and areosol-resistant filter pipette tips were used throughout.

(i) Estimating the utility of feathers for reliable genotyping I genotyped all DNA extracts (n=949) at all 10 loci twice. At least one additional PCR was carried out at a locus, when the individual was considered as homozygous after two PCR reactions. Samples, where less than 5 loci could be amplified or scoring of alleles was difficult due to weak gel bands were excluded from the further analysis.

(ii) To test genotyping reliability and allelic drop out rate, I used 20 randomly chosen samples, which consisted of more than two feathers from the same individual. Two DNA extractions of each individual were carried out and 2 genotyping experiments were performed using all 10 loci for each DNA extract, resulting in 4 independent genotyping experiments for each individual.

(iii) To test consistency of DNA extracts, five different DNA extracts were genotyped 10 times for each of the 10 loci (multiple-tube approach).

(iv) I used the number of successfully amplified loci as a measure of DNA quality to test the effects of different feather types and sampling on PCR amplification success with the Mann-Whitney-U Test (M-W-U).

Results

In typing all DNA extracts with 10 microsatellite loci, 16 samples (1.7%) yielded no PCR product. Forty percent of all typed samples (n=376) showed weak gel bands and less than 5 loci could be successfully scored per sample. These samples were excluded from further analysis.

Resulting genotypes for the 20 individuals, from which DNA had been extracted from two feathers, were identical for both extractions. Therefore I pooled all 4 genotypes for each individual and tested if allelic drop out could be detected. Out of 800 PCR reactions I detected 9 false homozygotes (1.1%). In 8 cases, three PCR's of the same individual showed two alleles whereas the fourth revealed only one allele. In one sample, 2 PCR products gave a single band whereas the other two showed two alleles. False homozygotes could be detected at only three loci, with one locus (TUT10) accounting for seven of the nine cases. No extra alleles and only one false homozygote at one locus could be detected when typing 5 individuals independently 10 times for all 10 loci. DNA extracted from feathers from shot individuals or carcasses yielded better genotyping results than did moulted feathers (M-W-U Test: $z=7.6$, $p < 0.001$; table 3). The number of loci typed was higher for DNA from larger feathers than from smaller feathers (M-W-U Test: $z=3.6$, $p < 0.001$; table 3).

Table 3 Effects of feather (small vs. large) and sampling type (moulted vs. plucked) for PCR amplification, shown by the number of successfully amplified loci.

Loci amplified	n (%)	Type of feather			
		Moulted	Plucked	Small feather	Large feather
<5	386 (41%)	386 (48%)	- (0%)	332 (44%)	54 (27%)
5	63 (7%)	61	2	54	9
6	50 (5%)	44	6	43	7
7	52 (5%)	48	3	42	9
8	75 (8%)	66	9	54	21
9	106 (11%)	88	18	76	30
10	218 (23%)	135 (17%)	83 (57%)	147 (20%)	71 (35%)
Total	949	804	145	748	201

Discussion

This study clearly demonstrates that feather samples, collected in the field, can be used as a valuable source of genetic information. However one should be aware of severe difficulties associated with samples with low DNA content, which are typical for non-invasive genetic studies (Taberlet *et al.* 1999). To reduce PCR errors and possible contaminations lab procedures were modified to reduce PCR errors and increase PCR amplification success (Segelbacher unpublished). Low DNA content of non-invasive samples makes PCR amplification of genomic DNA difficult and could cause erroneous results when one of two alleles at heterozygous microsatellite loci fails to be amplified (Goosens *et al*. 1998). In our study I used a similar approach as proposed by Morin *et al*. (2001) and pre-screened samples for DNA quantity. As DNA content of the feathers differed widely, I only used samples where I obtained clear scorable bands at least at 5 different loci. Allelic drop out rate was considerably low, when using only the high quantity DNA extracts. I therefore considered genotyping results as reliable, when two subsequent PCR reactions gave the same result for heterozygotes. For homozygous individuals at least three independent PCR reactions were carried out. One major disadvantage of this exclusion procedure is reduction of overall sample size to ~60% of the collected feathers in the case of this study.

Although even DNA from feathers that had been in the field for several months could be successfully genotyped, there had been a high degree of DNA degradation in moulted feathers, and PCR amplification success was reduced compared to freshly plucked feathers. About 50% of all moulted feathers could be used for reliable genotyping. In a genetic study based on moulted feathers I therefore recommend collecting twice as many feathers as the desired sample size. However the overall amplification success could be increased by using the multi-tube approach. It is therefore important to plan sampling strategy and analysis carefully.

Although non-invasive sampling in birds exhibits similar limitations as has been revealed in mammals (Taberlet *et al*. 1999), I could clearly demonstrate the use of feathers as an important genetic tool. Taking into consideration its possible limitations and expecting that some of the difficulties of non-invasive sampling and low DNA content will probably be overcome with improved methodology, non-invasive sampling will be a highly valuable tool for conservation genetics in the future.

4.1 Genetic diversity in Europe

The capercaillie is considered an indicator of healthy old growth coniferous forest communities. Thus it is highly susceptible to human-caused population fragmentation, mainly through forestry activities, and listed as threatened in western and central Europe. However the effect of anthropogenic pressure on the genetic structure and variation of capercaillie populations is unknown. In this study 557 individuals from 16 sample sites throughout Europe were typed at 10 polymorphic microsatellite loci to investigate the population structure of capercaillie from the Pyrenees to the Ural mountains. Levels of gene flow and population differentiation among the sampled regions were estimated using pairwise F_{ST} and genetic distances. Genetic diversity was significantly lower in the Pyrenees and in small isolated populations in central Europe than in more contiguous populations in the Alps and northern Europe. The data suggest that a reduction in the species' range may have led to population fragmentation in the Pyrenees and, more recently, to a break-up of the metapopulation structure and isolation of the remaining small populations in central Europe. The continued reduction of suitable habitat may lead to more populations becoming isolated remnants of a formerly widespread European distribution. Levels of genetic diversity can only be maintained through improving connections to neighbouring populations.

Introduction

The Capercaillie (*Tetrao urogallus*) is the largest and most sexually dimorphic member of all Tetraonidae (Johnsgard 1983) and is considered to be an indicator of ecosystem health and biodiversity in managed coniferous forests (Rolstadt 1989, Storch 2001). This species has a contiguous distribution in boreal forest from Scandinavia to eastern Siberia. The south-western part of the range in western and central Europe is fragmented due to the naturally patchy distribution of coniferous forests and secondarily due to habitat loss (Klaus *et al*. 1989, Storch 2001). The landscape of central Europe is dominated by farmland, and forests are distributed as isolated fragments within a matrix of open land. Altered habitat structure, excessive human disturbance and possibly increased predator numbers now render most lowland forests unsuitable for the capercaillie except in remote regions (Storch 2001). Its present distribution

mirrors that of coniferous habitats, that have remained after the last Ice Age, and its patchy distribution has possibly led to the formation of subspecies, as in the Pyrenees (Potapov & Flint 1989). More recently, since the 2nd half of the 20th century, the overall population decline throughout the western part of its range is mainly related to intensification of forestry and agriculture (Storch 2000). As a consequence the range of the capercaillie contracted along its edges, a process that contributed to further separation of the remaining populations. Most probably, capercaillie populations in central Europe became isolated from each other as the former metapopulation system broke up (Storch & Segelbacher 2000). Based on the historic and present distribution of capercaillie, we expect to find greater differentiation among isolated populations in Central Europe as compared to birds from various sites in the contiguous distribution range. The Capercaillie is listed in the Red Data Books in most western and central European countries (Storch 2000) and many populations are declining and threatened by extinction. But, even in remote areas of boreal Eurasia, capercaillie numbers have been declining in relation to increasing development and exploitation of their habitats, although the species generally is still common (Klaus *et al*. 1989, Storch 2000, 2001).

Our study areas (Figure 3) included populations in relatively pristine northern habitats as well as populations at the highly fragmented south-western edge of the range. We hypothesised that the genetic structuring among capercaillie populations will be greater in the south-west than among the birds from different sites within the contiguous and relatively undisturbed northern parts of the range. We assessed the population genetic structure of capercaillie based on 557 individuals collected across its European distribution. We used ten highly variable nuclear markers ($GATA_n$ microsatellites) (Segelbacher *et al.* 2000). Microsatellites have been successfully applied in population genetic studies of other bird species (Höglund *et al*. 1999, Lee *et al*. 2001, Shorey *et al*. 2000).

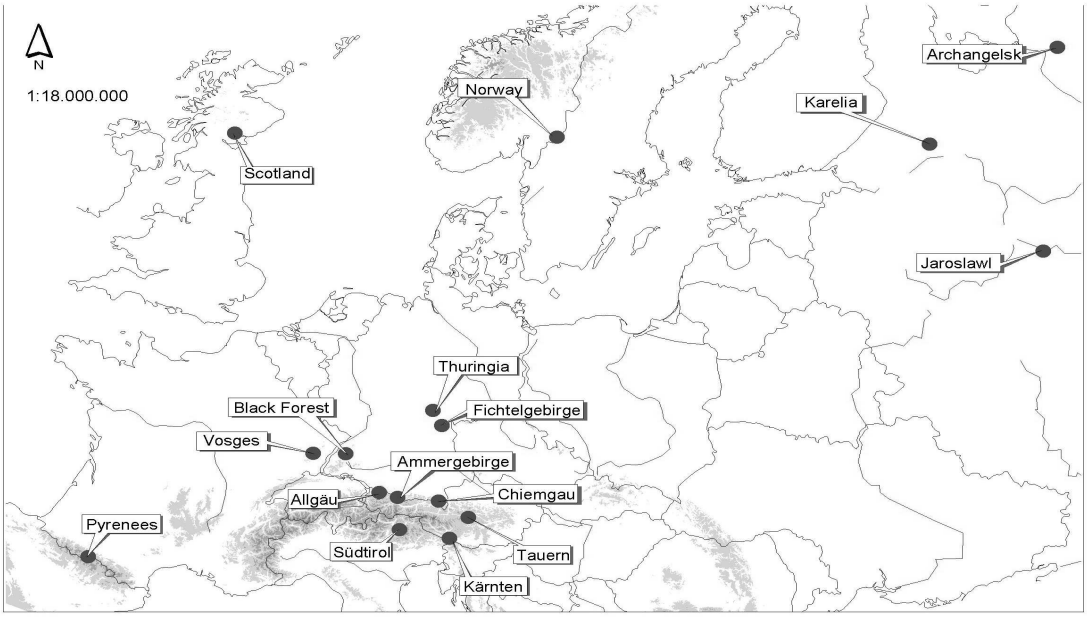

Figure 3 Map of the geographic locations of the sampled populations.

Material and Methods

Between 1997 and 2000, feathers were sampled as a source of genomic DNA at different locations over the entire European range of the capercaillie (Figure 3). Feathers from a total of 557 individuals were collected, put individually in envelopes or plastic bags and stored dry at room temperature for 1-20 months until analysis. Most samples obtained were freshly moulted feathers collected in the field during the summer months (n=412); others came from carcasses and birds killed by hunters (n=145). Genomic DNA was extracted from a circa 1cm segment at the root end of feathers using the DNeasy Tissue Kit (Qiagen) as described by Segelbacher (in press). Individual samples were genotyped at 10 tetranucleotide microsatellite loci (Tut1, Tut2, Tut3, Tut4, Tut10, BG4, BG5, BG6, BG15, BG18). PCR amplifications and genotyping were conducted as described elsewhere (Segelbacher *et al*. 2000, Piertney & Höglund 2001). PCR fragments were separated by electrophoresis on 6 % denaturing polyacrylamide gels and afterwards stained with silver. 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. Amplification of the cloned locus aided in size determination and also served as a positive control. To avoid contamination, DNA extractions, pre PCR and post PCR pipetting were carried out in different rooms and aerosol-resistant filter pipette tips were used throughout.

Data analysis

To investigate genetic structure within and between sample sites we used genotype and allele frequencies of the microsatellite loci.

Departures from Hardy-Weinberg equilibrium (HWE) were tested for each of the 10 loci by using GENEPOP Version 3.1d (Raymound & Rousset 1995b) which uses a Markov chain method following the algorithm of Guo & Thompson (1992). Relative genetic variation in each population was assessed using allele frequency data from which the mean number of alleles, allelic richness (Petit *et al*. 1998) and the unbiased expected heterozygosity H_e (Nei & Roychoudhury 1974) were determined using FSTAT 2.93 (Goudet 2001). Tests for differences among groups of populations in allelic richness, observed heterozygosity, gene diversity and F_{is} were calculated with the same program using 1000 permutations and a two-tailed test. Population differentiation can be assessed by investigating the distribution of allele frequencies across populations. An exact probability test for departures from random allelic frequencies (Raymond & Rousset 1995a) was carried out using GENEPOP. Genetic distances between populations were estimated using Cavalli-Sforza and Edwards` chord distance (Cavalli-Sforza & Edwards 1967) to reconstruct evolutionary relationships among the birds from our sample sites. It has been shown that this Euclidean distance is efficient in reconstructing phylogenetic trees when heterozygosities are high (Takezaki & Nei 1996). Genetic distances were calculated by PHYLIP 3.572 (Felsenstein 1995), as was an unrooted UPGMA and Neighbour-joining tree. In addition, individual genotypes were ordinated in a multi-dimensional space by principal-component analysis (PCA) using the program PCAGEN (http://www.unil.ch/izea/softwares/pcagen.html).

Geographic distance was the straight line distance between the centres of the different sampling areas. To examine isolation by distance we used Mantel tests within the sub-program Isolde (implemented in GENEPOP) assuming that $F_{ST}/ (1-F_{ST})$ was linearly related to the distance between the populations. Pairwise F_{ST} estimates were obtained from GENEPOP 3.1d (Raymound & Rousset 1995b; as per Weir & Cockerham 1984). To reduce the likelihood of Type I errors among multiple tests we applied a sequential Bonferroni correction (Sokal & Rohlf 1995). Populations that have experienced a recent bottleneck are assumed to display a reduction in both allele numbers and gene diversity. Excess heterozygosity in a population at mutation-drift equilibrium could indicate a recent population decline (Cornuet & Luikart 1996). To detect the genetic signature of a population decline we used the program BOTTLENECK (Cornuet & Luikart 1996). A two-phased model of mutation (TPM) was chosen, which is recommended by Luikart *et al*. (1998) for microsatellite data. Given the number of loci and sample sizes we followed the authors' recommendation in using the Wilcoxon sign-rank test to determine whether the number of loci with heterozygote excess was significant.

Based on the known distribution of capercaillie, we classified sampling locations as part of the contiguous boreal range (Archangelsk, Jaroslawl, Karelia, Norway), large ranges in central/western Europe (Alps – containing the local populations Allgäu, Ammergebirge, Chiemgau, Kärnten, Südtirol, Tauern - , Pyrenees) and small isolated ranges (Black Forest, Fichtelgebirge, Scotland, Thuringia, Vosges). We investigated genetic structure among all sampling locations. Local populations, either of the contiguous boreal distribution range or within the Alps, were regarded as connected to neighbouring populations. Using the maximum known dispersal distances (Storch & Segelbacher 2000) we treated any range as isolated, lacking a neighbouring population within 80 km.

Results

We used 10 loci in 16 sample sites, giving a potential total of 160 tests for Hardy-Weinberg equilibrium (per locus and per population). Eight loci-population combinations were monomorphic and could therefore not be tested. Of the remaining 152 tests, 35 instances with significant deviation at $p<0.05$ could be detected. These were distributed across all loci in no particular pattern and only 4 combinations were significant after Bonferroni correction. There was no evidence of significant heterozygote deficiency across all populations for any locus, indicating the absence of null alleles. Missing data were randomly distributed in the dataset and were therefore likely to be random occurrences due to a combination of failed PCR amplifications (most likely due to highly degraded DNA) and depletion of samples rather than caused by widespread null alleles. Significant heterozygote excess was detected for three populations (Table 4).

Table 4 Genetic variation estimates from the sampled regions including the mean number of alleles / locus (A), allelic richness (R), the mean observed (H_o) and expected heterozygosity (H_e) and F_{is}. Significant deviations from HWE are indicated by an asterisk.

	Abbreviation	n	A	R	H_o	H_e	F_{is}
Allgäu	Allg	50	5.8	2.45	0.666	0.637	-0.046
Ammergebirge	Amm	39	5.0	2.45	0.777	0.658	-0.184 *
Chiemgau	Chiem	94	6.1	2.48	0.749	0.658	-0.140 *
Fichtelgebirge	Ficht	11	3.8	2.29	0.741	0.599	-0.260 *
Karelia	Kar	3	2.8	2.41	0.700	0.653	-0.105
Norway	Nor	18	4.9	2.53	0.624	0.654	0.048
Pyrenees	Pyr	16	3.1	1.95	0.426	0.461	0.079
Archangelsk	Arch	43	5.3	2.39	0.599	0.606	0.010
Jaroslawl	Jaros	16	4.5	2.41	0.581	0.609	0.047
Tauern	Tau	36	5.4	2.32	0.595	0.600	0.008
Scotland	Scot	4	3.0	2.33	0.550	0.582	0.064
Black Forest	Black	78	5.2	2.26	0.566	0.558	-0.014
Kärnten	Kaern	25	5.1	2.42	0.663	0.646	-0.026
Südtirol	Süd	49	5.5	2.52	0.728	0.671	-0.086
Thuringia	Thur	18	4.6	2.47	0.608	0.641	0.053
Vosges	Vosg	57	4.4	2.16	0.583	0.544	-0.073

All microsatellite loci were polymorphic (4-10 alleles per locus) and observed heterozygosity was high (Table 4). The lowest degree of heterozygosity was detected in the Pyrenees, which showed also the lowest values of allelic richness and mean allele number. Alpine and northern populations showed greater levels of genetic diversity.

We pooled all isolated populations in Central Europe into one group (Ficht, Black, Thur, Vosg) and the remaining alpine sample sites (Allg, Amm, Chiem, Tau, Kärn, Süd) into a second group. All sample sites of the second group have geographically neighbouring local populations. Isolated populations showed a significant lower degree of allelic richness, heterozygosity and genetic diversity than the other local populations, which are part of a larger range (Table 5).

Table 5 Comparison of allelic richness, observed (H_o) and expected heterozygosity (H_e) between isolated and connected populations. Probability values are given. Departures from mutation-drift equilibrium under the IAM and TPM as given by P-values for the Wilcoxon sign-rank test (one-tailed) for heterozygote excess.

	Connected Populations	Isolated Populations	
Allelic richness	2.409	2.161	P= 0.009
H_o	0.634	0.541	P= 0.040
H_e	0.627	0.525	P= 0.006
IAM	0.002	<0.001	
TPM	0.215	0.003	

The exact tests revealed that the distribution of alleles was not identical across all the sample sites ($\chi^2=\infty$, $p<0.0001$). F_{ST} analysis across all populations and loci showed significant structuring, with $\theta=0.094$ ($p<0.001$). Both, isolated and connected populations, were not at mutation-drift equilibrium but exhibited heterozygote excess. Heterozygote excess as a transient effect of population decline has been demonstrated for loci evolving under the infinite allele model (IAM) (Maruyama & Fuerst 1985). As recommended by Spencer *et al*. (2000) we used the two phased model (TPM) for the detection of demographic bottlenecks and could therefore conclude that isolated populations showed stronger evidence of a recent population decline if TPM was the more appropriate model.

Pairwise F_{ST} values ranged from 0.009 to 0.242 (Table 6). The lowest F_{ST} values were found among the Alpine populations, suggesting high levels of gene flow between populations. The highest pairwise F_{ST} estimates were found between the Pyrenees, the Scottish, and the Russian populations. Those populations could be identified as the most genetically distinct relative to all other regions sampled.

Table 6 Pairwise F_{ST} values between populations. Bold values are significant after Bonferroni correction.

	Allg	Amm	Chiem	Ficht	Kar	Nor	Pyr	Arch	Jaros	Tau	Scot	Black	Kärn	Süd	Thur
Amm	**0.035**														
Chiem	**0.059**	**0.018**													
Ficht	**0.105**	**0.084**	**0.078**												
Kar	**0.138**	**0.116**	**0.108**	**0.175**											
Nor	**0.078**	**0.074**	**0.063**	**0.083**	**0.037**										
Pyr	**0.133**	**0.118**	**0.099**	**0.238**	**0.217**	**0.157**									
Arch	**0.139**	**0.135**	**0.132**	**0.159**	**0.019**	**0.086**	**0.194**								
Jaros	**0.154**	**0.130**	**0.129**	**0.135**	**0.061**	**0.076**	**0.174**	**0.025**							
Tau	**0.089**	**0.052**	**0.030**	**0.129**	**0.092**	**0.048**	**0.146**	**0.139**	**0.145**						
Scot	**0.110**	**0.128**	**0.112**	**0.177**	0.089	**0.069**	**0.212**	**0.103**	**0.112**	0.112					
Black	**0.098**	**0.092**	**0.092**	**0.118**	0.108	**0.093**	**0.176**	**0.118**	**0.132**	**0.083**	0.096				
Kär	**0.065**	0.024	0.010	**0.103**	**0.075**	**0.042**	**0.092**	**0.120**	**0.119**	0.009	0.104	**0.084**			
Süd	**0.069**	0.026	**0.021**	**0.082**	**0.085**	**0.036**	**0.147**	**0.122**	**0.112**	0.022	0.103	**0.077**	0.012		
Thur	**0.054**	**0.052**	**0.067**	**0.120**	**0.070**	**0.074**	**0.144**	**0.109**	**0.115**	0.063	0.086	**0.065**	0.048	**0.054**	
Vosg	**0.142**	**0.134**	**0.105**	**0.175**	**0.156**	**0.090**	**0.241**	**0.167**	**0.206**	**0.103**	0.213	**0.129**	**0.115**	**0.097**	**0.120**

Estimates of the chord distances paralleled the results from the pairwise F_{ST} values. An unrooted Neighbour-joining tree reveals the relationships of genetic distances to all populations (Figure 4), where the length of the tree branch is relative to the genetic distance. The phenogram suggests that northern and Alpine populations are distinct to the isolated central European populations. The tree also shows that the Alpine populations cluster together, the Russian populations cluster with each other and the other populations are each on their own branch. We found almost identical results analysing chords distance with an UPGMA clustering method. The results of PCA showed a similar pattern (Figure 5). Scores of the local populations were plotted on two principal axes (PC1 and PC2), which cumulatively explained 49% of the total genetic diversity. This plotting showed a clear separation of the local populations of the boreal range, the alpine populations and the birds of the small ranges.

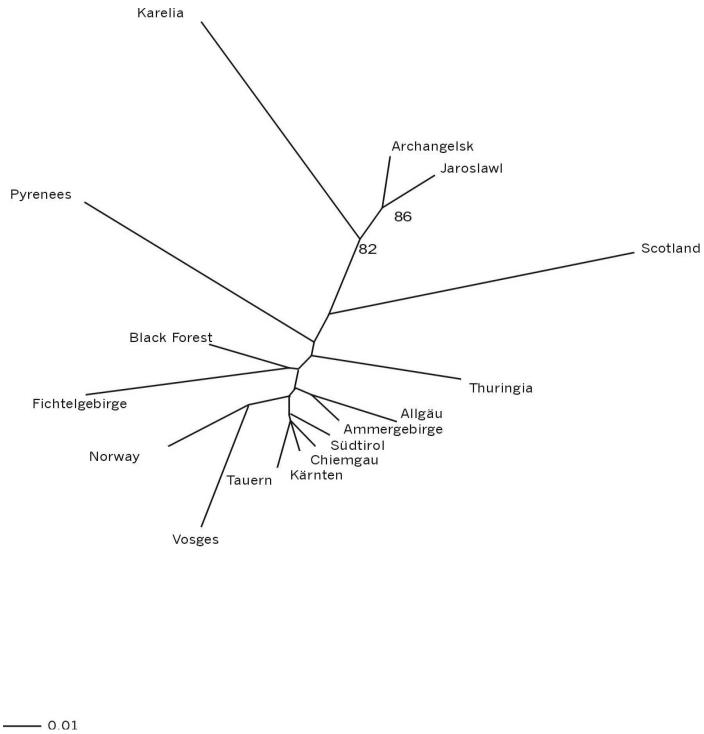

Figure 4 Unrooted Neighbour-joining phenogram depicting distance relationships based on Cavalli-Sforzas and Edwards chord distance among 16 capercaillie populations. Values on nodes represent the percentage of bootstrap replicates over loci (n=1000). Branch lengths are proportional to the genetic distance among populations.

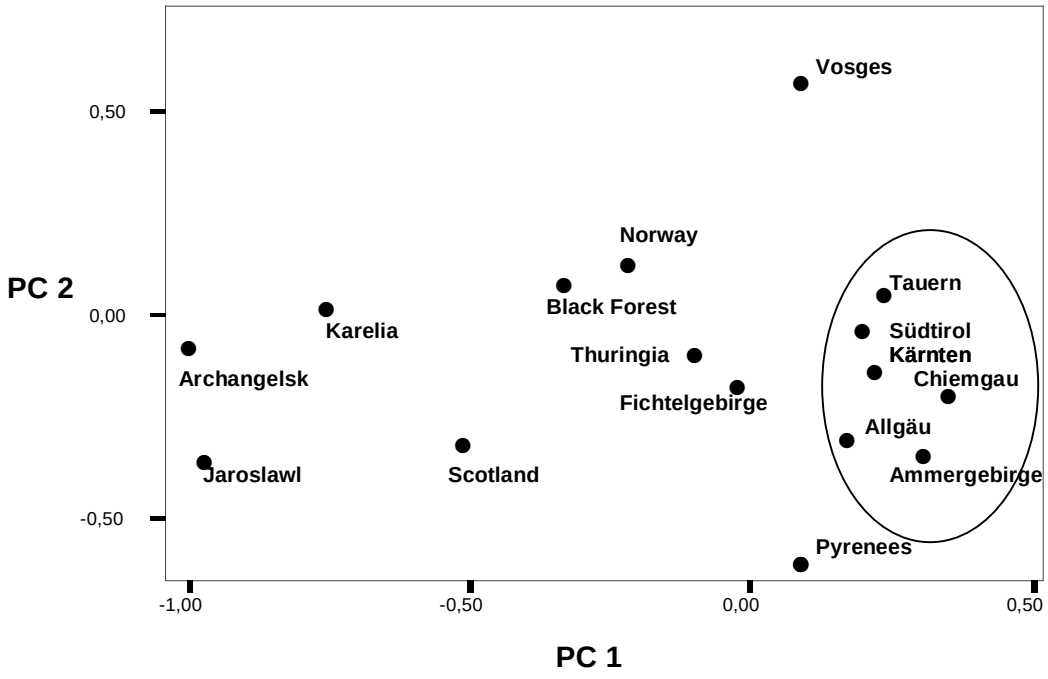

Figure 5 Scores of European capercaillie microsatellite genotypes plotted on the two first axes (PC1, PC2) of a principle component analysis performed using PCAGEN. Alpine Populations are circled.

The results from the F_{ST} pairwise comparisons were entered into a two-way Mantel test with geographical distance. This analysis of isolation by distance detected a strong effect of geographic distance on genetic distance between all sample sites (Mantel test, $p<0.001$) (Figure 6).

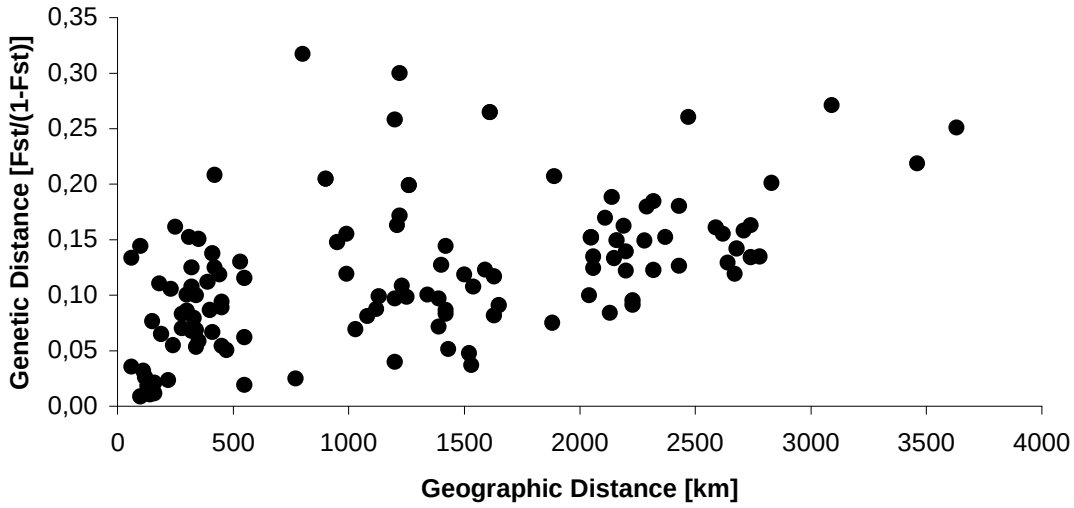

Figure 6 Genetic distance $F_{ST}/(1-F_{ST})$ is correlated to geographical distance.

Discussion

This study has elucidated the population genetic structure of capercaillie populations across large parts of the species' European range. Populations in Central Europe have become separated from the Alpine population and from each other during the 20th century and before, when many local populations disappeared (Storch 2001). Considering the relatively short mean dispersal distances of capercaillie (1-2km, Storch & Segelbacher 2000), it is likely that among these populations gene flow became restricted as their formerly neighbouring populations have become extinct (Storch 2000). Central European populations, except for those in the Alps, showed low genetic diversity, observed heterozygosity and allelic richness, suggesting that they are genetically isolated. Allelic richness is the most appropriate measure of genetic diversity for conservation purposes (Petit *et al*. 1998). It was lowest in isolated local populations, indicating the importance of connectedness to other populations to maintain high levels of genetic diversity. The decreased levels of H_e and increased levels of genetic structure based on F_{ST} and chord distance in the Pyrenean, Scottish and central European populations are consistent with other studies of insular populations. In these studies on brown bears (*Ursus arctos*) (Paetkau *et al*. 1998) and wolverines (*Gulo gulo*) (Kyle & Strobeck 2001) respectively, decreased genetic variability was attributed to population fragmentation from a previously larger contiguous population. Nucleotide diversity was lower in an isolated population of Siberian Jay (*Perisoreus*

infaustus) than in populations within its contiguous area of distribution, which suggests that isolation by anthropogenous habitat fragmentation reduces gene flow (Uimaniemi *et al*. 2000). To explain the generally low diversity observed in the central and southern European populations of the capercaillie requires consideration of both natural and anthropogenic factors. We suggest that the observed level of genetic structuring reflects low effective population sizes, restricted gene flow and potentially break-up of a metapopulation system, whereas the level of genetic structure in the northern and Alpine regions is consistent with high levels of gene flow. Genetic diversity was lowest in the Pyrenean population, which is situated at the south-western end of the capercaillie's distribution range. This population is geographically isolated, and unlike in the central European populations, no other populations have existed within 300km for centuries (Klaus *et al* 1989). The Scottish population, also geographically isolated, shows similarly low levels of genetic diversity, however the sample size was quite low in this case. Genetically the Scottish birds were closely related to the Karelian population, which may appear surprising. However, the autochtoneous birds became extinct in Scotland in the late 1700s and the recent population are descendants of birds re-introduced from Sweden (Lever 1977, Starling 1991).

All presently described subspecies of the capercaillie have been classified according to their variation in morphology and behaviour (Potapov & Flint 1989, see also del Hoyo *et al*. 1994). Considering the subspecies recognised by Potavov & Flint (1989), the sample site in Karelia belongs to the same subspecies (*Tetrao urogallus urogallus*) as Swedish birds.. However, morphological variation among subspecies is largely clinal. Our genetic data show that the Pyrenean birds form a genetically clearly distinct unit. Allozyme data (Linden & Teeri 1985) have demonstrated earlier that even within the subspecies classified in Finland, there was distinct genetic differentiation, supporting the notion of genetic gradients even within a contiguous distribution.

One surprising result of our analyses according to chord distances and multivariate analysis of F_{ST} was that Norwegian birds were quite distinct from Russian birds and more similar to those of central Europe, although geographic distances would have predicted the opposite. Since Fenno-Scandia was completely glaciated and later re-colonised in two routes, one from south-west and one from north-east, there is a well known "hybrid" zone in various taxa across Sweden/Norway (Taberlet *et al*. 1998). This has been demonstrated for rodents (Jaarola *et al*. 1999) and warblers (Bensch *et al*. 1999). As the sample site is located in southern Norway this could likely explain that those birds are more related, albeit distantly, with central Europe.

Pairwise tests of F_{ST} showed significant differences between most of the sample sites. Sample sizes of the Scotish and Karelian birds are quite small and this could explain that differences might not be significant although F_{ST} values are relatively high. Pairwise F_{ST} values are considerably lower between Alpine populations and gene flow is likely to be frequent amongst them. Although capercaillie numbers in the Alps may be at least locally declining local

populations seems to be still well connected. We therefore conclude that populations in the Alps still have a metapopulation structure, i.e. local populations are geographically distinct but connected by dispersal (Storch & Segelbacher 2000) and thus maintain genetic diversity across the Alps as a whole. Formerly connected populations as e.g. Black Forest and Vosges, or Fichtelgebirge and Thuringia are now also genetically isolated and therefore exhibit lower levels of genetic diversity.

A transient pattern of deviation from mutation-drift equilibrium in terms of excess heterozygosity will result from a population decline (Cornuet & Luikart 1996). Several studies have demonstrated excess heterozygosity in populations as evidence of bottlenecks (Dallas *et al*.1999, Pierson *et al*. 2000). Assuming that the TPM is the most appropriate model (Luikart *et al*. 1998) we could detect a decline in isolated populations as compared to those which are part of larger populations. However, even those populations exhibited a heterozygote excess under the IAM model, indicating that even the larger populations might show negative population trends. This is in accordance with reports of population declines throughout Europe based on field observations (Storch 2000, Storch 2001).

4.3 Metapopulation structure in the Alps

Habitat fragmentation may have significant consequences for population genetic structure because geographic distance and barriers may impede gene flow. In this study, we tested the relative influence of gene flow and drift on population structure of capercaillie both within and between regions in the Alps. We studied 18 regional populations across the northern and central Alpine range and estimated genetic variation with ten microsatellite loci. We discovered genetic variation within all populations, but no significant differences in numbers of alleles or heterozygosity between the northern and central group of populations. Geographic distance had a significant effect on genetic distance when comparing paired populations in the northern group, however in the central group, there was no evidence of isolation by distance. The strong relationship between gene flow and geographic distance in the northern group could be best explained by a stepping-stone model of dispersal. In the central group, however, genetic drift is the most likely explanation for the observed differentiation of populations.

Introduction

Fragmentation of habitats determines population structure as well as the distribution of gene frequencies within populations (Giles & Goudet 1997, Hale 2001). Loss of genetic diversity, increased inbreeding and stochastic demographic processes are suggested as the main causes for extinction of isolated populations of limited size (Soulé 1987, Frankham 1995a). Mathematical models predict a reduction of heterozygosity in metapopulations (Gilpin 1991). However, if metapopulations are connected by relatively high migration rates between populations, reduction in genetic diversity might not be great (Hanski 1999). As shown in wild populations of the butterfly *Melitaea cinxia* (Saccheri *et al.* 1998), genetic variation was not reduced in a metapopulation system due to a rather high rate of gene flow.

Among genetic models of metapopulation structure, the island model represents long distance gene flow; whereas the strict stepping-stone model is more useful for the study of short distance gene flow (Le Corre & Kremer 1998). The island metapopulation model describes a situation, where migration is assumed to be random within the entire metapopulation, and thus, gene flow is independent of geographic distance. In a stepping-stone model, patches are thought to be

more likely colonised by individuals originating from nearby rather than from distant populations. As the dispersal ability of individuals is limited, natural populations are more realistically described by the stepping-stone model (Le Corre & Kremer 1998).

Observational data suggest that gene flow is likely to be limited among capercaillie populations due to short mean dispersal distances (Storch & Segelbacher 2000). Capercaillie (*Tetrao urogallus*) inhabit boreal and montane forests of Eurasia. In the Alps they occupy the upper slopes of forested mountain ranges (Storch 2001). The Alpine landscape is characterised by mountain ranges reaching altitudes of 2,000 to 4,000 m and covering areas of typically 100–400 km^2. Thus, mountain ranges form patches of contiguous forests separated by open valleys a few kilometres wide and interspersed by alpine habitats above the treeline. In this landscape the distribution of capercaillie is spatially structured, with local populations on separate mountain ranges. Juvenile dispersal and adult movement distances reported from the entire range (Storch & Segelbacher 2000) and telemetry results from the Bavarian Alps (Storch 1995) suggest that only few capercaillie move between habitat patches. Therefore, a metapopulation structure has been assumed for the capercaillie in the Alps (Storch 1993, 1997, Storch & Segelbacher 2000).

Because of their relatively large spatial requirements with annual home ranges of several hundred hectares in size, capercaillie are highly susceptible to habitat fragmentation (Rolstad and Wegge 1987, 1989, Storch 1995). Altered habitat structure and excessive human disturbance render many forests unsuitable for the species now and capercaillie have disappeared from most lowland forest during the 20^{th} century (Klaus and Bergmann 1994, Storch 2001). In the Alps, the population density of capercaillie varies locally between mountain ranges, and landscape-scale habitat variables such as the spatial dispersion of habitats are thought to play an important role for the size and dynamics of populations (Storch 2001).

After the last Ice Age the central European range of the capercaillie has been naturally fragmented due to the restriction of its habitat, coniferous forest, to mountainous areas (Klaus *et al.* 1989). In the Alps, forests inhabited by capercaillie are separated by high-altitude areas above treeline. Further fragmentation resulted from forest clearings in most Alpine valleys for farming, which date back several hundred to thousand years and more (Bätzing 1991). The spatial distribution of forest, and thus of capercaillie habitats, in the Alpine landscape has been more or less constant during the last hundreds of years, but suitability of habitat patches for capercaillie has probably varied over time due to abiotic and anthropogenic changes (see Klaus *et al.* 1989, Storch 2001). During the 20^{th} century and particularly since 1950 a severe population decline has been observed throughout Europe, that coincided with a contraction of the species´ r ange along the northern edge of its Alpine distribution. At the beginning of the 21^{st} century, capercaillie population in the Alps are generally assumed to be declining at various rates, although locally stable or even increasing populations have been reported (Storch 2000, 2001). In the northern Alps of Switzerland, Austria and Germany, declines appear to be most

pronounced along the edge of the range (Storch 2002, K. Bollmann, pers. comm, H. Zeiler, pers. comm., A. Zeitler, pers. comm.).
In this paper, we compare the genetic structure of populations along the northern edge of the Alpine distributional range with populations from other parts of the Alps.
We used microsatellite DNA typing techniques to measure the effects of geographic distance on genetic differentiation between populations of *Tetrao urogallus*. We asked specifically: what are the consequences of the spatial metapopulation structure on genetic diversity and gene flow? Which model, island or stepping-stone model, describes best dispersal between neighbouring populations? What are the differences in gene flow among peripheral populations of the capercaillie´ s Alpine range compared to populations living in the centre of the range ?

Material & Methods

Sampling

We sampled feathers as a source of genomic DNA from different locations over the Alpine range of the species from 1997-2000 (Figure 7). Feathers from a total of 296 individuals (201 males/ 91 females) were collected, put individually in envelopes or plastic bags and stored dry until analysis (1-20 months). Extraction of genomic DNA, polymerase chain reaction (PCR) amplification and genotyping were conducted as described in Segelbacher (in press). PCR fragments for ten microsatellite loci were then separated by electrophoresis on 6 % denaturing polyacrylamide gels and made visible afterwards with the silver staining method. Only individuals with ≥ 7 scorable loci were included in the analysis.
To assess metapopulation structure at the northern edge of the Alpine distribution range we sampled feathers across the German Alps and Vorarlberg (western Austria). We grouped sample sites into geographically separated units 10x10 km^2 – 20x20 km^2 in extent that comprised several mountain ranges. In the following, we will term these spatial units “study areas”, and the birds from each study area a “population”. As control populations, defined as above, we sampled feathers in different study areas in the central (Italy) and eastern (Austria, Slovenia) parts of the Alpine range of the capercaillie.
The group of Northern populations (Northern, N) includes all those located at the northern edge of the Alpine distributional range where connectivity to other populations is geographically limited. These are the populations Vorarlberg, Allgäu East, Allgäu West, Ammer, Ester, Mangfall, Chiemgau Middle and Chiemgau East. Populations from areas in the southern and eastern parts of the alpine distribution range comprises the second group (Central, C) and were all surrounded by neighbouring populations (Dachstein, Schladming, Tauern, Nockberge, Reisseckgruppe, Sextner Dolomiten, Aldein and Trudner Horn) (see Figure 7). Geographic

distance refers to the straight line distance between the centres of the different study areas and distances between the sampled populations are in the same order of magnitude for central and northern populations. Populations Gleinalm and Slowenien were excluded in the group comparisons, as they were located at the southern edge of the Alpine distribution.

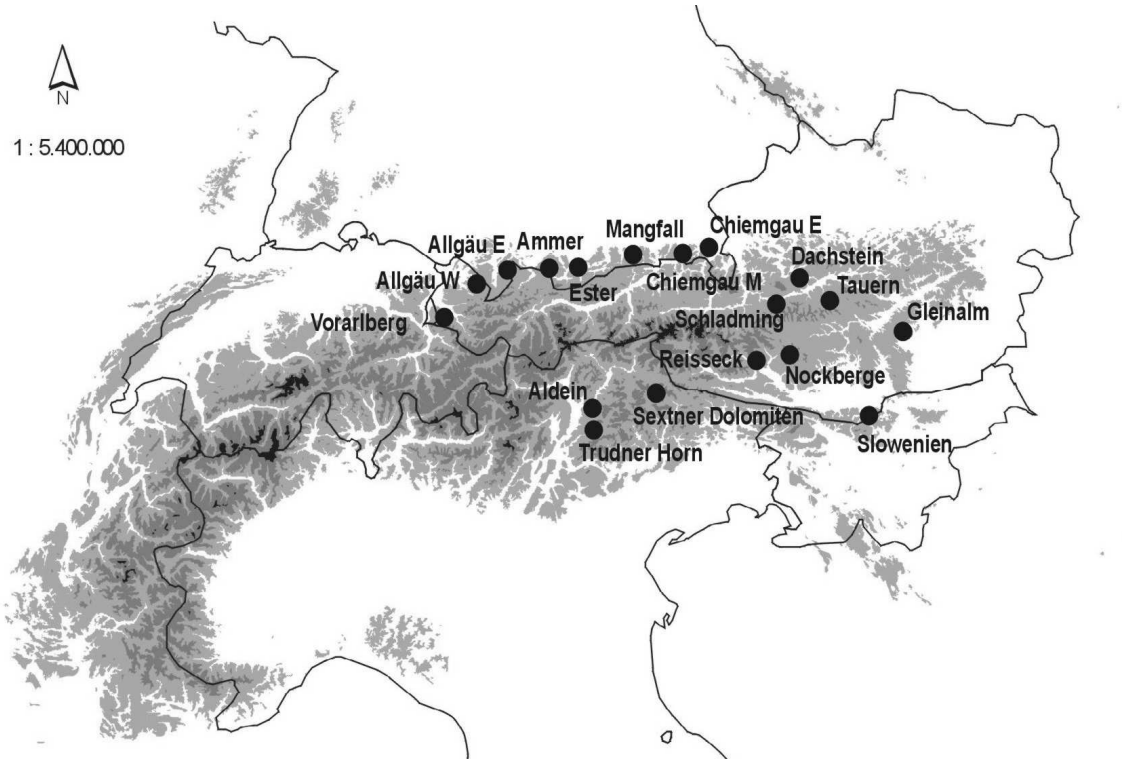

Figure 7 Map of the geographic locations of the sampled populations.

Data analysis

We investigated genetic variation of all sampled populations by analysing departures from Hardy-Weinberg distribution and linkage disequilibrium and levels of genetic diversity within populations. Two tests for departures from Hardy-Weinberg equilibrium were used. The first test took account of both heterozygote deficit and heterozygote excess (HW). The second test took account only of heterozygote deficit (HD) or heterozygote excess (HE) respectively. Departures from Hardy-Weinberg equilibrium (HWE) were tested for each of the 10 loci, as assessed by the computer program GENEPOP Version 3.1d (Raymound & Rousset 1995b), which uses a Markov chain method following the algorithm of Guo & Thompson (1992). Amount of relative genetic variation in each population was assessed using allele frequency data from which the mean number of alleles per locus, allelic richness (Petit *et al.* 1998) and the unbiased expected heterozygosity H_e (Nei & Roychoudhury 1974) were determined using the program FSTAT 2.93 (Goudet 2001). Tests for significance of differences among groups of populations for allelic

richness, observed heterozygosity, gene diversity and F_{ST} were calculated with the same program using 1000 permutations and a two-tailed test. Population differentiation can be assessed by investigating the distribution of allelic frequencies across populations. An exact probability test of differences in the allelic frequencies (Raymond & Rousset 1995a) was carried out using the program GENEPOP. Pairwise F_{ST} estimates (θ) were also obtained from GENEPOP (Raymound & Rousset 1995b; as per Weir & Cockerham 1984).

To reduce the likelihood of Type I errors among the multiple tests we applied a Bonferroni correction (Sokal & Rohlf 1995) whenever appropriate.

Genetic distances between populations were estimated using Cavalli-Sforza and Edwards` chord distances (Cavalli-Sforza & Edwards 1967) to reconstruct evolutionary relationships among the populations from our study areas. Calculations of these genetic distances were performed by PHYLIP 3.572 (Felsenstein 1995). The same program was used for the construction of the unrooted Neighbour-joining tree. The relative degree of genetic divergence between all 18 local populations studied was examined by multidimensional scaling of Nei´ s standard genetic distance (Nei 1972) using SPSS. Paetkau *et al.* (1997) recommend the use of this distance over small geographical scales for microsatellite data. To examine isolation by distance we used Mantel tests, assuming that $F_{ST}/(1-F_{ST})$ was linearly related to the distance between the populations. The significance of the correlation between genetic distance (θ) and geographic distance was tested using a Mantel test implemented in GENEPOP. The value of the proportion of 1000 permutations of the Mantel statistic Z that was greater than the observed value was assumed as a measure of the probability value for the deviation from null hypothesis assumed that no association exists between θ and geographic distance. If pairwise population tests gave zero values, they were excluded from the scatterplots, as they do not contribute any information. Additionally, we performed a permutation test (Schönfisch 2001 unpublished) to test significance of the correlation between Nei´ s standard genetic distance and geographic distance.

Furthermore, we tested population genetic structure by means of a hierarchical analysis of molecular variance (AMOVA) using ARLEQUIN 2.0 program (Schneider *et al.* 2000) with three levels of population structure: total of populations, groups (northern, central) and local populations. We also calculated probabilities of individuals belonging to a population using a Bayesian approach. These analyses were performed using the program GENECLASS 1.0.02 (Cornuet *et al.* 1999). For each individual, the marginal probability of each individual in each population was calculated, based on the allele frequencies of the samples, and an individual was assigned to the population in which it had the highest marginal probability.

Populations that have experienced a recent bottleneck will display a reduction in allele number and more slowly, in heterozygosity. Excess heterozygosity in a population at mutation-drift equilibrium could therefore indicate a recent population decline (Cornuet & Luikart 1996). To detect the genetic signature of a population decline we used the program BOTTLENECK

(Cornuet & Luikart 1996). Given the number of loci and sample sizes in our data set, we followed the authors´ recommendation in using the Wilcoxon sign-rank test to determine if significant numbers of loci showed heterozygote excess.

Results

Genetic variation within populations

No population from any study area exhibited significant heterozygote deficit. All but one population of the northern Alpine group displayed non-equilibrium proportions of genotypes according to one or more tests (Table 7). Deviation from Hardy-Weinberg equilibrium seems to be a common characteristic of local capercaillie populations. Alleles of only three pairs of loci (out of 846) were significantly deviant from linkage equilibrium following a Bonferroni correction. Significant linkage occurred in different locus pairs, suggesting that physical proximity of the loci was not responsible for linkage.

Levels of heterozygosity, allelic richness and mean number of alleles displayed a similar pattern within all investigated populations and were all quite homogenous (Table 7).

Table 7 Genetic diversity of 19 populations of Capercaillie: n, number of individuals, M/F, number of males/females, A, mean number of alleles per locus, R, allelic richness, (H_o), the mean observed heterozygosity, (H_e), expected heterozygosity and $F_{is.}$ The probability of departure from Hardy-Weinberg equilibrium is indicated HW (heterozygote deficit/excess) and HE (heterozygote excess), * = p>0.5.

	Abbrev.	n	Sex M/F	A	R	H_e	H_o	HW	HE	F_{is}
Vorarlberg	Vor	12	7/5	4.0	2.18	0.572	0.689	NS	*	-0.149
AllgäuE	AllgE	17	7/10	4.2	2.42	0.603	0.625	*	NS	0.080
AllgäuW	AllgW	18	14/4	4.4	2.35	0.613	0.695	NS	*	-0.098
Ammer	Amm	20	11/9	4.3	2.43	0.647	0.781	*	*	-0.178
Ester	Est	9	8/1	3.2	2.38	0.581	0.855	NS	*	-0.406
Mangfall	Mang	10	5/5	4.0	2.49	0.604	0.710	NS	NS	-0.107
ChiemgauM	ChgM	21	18/3	4.4	2.28	0.596	0.713	NS	*	-0.159
ChiemgauE	ChgE	73	41/32	5.7	2.49	0.659	0.759	*	*	-0.143
Dachstein	Dach	5	3/2	2.7	2.29	0.519	0.558	NS	NS	0.075
Schladming	Schl	25	21/4	4.9	2.34	0.595	0.612	NS	NS	-0.006
Tauern	Tauer	11	10/1	4.0	2.34	0.548	0.557	NS	NS	0.033
Nockberge	Nock	4	4/0	3.3	2.46	0.559	0.675	NS	NS	-0.066
Reisseckgruppe	Reiss	5	4/1	3.1	2.40	0.564	0.777	NS	NS	-0.021
Slowenien	Slow	6	6/0	3.7	2.46	0.594	0.590	NS	NS	0.104
Gleinalm	Glein	4	2/2	2.7	2.19	0.491	0.575	NS	NS	-0.015
Sextner Dolomiten	Sext	25	21/4	4.7	2.55	0.662	0.765	*	*	-0.129
Aldein	Ald	18	15/3	4.0	2.39	0.618	0.674	NS	NS	-0.052
Trudner Horn	Trud	6	5/1	3.2	2.46	0.570	0.725	NS	*	-0.168

Population differentiation

Five analyses of the spatial pattern of genetic differentiation were performed. In a first analysis, levels of genetic differentiation were estimated between all study areas (Table 8).

Among all populations, there are significant differences in allele frequencies among populations, but the magnitude of the differentiation is generally low or moderate, as measured by F_{ST}. Pairwise tests of genotypic differentiation among all samples are indications of significant differentiation between most of the populations in their genotypic proportions (Table 8). Overall θ across loci is 0.046 ($p<0.001$) indicating significant differentiation among populations. Pairwise F_{ST} values range from 0 to 0.143 (Table 8). Neighbouring populations generally showed low degrees of differentiation whereas more distant populations exhibited higher genetic differentiation.

Table 8 Pairwise F_{ST} (above diagonal) values and genotypic differentiation for each population pair using Fishers exact test (below diagonal). Underlined values are significant after Bonferroni correction ($p<0.05$). Negative values due to sampling errors and indicate gene flow without limitation.

		Northern Populations								*Central Populations*									
		Vor	AllgW	AllgE	Amm	Est	Mang	ChgM	ChgE	Dach	Schl	Tauer	Nock	Reiss	Slow	Glein	Sext	Ald	Trud
Northern	Vorl	—	0.015	0.048	0.083	0.052	0.032	0.073	0.078	0.109	0.096	0.113	0.099	0.036	0.096	0.143	0.098	0.092	0.075
	AllgW	*	—	0.014	0.049	0.044	0.035	0.053	0.069	0.092	0.096	0.095	0.073	0.020	0.098	0.126	0.075	0.076	0.081
	AllgE	***	*	—	0.058	0.044	0.039	0.048	0.081	0.110	0.098	0.115	0.098	-0.007	0.097	0.119	0.099	0.094	0.092
	Amm	***	***	***	—	0.032	0.012	0.037	0.028	0.050	0.066	0.066	0.033	0.010	0.046	0.058	0.048	0.033	0.053
	Est	***	**	**	**	—	0.019	0.010	0.019	0.065	0.041	0.059	0.057	0.039	0.046	0.115	0.037	0.040	0.015
	Mang	*	*	**	n.s	*	—	0.013	0.029	0.057	0.042	0.073	0.066	0.005	0.032	0.067	0.053	0.020	0.029
	ChgM	***	***	***	***	n.s	*	—	0.019	0.047	0.032	0.045	0.026	-0.008	0.034	0.064	0.051	0.046	0.034
	ChgE	***	***	***	***	n.s.	n.s	**	—	0.013	0.033	0.034	0.002	-0.015	0.016	0.072	0.026	0.028	0.038
Central	Dach	***	***	***	**	**	*	*	n.s.	—	0.027	-0.016	-0.008	-0.035	-0.005	0.106	0.024	0.027	0.068
	Schl	***	***	***	***	0.001	***	***	***	n.s.	—	0.006	0.025	0.010	-0.024	0.043	0.019	0.044	0.029
	Tauer	***	***	***	***	***	***	***	***	n.s.	*	—	-0.015	0.047	-0.001	0.066	0.026	0.049	0.051
	Nock	***	**	***	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	—	-0.009	-0.007	0.057	0.022	0.022	0.061
	Reiss	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	—	-0.024	0.014	-0.010	-0.025	0.026
	Slow	***	***	***	***	*	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	—	0.043	-0.007	0.018	0.004
	Glein	***	***	***	**	***	n.s.	**	***	n.s.	*	*	n.s.	n.s.	n.s.	—	0.085	0.051	0.028
	Sext	***	***	***	***	**	***	***	***	n.s.	***	***	*	n.s.	n.s.	***	—	0.028	0.035
	Ald	***	***	***	***	**	n.s.	***	***	n.s.	***	***	*	n.s.	*	**	***	—	0.024
	Trud	***	***	***	***	n.s.	n.s.	***	***	n.s.	***	**	n.s.	n.s.	n.s.	n.s.	*	*	—

Northern populations differed significantly from central populations (θ= 0.023, p<0.001). Although northern and central populations did not differ in allelic richness and genetic diversity, genetic structure of the northern populations was more distinct than in the central ones (Table 9).

Table 9 Comparison of allelic richness, observed (H_o), expected heterozygosity (H_e) and F_{ST} between northern and central populations. Probability values are given. Departures from mutation-drift equilibrium under the TPM are given as P-values for the Wilcoxon sign-rank test (one-tailed) for heterozygote excess.

	Northern populations	Central populations	
Allelic richness	2.37	2.37	n.s.
H_o	0.73	0.66	P=0.04
H_e	0.64	0.63	n.s.
F_{ST}	0.046	0.025	P=0.11
TPM	0.003	0.133	

A second analysis investigated the evolutionary relationship between the birds from our study areas (Figure 8). Using chord distances, the resolved consensus tree reflected only partly the spatial pattern of the study areas. Northern populations group together and are distinct from the central group. The geographic distribution of the central group in particular, could not be truly recovered suggesting that, in this group gene flow is higher than in the northern group. This observation is supported by the results of the pairwise differences in F_{ST}.

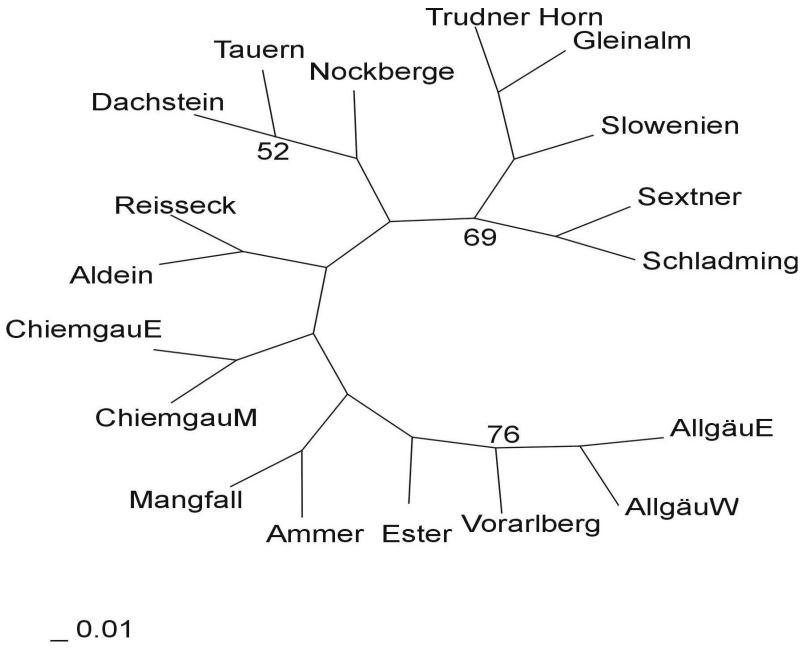

Figure 8 Neighbor-joining tree based on Cavallis-Sforza & Edward´ s chord distance. Consensus Tree with bootstrap values. Only values shown when P>50%.

In a third analysis, we assessed the relative degree of genetic differentiation between local populations using multi-dimensional scaling of Nei´ s (1972) genetic distance (Figure 9). Two features become apparent. Firstly, populations of the two groups form two distinguishable units. Secondly, the first dimension separates the two groups of populations and differentiates the northern group of populations. The second dimension separates the central populations from one another but does not differentiate between the two groups.

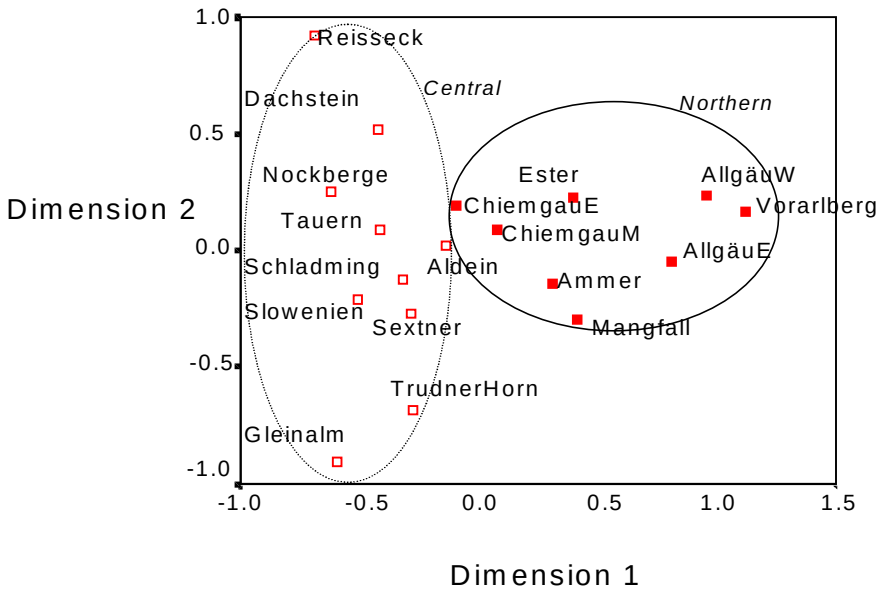

Figure 9 Multidimensional scaling plot of Nei´ s distance among the sample sites. Northern and central populations are enclosed by ellipses.

In a fourth analysis, we investigated genetic variation between populations with an analysis of molecular variance (AMOVA). We found significant effects among groups, among populations within groups, and among individuals within populations. However, most of the variance could be explained by the variation among birds within populations (Table 10).

Table 10 Analysis of molecular variance (AMOVA) based on the number of different alleles among microsatellite loci. (Significant test were based on 1023 permutations. AMOVA based on the sum of squared differences yielded almost identical p-values (not shown).)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.66	0.000 V_a	0.09
Among populations within groups	16	8.57	0.002 V_b	0.35
Within populations	416	206.78	0.497 V_c	99.56
total	433	216.01	0.50	

Fixation indices: F_{SC}=0.003. F_{ST}=0.004. F_{CT}=0.001

V_c and F_{ST}: P(random value ≤ observed value) = 0.0000+-0.0000
V_b and F_{SC}: P(random value ≥ observed value) = 0.0019+-0.0014
V_a and F_{CT}: P(random value ≥ observed value) = 0.0196+-0.0041

Most of the individuals studied could be attributed to the population of origin using an assignment test, although a substantial number of misclassified individuals remained (data not shown). As small sample sizes and missing values could lead to erroneous results, we repeated the assignment using only individuals with complete genotype data (10 loci typed) and populations for which sample sizes were larger than 5 individuals. Although this reduced overall sample size, nearly all individuals could be classified to their population of sampling; in this case misclassified birds indicated migration between neighbouring populations (Table 11).

Table 11 Assignment of individuals to populations (using only individuals with 10 loci typed and populations with at least 5 individuals).

	Vor	AllgW	AllgE	Amm	ChgE	Schl	Tauer	Ald
Vor	**5**							
AllgW	**1**	**6**						
AllgE		**1**	**7**					
Amm				**8**	**1**			
ChgE					**23**	**1**	**1**	
Schl	**1**				**2**	**18**	**3**	**1**
Tauer							**9**	
Ald								**11**

Isolation by distance

Over all populations, a significant positive relationship between pairwise F_{ST} or $F_{ST}/(1-F_{ST})$ (Rousset 1997) and geographical distance could be observed (Table 12). A group-wise analysis revealed a strongly significant correlation between F_{ST} and geographic distance for the northern populations; the central populations, although distributed along a similar geographic gradient, showed only a weak and non-significant correlation between genetic differentiation and geographic distance. We obtained similar results when testing the significance of the correlation between Nei´ s standad genetic distance and geographic distance (Figure 10).

Table 12 Mantel statistics: g, Standard normal variate, Z, Mantel statistic, r, product-moment correlation, p, significance values.

Populations analysed	g	Z	r	p
All populations	5.88	3312	0.609	<0.001
Northern populations	3.36	284	0.653	<0.001
Central populations	0.31	473	0.056	n.s.

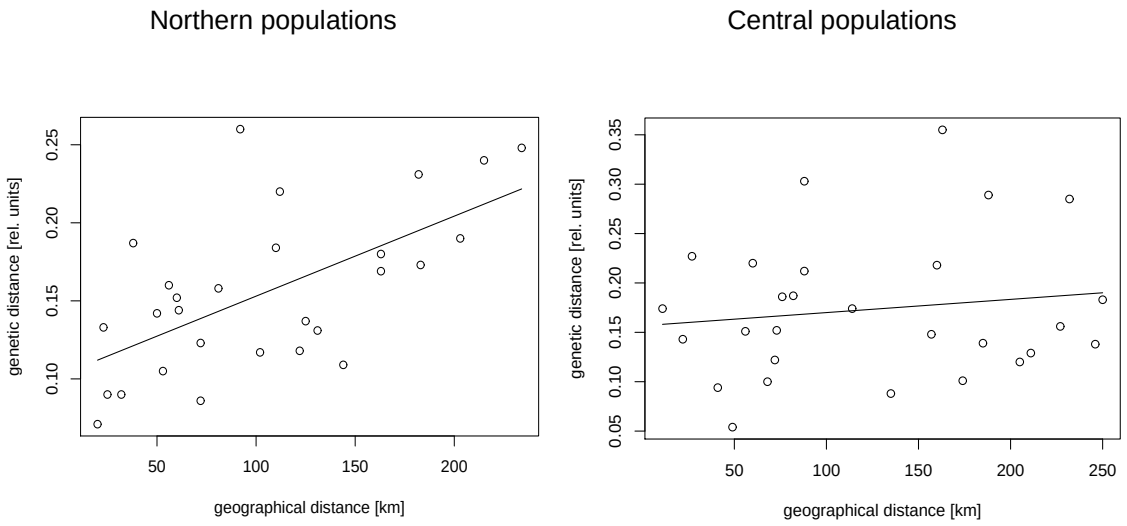

Figure 10 Genetic distance (Nei) plotted against geographical distance (km) for the northern alpine and central alpine populations. Northern populations r=0.58 (p=0.012), Central Populations r=0.11(p=0.250).

Population decline

Assuming a two-phased model (TPM), populations of the northern group showed a highly significant difference between observed and expected levels of heterozygosity under the assumption of mutation-drift equilibrium (Table 9). In central populations, however, we could not detect a significant difference between the observed and expected values. We therefore assume a stronger recent decline in population size in the northern populations than in the populations of the central group.

Discussion

Genetic variation within populations

Populations of capercaillie show a metapopulation structure in the Alps. They occur in spatially distinct habitat patches and the aim of our study was to assess some of the genetic consequences of this spatial distribution pattern.

We could show that many (9 out of 18) of the capercaillie populations studied were not in Hardy-Weinberg equilibrium. While the basis for heterozygote deficiency in populations has been theoretically and experimentally described in literature, a heterozygote excess in populations that is not as common has not been fully addressed in the literature. Deviations from Hardy-Weinberg equilibrium have been detected in a metapopulation of water voles *Arvicola terrestris* and could be explained through fluctuations in the genetic composition of populations (Stewart *et al.* 1999) and might be characteristic for metapopulations. If genes are transmitted from small numbers of parents to their offspring, deviation from HW equilibrium are expected. Siblings will show heterozygote excess when the two parental genotypes happen to differ. Mating system, a limited number of parents and a great reproductive skew within local populations might all be possible explanations for heterozygote excess. Prout (1981) predicted heterozygote excess as a result of sex-biased dispersal. Female-biased dispersal is assumed to be the case in capercaillie (see Storch & Segelbacher 2000) and our microsatellite analysis (chapter 4.3) supports these findings. Pudovkin *et al.* (1996) demonstrated another mechanism leading to an excess of heterozygotes. When the number of breeding individuals producing the next generation is small, the allele frequencies in males and females will be different due to binomial sampling errors. This difference generates an excess of heterozygotes in the progeny relative to the proportion expected under HW equilibrium (Luikart & Cornuet 1999). This has been shown for basidiomycetes (Rosewich *et al.* 1999). As our northern populations showed

mostly low population densities and since they may have declined in size during the last decades, low numbers of parental individuals could explain the observed heterozygote excess.
All local populations showed high levels of genetic diversity and we could not detect any indication of inbreeding, leading us to the conclusion that among populations there are considerably high levels of gene flow and that individuals avoid mating with kin. Gene flow could be mediated either by migration of individuals or by recolonization of vacant habitat patches (Wade 2001). The latter theory can be excluded as it is expected and was observed for a propagule-pool recolonization (Slatkin 1977), that genetic diversity would be much lower than found in our study. Also, there is no indication of local extinction and recolonization in Alpine capercaillie from field observations. We therefore conclude that migration of individuals is the major cause of gene flow in our study. The high level of genetic diversity observed within populations suggests that all populations are exposed to gene flow and emphasises the assumption that these populations are determined by contemporary processes, i.e. their genetic structure is unlikely to be a relict of historical factors.

Population differentiation

Gene flow among the Alpine populations of capercaillie may be mediated by migration of individuals and can be best explained by a stepping-stone model. According to the island model, gene flow operates between populations independently of their distance from each other, and genetic differentiation between populations will not be correlated with geographic distance. In the stepping-stone model, gene flow operates only between adjacent sites (Kimura & Weiss 1964) and genetic differentiation between populations will increase with distance. Patterns of isolation by distance result from the changing relative influences of gene flow and drift as populations become more geographically separated (Hutchinson & Templeton 1999). As we could show, local populations at the northern edge of the capercaillie´ s Alpine distribution displayed a highly significant correlation between geographic and genetic distance. Gene flow according to the stepping-stone model is therefore the most likely explanation in those populations. As mean dispersal distances of capercaillie are in an order of 5-10 km (Storch & Segelbacher 2000) the great majority of dispersing individuals move mainly to neighbouring patches and only a few birds will move over longer distances. In central populations, however, we found only an insignificant association between genetic and geographic distances. The observed pattern could be explained by isolation, allowing allele frequencies to drift independently in each population without relation to geographic distances (Hutchinson & Templeton 1999). Drift seems to have a much greater influence to populations than has gene flow. Capercaillie populations of the central Alpine group probably have experienced habitat fragmentation for a substantially longer period of time than the northern group. In the central Alps, much of the fragmentation is due to geographic barriers (high mountain ranges, low

amount of forest) whereas anthropogenic causes of fragmentation could dominate in the northern foothills. The population differentiation observed along the northern Alpine range is most likely a very recent process. During the past few decades deterioration of the habitat, mainly due to forestry practices, has led to declining population sizes (Storch 2001), and thus, reduced dispersal and gene flow. How potential barriers effect dispersal on a local scale in the central Alpine range needs to be assessed in future studies.

Significant differences in allele frequency distributions among the populations were observed. Nevertheless, the observed heterozygosity, averaged over all loci, was not statistically different among these populations. Pairwise F_{ST} comparisons also showed statistically significant differences only among some populations. Changes in allelic diversity are probably occurring faster in more isolated populations and therefore causing stronger differences between those populations. Maruyama & Fuerst (1985) predicted that allelic variation is probably declining faster than heterozygosity. This was found in a study of three small mammal species isolated on forest fragments in Thailand (Srikwan & Woodruff 2000), in fragmented populations of tamarins *Leontopithecus rosalia* in Brazil (Grativol *et al.* 2001) and in mosquitos *Culex quinquefaciatus* on the Hawaiian Islands (Fonseca *et al.* 2000). Loss of heterozygosity through drift alone occurs very slowly (Nei *et al.* 1975). Therefore, loss of allelic diversity might be as serious a concern to endangered species as a loss of heterozygosity or an increase in inbreeding values.

From reported juvenile dispersal distances (mean: 5 km, Storch & Segelbacher 2000), we would expect limited gene flow and considerable genetic structuring among local capercaillie populations. However, we found that capercaillie populations exhibited only low to moderate genetic structuring in the Alps, implying gene flow over much larger distances or more gene flow than observational data suggest. Such discrepancies between direct and indirect estimates of dispersal have been also reported in other sedentary bird species. A genetic study by Baker *et al.* (2001) reported evidence for long-distance dispersal in Australian magpies *Gymnorhina tibicen* although behavioural data suggested very short dispersal distances. Kvist *et al.* (1998) also demonstrated high levels of gene flow between two geographically distant populations of willow tits *Parus montanus borealis*. Gene flow occurring over greater distances than those measured by dispersing individuals can not be explained by direct gene flow between populations, but rather by genes being dispersed according to a stepping-stone model (Kimura & Weiss 1964). Continued gene flow between neighbouring populations spreads genes over larger distances than the dispersal ability of the individuals might predict.

Population decline

Excess heterozygosity will result from population size decline (Cornuet & Luikart 1996) and has been detected already in some natural populations (Dallas et al. 1999, Luikart & Cornuet 1998, Lee et al. 2001) but not in others (Luikart & Cornuet 1998, Rooney et al. 1999). Although some populations displayed deviations from HWE, which could bias the result of this test (Cornuet et Luikart 1996), we did not find any significant deviation from HWE in a test over all loci and populations. If excess heterozygosity, as observed for capercaillie populations in the northern Alps, is an indicator of recent size decline, we can assume populations of the northern Alpine range to have experienced a more severe decline than central Alpine populations. Field data also suggest that population density in the north is lower than in the centre (Storch 2001). Climatic differences between northern and central populations (e.g. higher precipitation rate in the North) may have led to lower reproductive success in the northern capercaillie populations.

Conclusions

Capercaillie populations in the northern Alps are restricted to forested mountain ranges, separated by densely populated farmland valleys. The interspersion of forest and farmland may lead to large-scale edge effects (see Laurance 2000) that affect capercaillie population density (Storch 2002). This patchy distribution of capercaillie populations requires migration of individual birds between patches to sustain the metapopulation structure. According to our results, a stepping-stone model best explains gene flow among Alpine capercaillie populations. Local habitat patches serve as stepping-stones that maintain gene flow within the metapopulation system. For a patch to function as a stepping-stone in a population genetic sense it appears to be vital that habitat conditions suitable for the successful raising of offspring, and thus, production of potential dispersers, are maintained. Small local populations might be able to persist if they remain interconnected by dispersal. Therefore even small suitable patches may be important for metapopulation persistence (Moilanen *et al.* 1998). However, in many of these patches along the northern edge of the Alps, capercaillie habitat suitability is limited by forestry practices. In addition to the amount of habitat destroyed, metapopulation persistence and extinction are strongly influenced by the rate at which the landscape changes (Keymer *et al.* 2000). Changes in landscape patterns will lead to extinction of populations more often than expected by considering only the effects of reductions in area, increase in isolation and loss of connectivity. Managing capercaillie population in the Alps should therefore take into account landscape patterns and dynamics.

This study supports our argument that metapopulation connectivity is a key issue for capercaillie conservation (Storch 1993, 1995, 1997, Storch & Segelbacher 2000). Especially in the northern Alpine populations, where population decline in size seems to be most severe, conservation management should focus on the protection of subpopulations to promote regional population persistence. According to present knowledge, maintaining a dense network of suitable patches and stepping stones, and maximising local carrying capacity in order to maximise reproductive output and thus, the number of potential dispersers, are the most promising approaches to secure the persistence of the capercaillie metapopulation in the Alps and in other, similarly fragmented landscapes.

4.3 Source-sink populations

Habitat fragmentation can significantly alter population genetic structure as geographic distance and potential physical barriers affect gene flow. The aim of this study was to identify genetic evidence for source-sink dynamics between local capercaillie populations (*Tetra urogallus*) in the Bavarian Alps, Germany. We studied five local populations and estimated genetic variation using ten polymorphic microsatellite markers. We found no significant differences in the number of alleles per locus or the degree of heterozygosity between populations, but significant genetic variation among all populations. Significant genetic differentiation was detected for given population pairs even at short distances (~10 km). Effective population size was lowest in the study area Ammergebirge, which bordered dairy farmland. This population was identified as a sink population, as immigration clearly exceeded emigration. This study confirmed that microsatellites have the potential to detect differences among populations and source-sink dynamics at a local scale. Results of such studies will help to develop improved, effective management and conservation plans for capercaillie.

Introduction

In landscapes altered by human activities, many species are restricted to small patches of habitat. Species that were once common have decreased both in distribution range and density, and many have become extinct (Hilton-Taylor 2000).

A main cause is habitat fragmentation, which subdivides previously contiguous populations into a number of local populations, turning them into metapopulations (Hanski & Gilpin 1991). As pointed out by Harrison (1991), source-sink metapopulations dominate when humans have induced fragmentation. In this type of metapopulation, the sink populations have little effect upon regional persistence, since the reproductive rates in these patches by definition is negative. Hence the source populations are the major providers of colonists. Sink habitats are defined as unsuitable for long term survival and reproduction, and thus, are assumed to be qualitatively different from source habitats. In a source-sink metapopulation system, there is a net flow of migrants from populations in good habitats (sources) to populations in inferior

habitats (sinks). Gaggiotti (1996) theoretically showed that a collection of interconnected sinks, however, can maintain a substantial fraction of the genetic variability observed in source populations, particularly where migration from the source population is continuous over time. The degree of the genetic differentiation among the sink populations might be large, especially if migration patterns from the source populations are stochastic. However, genetic differentiation is considered to be small when most of the sink populations are composed of recent migrants from the source or when there is an increased effect of migration among sink populations (Gaggiotti 1996).

The Alps contain the largest distribution range of Capercaillie (*Tetrao urogallus*) in Central Europe. In the northern parts of the Alps, the birds occupy patches of contiguous mountain forests separated by open valleys and interspersed by alpine habitats above the treeline (Storch 2001). In this landscape, capercaillie are distributed in a metapopulation fashion (Storch 1993, Storch & Segelbacher 2000). Parallel to an ongoing population decline during the 20th century throughout Europe, the Alpine range of the capercaillie has contracted (Storch 2001). Due to generally poor habitat structure (Storch 2002) and great predation pressure in vicinity to the farmland lowlands (Storch 2001), local populations at the edge of the range may function as sink populations, which are not self-sustaining, but persist owing to immigration. Mountain ranges surrounded by suitable capercaillie habitats to all sides, where extended farmland is absent and habitat structure is better (Storch 2002), however, may serve as source populations and may thus be essential for the persistence of the metapopulation system.

In this study, we aim to identify genetic evidence of source-sink dynamics between local capercaillie populations in the Bavarian Alps, Germany. For this purpose we investigated metapopulation structure and gene flow for five mountain ranges using ten highly polymorphic microsatellite markers. We expected populations at the edge of the range to show indication of greater population decline, and specific characteristics of sink populations, such as low population size and high immigration and low emigration rates.

Methods

Fieldwork

To investigate fine-scale genetic structure of capercaillie populations capercaillie feathers were sampled from two regions in the Bavarian Alps, Germany (Figure 11) during the summers 1997-2001. The western group consisted of three neighbouring mountain ranges (Ammergebirge, Estergebirge, Wetterstein). We collected 24 male and 32 female feather samples in the Ammergebirge, 16 male and 6 female feathers in the Estergebirge and 52 feathers of males and 12 feathers of females in the Wetterstein. The eastern group consisted of the mountain

ranges Sulzberg and Teisenberg, where 22 male and 5 female and 58 male and 42 female feathers were collected, respectively.

The ranges Ammergebirge, Sulzberg and Teisenberg bordered the dairy-farming lowlands to the north of the Alps; this borderline coincided with the northern edge of the Alpine range of the capercaillie. The other ranges (Estergebirge, Wetterstein) were surrounded by forests inhabited by capercaillie to all sides. On each range, a study area of about 2,000 ha with about 500 sample points regularly spaced at 200 m distances was established (Storch 2002); at each sample point, habitat suitability for capercaillie was assessed within a 20 m radius using a habitat suitability index (HSI) model, and incidence of indirect sign of capercaillie use such as faeces and moulted feathers was recorded within a 5 m radius. The mean HSI score calculated over all sample points was used as an index of a study areas' capercaillie habitat suitability; the proportion of sample points with capercaillie signs was used as an index of capercaillie population abundance (see Storch 2002 for methods). As capercaillie abundance, and thus, the number of feather samples, was very low in the Ammergebirge we included four additional individuals from outside the 2000 ha study area in the genetic analysis. We grouped all Ammergebirge samples into one study population as we could not detect any significant genotypic or genic differences between these four extra individuals and those from within the 2000ha study area.

Capercaillie densities on the five ranges spanned from low (Ammergebirge) to high (Teisenberg, Wetterstein) for the Bavarian Alps (Table 13). For the purpose of this paper, we use the term population for the birds on each of the mountain ranges studied, although they were clearly interconnected by gene flow and thus belonged to the same metapopulation system.

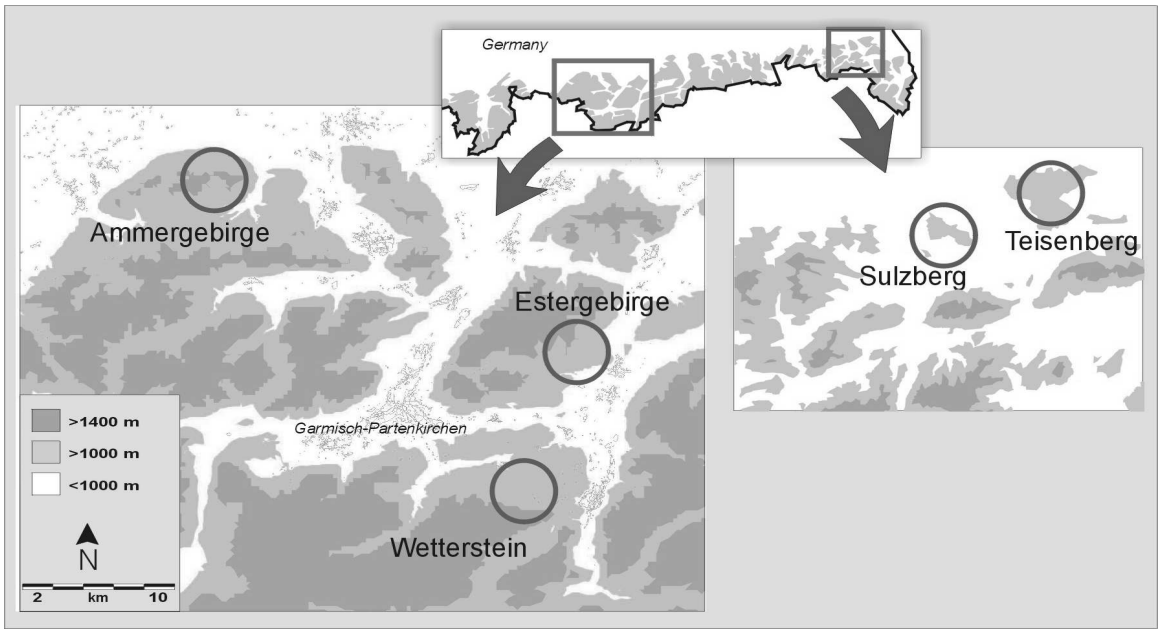

Figure 11 Location of the three subpopulations in the Bavarian Alps. Circles indicate the sampling areas. Shaded areas are above the 1000m line.

Genotyping

Genomic DNA was extracted from all sampled feathers using the DNeasy Tissue Kit (Qiagen). Polymerase chain reaction (PCR) amplification and genotyping were conducted for 10 microsatellite loci as described in Segelbacher (2002).

Statistical analysis

Genotype and allele frequencies of the microsatellite loci were used to estimate genetic variance within and among populations. Statistical analyses were performed by using various computer programs: Allele frequencies, observed and expected heterozygosity and mean number of loci were calculated with GENETIX version 4.02. Deviations from Hardy-Weinberg equilibrium were tested by using GENEPOP version 3.1d (Raymound & Rousset 1995b) using a Markov chain method following the algorithm of Guo & Thompson (1992). Allelic richness (Petit *et al.* 1998) and F_{is} was determined using FSTAT 2.93 (Goudet 2001). Population differentiation was assessed by investigating the distribution of allele frequencies across populations using the log-likelihood statistics G (Goudet *et al.* 1996). If Hardy-Weinberg equilibrium is rejected within populations this log-likelihood statistics is recommended (Goudet *et al.* 1996). Genetic distances (D_{TL}, Tomiuk & Loeschcke 1991, 1995) were calculated using the program POPDIST, which is available under http://genetics.sh.dk/~popgen. This measure is efficient in obtaining the

correct phylogenetic topology of related populations and very robust against non-equilibrium conditions (Tomiuk *et al.* 1998).

Pairwise F_{ST} estimates were obtained from Genepop 3.1d (Raymound & Rousset 1995b; as per Weir & Cockerham 1984). To reduce the likelihood of Type I errors among multiple tests we applied a sequential Bonferroni correction (Sokal & Rohlf 1995).

Based on coalescence theory, bi-directional gene flow (N*m*) and effective population sizes (N_e) were calculated by running MIGRATE (Beerli & Felsenstein 1999) ten times and using the estimates of each run as starting values for the consecutive one. Unlike F_{ST}, MIGRATE accounts for directional gene flow and differences in sample size.

Estimates of the effective population size largely depend on the presumed underlying mutation rate. A mutation rate of 5×10^{-4} is commonly accepted for microsatellites across a wide range of animal taxa (Ellegren *et al.* 1995). However Primmer *et al.* (1998) observed 44 mutations among 1209 meioses in a tetranucleotide repeat of the barn swallow (*Hirundo rustica*), equivalent to a mutation rate of 3.6×10^{-2}. Given the uncertainty about the true mutation rate, we calculated the effective population size N_e for both mutation rates.

We furthermore estimated the number of immigrants and/or their descendants by the assignment test STRUCTURE (Pritchard *et al.* 2000). This method uses a Gibbs sampler to implement a Bayesian clustering algorithm. Individuals were assigned probabilistically to one or more subpopulations on the basis of their genotypes and the estimated allele frequencies per subpopulation. Additionally we used the program GENECLASS (Cornuet *et al.* 1999) with a Bayesian approach (Rannala & Mountain 1997) to assign individuals to the population in which their probability of belonging was highest using threshold values of $p<0.01$.

To assess kinship among males and females in the subpopulations we used two different approaches. Firstly, we tested genetic similarity within the groups of individuals using BURIAL version 1.0 (Schönfisch *et al.* 2001), which uses a permutation test based on a multilocus model. Secondly, we assessed kinship with the program RELATEDNESS, version 5.0 (Queller & Goodnight 1989).

Results

Habitat suitability and abundance

The chances of finding signs of capercaillie was lowest at Ammergebirge and highest at Wetterstein (Table 13). The data confirmed that there is a general trend for poorer habitats and lower abundance on mountains at the edge of the range as compared with more central areas such as Wetterstein.

Table 13 Standardized index of abundance and habitat suitability index (HSI) for five local capercaillie populations in the Bavarian Alps.

	Index of abundance	Habitat suitability index
Ammergebirge	1.8	0.31
Estergebirge	6.4	0.46
Wetterstein	18.5	0.45
Sulzberg	2.8	0.25
Teisenberg	9.2	0.34

Genetic variability within populations

The global probability test for HW-equilibrium revealed that the genotypic structure of populations from Ammergebirge and Teisenberg significantly deviated from HW-equilibrium. No pair of loci indicated significant departure of linkage disequilibrium.

We found a high degree of genetic variation within populations, both in terms of average number of alleles and allelic richness per population. Furthermore, comparing observed and expected heterozygosity revealed that populations displayed high genetic diversity (Table 14).

Table 14 Genetic diversity of five local populations of capercaillie. n, number of individuals analysed (number of males/females in brackets), A, mean number of alleles per locus, R, allelic richness, (H_o), the mean observed heterozygosity, (H_e), expected heterozygosity and $F_{is.}$

	N	**A**	**R**	**H_o**	**H_e**	**Fis**
Ammergebirge	15 (9/6)	3.90	3.13	0.78	0.63	-0.21
Estergebirge	6 (5/-)	3.00	2.97	0.89	0.59	-0.42
Wetterstein	16 (13/3)	4.30	3.23	0.70	0.64	-0.07
Sulzberg	7 (7/-)	3.80	3.51	0.76	0.59	-0.21
Teisenberg	33 (19/14)	5.30	3.67	0.75	0.66	-0.11

Genetic variability among populations

We found significant genetic differentiation across all populations both within the western group ($p<0.002$) and within in the eastern group ($p<0.004$). The multilocus estimate of genetic differentiation was F_{ST} = 0.059 ($p<0.001$) for the western group, indicating moderate genetic differentiation between populations separated by a maximum distance of 25 km. Pairwise F_{ST} estimates varied from 0.015–0.071 (Table 15) and the degree of genetic differentiation between pairs of populations correlates with Tomiuk & Loeschcke`s distance (D_{TL}). Genetic differences between the populations of Ammergebirge and Estergebirge, although separated by a valley of

up to 5 km with unsuitable habitat, were lowest. The populations of Sulzberg and Teisenberg in the eastern group were significantly different from each other comparing with pairwise F_{ST} (θ=0.03, p<0.05), but genetic differentiation was lower than in the western group, which is supported by Tomiuk & Loeschcke`s distance (D_{TL} =0.082).

Table 15 Pairwise F_{ST} (above diagonal) values and genetic distance (D_{TL})for the western population pairs (below diagonal). Bold values are significant after Bonferroni correction (p<0.05).

	Ammer	Ester	Wetterstein
Ammergebirge	-	0.015	**0.067**
Estergebirge	0.094	-	**0.071**
Wetterstein	0.097	0.140	-

Population size and gene flow

Under the assumption of mutation-drift equilibrium, effective population sizes were estimated to be 5 (Ammergebirge), 45 (Estergebirge), 680 (Wetterstein), 290 (Sulzberg) and 545 (Teisenberg) birds assuming a mutation rate of 5×10^{-4} , but only 0.1 (Ammergebirge), 0.6 (Estergebirge), 9 (Wetterstein), 4 (Sulzberg) and 8 (Teisenberg) birds assuming a mutation rate of 3.6×10^{-2}. We assumed a ratio of effective to estimated population size of 0.06 (Nei & Graur 1984). This ratio is similar to that found in a population of the taita trush (Galbusera *et al.* 2000) and well in accordance with the range for several other empirical studies (Frankham 1995b). From field data we estimated the population size of the Teisenberg mountain range to be approximately 100-200 birds (Storch 1993 and unpublished) that yields the estimate of the effective population size of nine individuals. We therefore conclude that the high mutation rate of 3.6×10^{-2} is the more realistic model for the microsatellites in our capercaillie study.

Uni-directional migration rates ranged between 0.0 and 13.2 individuals per generation (Table 16). Our results indicated immigration to but no emigration from Ammergebirge. In the Wetterstein population, emigration to Ammergebirge and Estergebirge clearly exceeded immigration from these two areas. Between the populations of Teisenberg and Sulzberg gene flow seemed to be frequent.

Table 16 Uni-directional estimates of gene flow (Nm) between three capercaillie subpopulations in the western and two subpopulations in the eastern group as calculated with MIGRATE (95% interval in brackets).

Western Group

		from		
	Subpopulation	Ammergebirge	Estergebirge	Wetterstein
to	Ammergebirge	—	13.20	4.81
			(11.43-15.20)	(3.51-6.51)
	Estergebirge	0.33	—	11.30
		(0.19-0.51)		(10.18-12.69)
	Wetterstein	0.00	7.54	—
		(0.00-0.03)	(6.81-8.25)	

Eastern Group

		from	
	Subpopulation	Sulzberg	Teisenberg
to	Sulzberg	—	9.10
			(8.22-10.08)
	Teisenberg	5.40	—
		(4.81-6.03)	

We additionally applied two assignment tests to identify migrants and obtain an accurate estimation of current gene flow. All individuals clustered clearly to the population where they had been sampled with the Bayesian clustering method (STRUCTURE) and we could not identify any individual as an immigrant from one of the other populations in the western (Figure 12) and eastern group; the test indicated that up to five generations ago no individual immigrated from another population. For the Bayesian method (GENECLASS) we got identical results for the western group. In the eastern group, however, this method identified three individuals from the Sulzberg population belonging to the Teisenberg population.

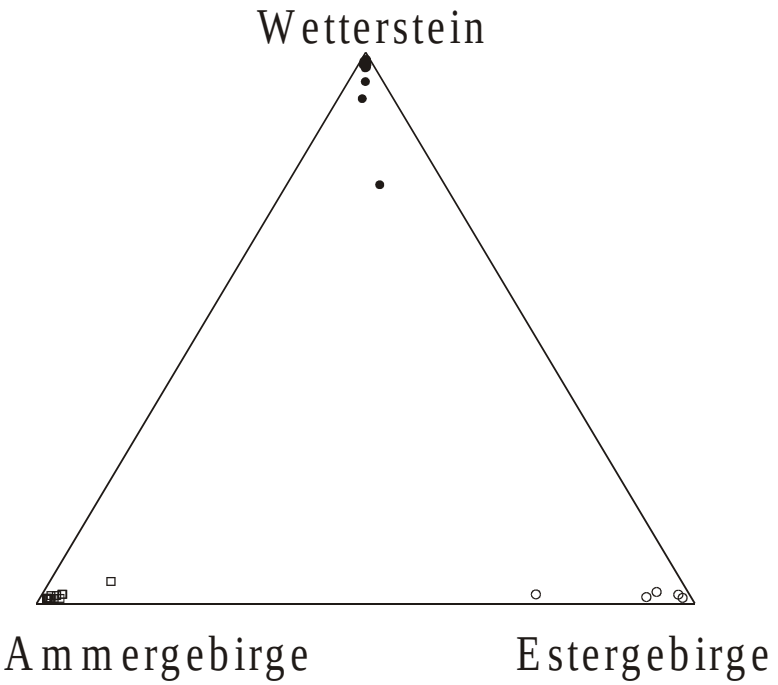

Figure 12 Assignment of capercaillie to their populations of origin, based on individual genotypes and the estimated allele frequencies per population. Position on the x axes refers to the genetic affiliation of each individual, symbols refer to the study areas. Ammergebirge (squares), Estergebirge (open circles), Wetterstein (filled circles).

Relatedness among individuals

With the programme BURIAL, we tested the genetic relationships within populations to determine whether individuals were genetically related with the programme BURIAL. The observed grouping of individuals was not random (p=0.0002). Individuals were related in the populations of Ammergebirge (p=0.01) and Estergebirge (p=0.005), but not in the Wetterstein population (p=0.4). Testing for sex differences, we found males in the populations of Ammergebirge (p=0.001) and Estergebirge (p=0.001), respectively, highly related to each other. Males of the Wetterstein population, however, showed no significant relationships (p=0.9). Females in the populations of Ammergebirge and Wetterstein were not related (p=0.12; p=0.77). Relatedness among males was significant for the Teisenberg population (p=0.01), but not for the Sulzberg population (p=0.1). Females of the Teisenberg population were unrelated (p=0.9).

Assessing relatedness among males and females within each of the three western populations gave similar results with the programme RELATEDNESS: Ammergebirge (males r=0.142, females r=0.047), Estergebirge (males r=0.207), Wetterstein (males r=0.142, females r=0.032). In the western populations, we found also higher relatedness among males (Sulzberg r=0.180, Teisenberg r=0.030), than among females (Teisenberg r=0.004). However, the observed differences between sexes were not significant.

Discussion

Genetic variability within and among populations

This study clearly demonstrates the potential of DNA microsatellite markers to analyse local-scale genetic variation within and among capercaillie populations. Local populations separated by as little as 10 km (see Fig 1) exhibited significant genetic differentiation. However, between two of the five populations studied, Ammergebirge and Estergebirge, we did not detect any significant differentiation, although these study areas were separated geographically by approximately 20 km including a 5 km wide, open valley. Genetic homogeneity at a landscape level can be explained by demographic sinks that persist because of recurrent dispersal from a common source population (Gaggiotti 1996). The populations of Ammergebirge and Estergebirge depended on immigrants from the Wetterstein population and genetic composition of the populations was therefore quite similar.

Apparently, genetic variation within populations of capercaillie was not reduced by the present degree of spatial separation. The number of alleles and the degree of genetic heterozygosity were consistently high in all populations and none of the investigated populations showed evidence of a bottleneck. As has been shown in pika *Ochonta princeps* (Peacock & Ray 2001) high levels of heterozygosity in a metapopulation can be explained by highly subdivided populations.

Previously, decreasing genetic differentiation was considered to be the major effect of habitat fragmentation and subsequent spatial separation of populations. More recently, some authors have suggested that in metapopulation systems, the overall genetic variation may be maintained due to genetic differentiation among local populations (Harrison & Hastings 1996, Gaggiotti 1996). Gaggiotti (1996) demonstrated that a collection of sinks can maintain a substantial proportion of the genetic variability observed in the source population. The amount of genetic variability maintained within each sink population is a function of the rate of migration among sinks (Gaggiotti 1996). Sinks may have positive effects on the genetic structure of metapopulations and thus, profound consequences for the populations in fragmented habitats.

Population size and gene flow

We estimated dispersal between populations using two different procedures. F_{ST} -and MIGRATE-based values mainly reflect long term gene flow, whereas non-equilibrium assignment test reflect current dispersal events. We found fairly high estimates, based on F_{ST} - and Migrate-values, reflecting high ancestral rates of dispersal. However, we could not detect any individual dispersing between populations over the last five generations with the Bayesian

clustering method (STRUCTURE). However, the Bayesian method (GENECLASS) identified three immigrated individuals at Sulzberg. Similar differences between past and recent dispersal rates have been found between subpopulations of the taita trush *Turdus helleri* (Galbusera *et al.* 2000). This could be an effect of sample size, but may also reflect recent fragmentation processes

Uni-directional gene flow estimates revealed that individuals of the Ammergebirge population did not emigrate, but were most likely offspring of birds, which have immigrated from elsewhere. The Ammergebirge population could therefore be identified as a sink population. Sink populations are typically associated with inferior habitat quality relative to the sources (Dias 1996). Our field data (Table 1) further support this hypothesis. The chances of finding capercaillie signs at the scale of individual sample plots were strongly related to habitat suitability scores according to the HSI model (see Storch 2002). The Wetterstein population to the south of Ammergebirge served as a source population, where more individuals emigrate, than immigrate. As this population is surrounded by other populations, whereas Ammergebirge is situated at the northern edge of the Alpine distribution range, possibly edge effects can also influence the population dynamics (Storch 2002). Further support for the edge effect hypothesis comes from the analysis of effective population sizes. The Ammergebirge population had the lowest and the Wetterstein population the greatest number of possible breeders. The genetic data support the assumption that the Estergebirge population functioned as a stepping stone between these two populations, which is in congruence with its geographic location.

Estimates of effective population sizes N_e depend on the presumed underlying mutation rate. We therefore adopted two different mutation rates for our data. Both mutation models, however, estimated the lowest effective population size for Ammergebirge.

In the eastern group, we found a different pattern. Both populations were located at the edge of the Alpine range of the capercaillie and border the farming lowlands north of the Alps. Based on field studies, the capercaillie population on the 50 km² Teisenberg mountain range has been estimated to 100-200 birds in the early 1990s (Storch 1993); repeated assessments of capercaillie abundance with the method of Storch (2002) indicated that the population has remained at this level until the years of this study (Storch 2002 and unpublished data). On Sulzberg, capercaillie habitat suitability was poorer, and capercaillie abundance lower (see table 1). Our study shows consistent gene flow between the two areas. Although we could distinguish genetically the Sulzberg from the Teisenberg birds, the results on effective population sizes showed that both local populations serve as a single metapopulation unit.

Relatedness

Males in all populations tended to be closer related than females. Sex-biased dispersal with strong natal philopatry in males seems to be a common pattern in birds (Greenwood 1980, Greenwood & Harvey 1982) and has been described in studies of black grouse *Tetrao tetrix* (Höglund *et al.* 1999) and red grouse *Lagopus lagopus scoticus* (Piertney *et al.* 1999, Piertney *et al.* 2000). This is in good congruence with field data on capercaillie dispersal distances (Storch & Segelbacher 2000).

Implications for conservation of capercaillie

The effects of fragmentation on dispersal may be altered either by changes in the landscape mosaic or by changes in the dispersal behaviour of individuals (Wiens 2001). Dispersal movements between patches might be facilitated by actively managing the landscape, reducing the actual effects of fragmentation. (Wiens 2001). On the other side dispersal distances could become greater with increasing distance between suitable habitat patches, as has been shown for nuthatches *Sitta europaea* (Matthysen *et al.* 1995). There are a few reports of capercaillie, mainly females, dispersing over longer distances, probably individuals searching for a suitable habitat (Klaus *et al.* 1989, Storch & Segelbacher 2000) . Contraction of the capercaillies range at the northern edge of the Alpine distribution occurred during the 20th century and particularly since 1950 (Storch 2001). Our findings suggest that a network of suitable habitat patches within the capercaillies mean dispersal distance of 5 km is essential to provide gene flow through highly fragmented landscapes. Although we know from field observations, that birds regularly cross valleys and areas of unsuitable habitat (Klaus *et al.* 1989), we found genetic differentiation and subdivision at distances of about 10km.

Gene flow among local populations inhabiting spatially distinct habitat patches, however, greatly depends on local metapopulation structure and dynamics. Therefore, any given distance within the dispersal ability of the species, between two neighbouring populations, may or may not result in genetic differentiation. Between the populations of the mountain ranges Sulzberg and Teisenberg, separated by 5 km measured from edge to edge, we found high levels of gene flow. Habitat quality and capercaillie abundance at Sulzberg were low. Some of our feather samples collected on Sulzberg were most likely from individuals coming seasonally from Teisenberg as indicated by the Bayesian method. Also, radio-tracking had shown that 4 out of 40 adult capercaillie caught on Teisenberg migrated to Sulzberg on a seasonal basis (Storch 1993). Effective population size at Sulzberg was therefore considerably high despite the fact that local abundance was low. Thus the Teisenberg birds can be considered the source for the Sulzberg population. At the time of this study, there was evidence of regular exchange between the two

ranges. However, if the number of capercaillie on Teisenberg should decrease, isolation of the Sulzberg birds is a likely consequence, although the geographic distance between the two patches has remained the same. Reduced dispersal between local mountain ranges can occur in other parts of the Alps and for better understanding these patterns of local gene flow, further studies in other areas will be needed.

Considering the small patches of suitable capercaillie habitat in the Bavarian Alps, many of those small populations only persist due to their connectivity with other sink or source populations. Especially populations which border the extended farming landscapes surrounding the Alps are most sensitive to population decline, as they may act as sinks. The amount of suitable habitat has to be maintained or restored in those edge populations to maintain the carrying capacity of the whole metapopulation system. It has to be stressed that not only source populations have to be preserved, but also sink populations should be managed to ensure the persistence of the metapopulation system. Even small local populations, which act as sinks, could help to maintain the persistence of the metapopulation system as a whole (Gaggiotti 1996) as they maintain a substantial fraction of the genetic variability.

5 Conclusions

Habitat fragmentation is a characteristic feature of present day forest ecosystems, and its effects on viability and persistence of populations and metapopulations have been widely discussed (Soulé 1986, Morrison *et al.* 1992). Different concepts have been developed to illustrate and explore the dynamics of patchily distributed populations, among them metapopulation theory and source-sink models (Pulliam 1988, Hanski 1999).

The total amount of habitat in a fragmented landscape is considered a good predictor of long-term metapopulation persistence (Hanski 1998). The primary aim in conservation should therefore be simply to preserve as much habitat as possible. However, many habitats have become so fragmented that isolated populations cannot be expected to survive for long. Long term persistence can then only occur within a metapopulation system (Hanski 1998). Generally, even small local populations can contribute to maintain the persistence of the metapopulation system as a whole as they maintain a substantial fraction of the genetic variability (Gaggiotti 1996). Decreasing areas of existing fragments are often more harmful to metapopulation persistence than random loss of entire patches (Hanski & Ovaskainen 2000). Thus it should be stressed that, for capercaillie as well as for other threatened species, as many habitat patches as possible should be maintained and further loss or reduction in size and quality of these patches should be prevented in order to maintain viable populations.

Many forest bird populations have declined as an effect of human-induced habitat fragmentation, due to reduction in the amount of habitat, reduction of habitat patch size, and increasing isolation of patches, or a combination of all these effects (Wilcove *et al.* 1986, Saunders *et al.* 1991). Loss of habitat continuity is the main impact of habitat fragmentation on populations, and has to be distinguished from habitat loss *per se* (Wilcove *et al.* 1986, Rolstadt 1991, Andrén 1994). The species that are most sensitive to changes in the structure of the habitat and landscape are those that occur at relatively low densities (Wilcox 1980), that are sedentary habitat specialists (Opdam 1990), that occupy late successional forest habitats (Gotteli & Graves 1990), or that have a low dispersal ability (Wiens 1995, Pimm *et al.* 1988). Habitat specialists living in a landscape with low proportions of suitable habitat are therefore expected to suffer most from habitat fragmentation (Andrén 1996). The Capercaillie meets most of the above criteria and it is known from field studies that the species is severely affected by habitat fragmentation (Rolstadt 1989, Storch 1993, Kurki *et al.* 2000).

Capercaillie habitats are fragmented at several hierarchical levels of spatial scale. The largest scale concerns the central European distribution of the species. The range is subdivided into isolated fragments of different size, separated by up to several hundred kilometres (Storch &

Segelbacher 2000; see Klaus *et al.* 1989). Formerly connected populations, for example those of Black Forest and Vosges or Fichtelgebirge and Thuringian Forest, are now genetically isolated and exhibit lower levels of genetic diversity. In these isolated populations I found stronger evidence for a decline in population size as in those populations which are part of larger populations. Continued deterioration of habitat may lead to more populations becoming isolated remnants of a formerly widespread European distribution. Conservation plans should therefore focus on the protection of subpopulations within these ranges to promote regional population persistence and on the improvement of connections among neighbouring populations to maintain high levels of genetic diversity and prevent further reductions in population size.

Within regional distribution ranges, for example in the Alps, capercaillie habitats are restricted to forested mountain ranges, often separated by several kilometres of unsuitable habitat. Among these regional populations, a metapopulation structure was assumed (Storch & Segelbacher 2000) and could clearly be confirmed genetically by this study. According to my results, a stepping-stone model best explained gene flow among capercaillie populations along the northern edge of the Alps. Local habitat patches serve as stepping-stones that maintain gene flow within the metapopulation system. However, in much of the Alpine forests capercaillie habitat suitability is limited by forestry practices. According to present knowledge, maintaining a dense network of suitable patches and maximising local carrying capacity in order to maximise reproductive output, and thus the number of potential dispersers, are the most promising management goals to secure the persistence of the capercaillie metapopulation in the Alps and other, similarly fragmented landscapes. Small local populations might be able to persist if they remain interconnected by dispersal. My findings suggest that a network of suitable habitats within the dispersal distance of capercaillie is essential to provide gene flow through the highly fragmented Alpine landscape.

The levels of fragmentation, speed of fragmentation and dynamics of the original vegetation will have crucial effects on the relationship between migration rates and the probability of persistence in a metapopulation (Thomas 1999). Species become extinct long before the last fragment of suitable habitat is lost, both because of metapopulation dynamics and because of emigrating individuals which fail to arrive and reproduce successfully in other patches. The landscape structure, however, can mitigate the effects of habitat loss and enhance metapopulation persistence (With & King 2001).

Considering the small size of suitable capercaillie habitat patches in the Bavarian Alps and the distances among these patches, it is likely that many of those small populations might only exist due to their connectivity to other populations. I demonstrated source-sink dynamics among populations at local mountain ranges. Populations at the edge of the range are most sensitive to population decline as they exist only through immigration of birds from neighbouring populations. This result agrees with the hypothesis that edge populations may act as sinks. The

amount of suitable habitat has to be maintained or restored in edge populations as well as in source populations to maintain the carrying capacity of the whole metapopulation system. It has to be stressed that not only source populations have to be preserved but also sink populations should be managed to secure the persistence of the metapopulation system. An increase of productivity could even turn small habitat patches that would normally be population sinks into source habitats (Walters 2001).

One prediction of demographic metapopulation models with great importance for conservation is the time delay with which species are expected to track changes in the structure of fragmented landscapes (Hanski 1998) which is called the extinction debt (Tilman *et al.* 1994). Especially in cases of metapopulation break-up there will be a time delay until population extinction becomes evident. In a worst-case scenario an observed, seemingly stable metapopulation system might merely reflect a pattern of the recent past. Many species might be present for a long time in landscapes that have already lost their capacity to support these species in the long term (Hanski 1998). Thus, the Alpine capercaillie metapopulation may be less secure than it may seem, and measures for large-scale improvement of habitat suitability and carrying capacity are strongly recommended to reduce the risk of metapopulation break-up and subsequent extinction.

Considering the short evolutionary time-scales conservation biologists have to deal with, genetic diversity in wild populations in fragmented landscapes can be best conserved by maintaining self-sustaining local populations well-distributed among a network of infrequently disturbed habitats (Gibbs 2001). To retain genetic diversity in local populations, it is important to maintain suitable habitat patches in the landscape and also to reduce the frequency of human disturbance to local populations (Gibbs 2001). Management plans for capercaillie should therefore aim to maintain and restore as many habitat patches as possible to ensure viability of the metapopulation as a whole. If isolated patches are managed individually, then the long-term danger is that it will be necessary to independently manage a number of small vulnerable populations that require high levels of intervention instead of managing a single large resilient metapopulation (Nunney 2000). This is particularly important for capercaillie populations as effective population size in many local populations is already low. The effective population size (N_e) is a very valuable parameter for predicting the level of threat facing a population (Mace & Lande, Nunney 2000). Capercaillie populations displayed signs of an ongoing decline in population size and to prevent further reduction in population size and to ensure the viability of the metapopulation system any further loss of suitable habitat patches has to be avoided.

The new developed microsatellite markers described in this study are well suited as genetic markers in regard to the conservation and management of capercaillie. I demonstrated that even minute amounts of DNA, extracted from feathers or faeces of birds, can be used for reliable genotyping and population genetic studies. I showed that non-invasive sampling (collecting moulting feathers or faeces in the field) could be used for endangered bird species to

detect population structure, to estimate gene flow, to assess effective population size and to determine population trends. With these genetic markers, I provided a tool to investigate the influence of habitat fragmentation on the genetic structure of capercaillie. It is now possible to identify individual birds in the field from indirect signs such as feathers or faeces. The genetic analysis of these non-invasive sample provides important indications about demographic factors. This knowledge can be very useful to develop concrete management plans for the conservation of capercaillie.

6 Summary

In conservation biology, the effects of habitat fragmentation on population genetic structure are a major focus of concern. Increasing anthropogenic fragmentation of the natural landscape will form metapopulations and with decreasing connectivity lead to isolated populations. A metapopulation is a system of geographically or ecologically isolated populations. Among these populations there is sufficient migration to have a significant impact on either the demography or genetic structure of each component population. In this study I describe a new approach to study dispersal between populations in an endangered bird species using non-invasive genetic sampling.

The newly developed microsatellites described in this study (chapter 3.1) are well suited as genetic markers to study levels of gene flow and population differentiation in regard to conservation of capercaillie. For this study, I collected moulted feathers and faeces in the field and described methods for reliable genetic analysis of these non-invasive samples (chapter 3.2, 3.3). Although, in some cases, DNA from feathers that had been in the field for several months after moulting could be successfully genotyped, there was a high degree of DNA degradation in moulted feathers, and thus PCR amplification success was reduced compared to freshly plucked feathers. DNA quantity of larger feathers was greater than that of smaller feathers and about 50% of all moulted feathers could be used for reliable genotyping. In genetic studies based on moulted feathers I therefore recommend collecting twice as many feathers as the desired sample size. I showed that moulted feathers and bird faeces can be successfully used for genetic studies and offer a new perspective in ecological and population studies of birds. In general, my study clearly demonstrated the potential of non-invasive genetic sampling for studying endangered bird populations.

The capercaillie (*Tetrao urogallus*) is considered an indicator of old growth coniferous forest communities and is listed as threatened in western and central Europe. It is highly susceptible to human-caused habitat fragmentation, mainly through forestry activities, and habitats are fragmented at hierarchical levels of spatial scale. I investigated the consequences of this habitat fragmentation on genetic structure and variation of capercaillie populations at multiple scales.

The largest scale studied concerned the Central European distribution of the species (chapter 4.1). The range is subdivided into isolated fragments of different size, separated by up to several hundred kilometres. I found significant isolation among the capercaillie populations inhabiting these fragments. Genetic diversity was significantly lower in the Pyrenees and in isolated populations in central Europe (e.g. Black Forest, Vosges) than in more contiguous populations in the Alps and northern Europe. The data suggested that a reduction in the

species' range has led to population fragmentation in the Pyrenees and, more recently, to a collapse of the metapopulation structure and isolation of the remaining small populations in central Europe. The continued reduction of suitable habitat leads to more populations becoming isolated remnants of a formerly widespread European distribution. High levels of genetic diversity can only be maintained by improving connections among neighbouring populations.

At a regional scale, i.e. in the Alps, populations of capercaillie occur in spatially distinct habitat patches and show a metapopulation structure. One aim of my study was to assess some of the genetic consequences of this spatial distribution pattern. I investigated relative influence of gene flow and drift on population structure in a northern group of populations, where connectivity to other populations was limited, and in a second group of central populations, which were surrounded by neighbouring populations. I discovered genetic variation within all populations, but no significant differences in the number of alleles or heterozygosity between the northern and central group. Geographic distance had a significant effect on genetic distance when comparing pairs of populations in the northern group, however in the central group, there was no evidence of isolation by distance. The strong relationship between gene flow and geographic distance in the northern group could be best explained by a stepping-stone model of dispersal. In the central group, however, differentiation due to genetic drift was the most likely explanation.

I found genetic evidence for source-sink dynamics between local capercaillie populations in the Bavarian Alps (chapter 4.3). Birds from five populations displayed no significant differences in number of alleles per locus or heterozygosity between populations, but significant genetic variation among all populations. Significant differentiation was already detected between populations separated by distances as short as 10 km. Effective population size was low in a population which bordered farmland and where immigration clearly exceeds emigration. This result agrees with the hypothesis that edge population are sinks. My findings suggest that a network of suitable habitat patches within the capercaillies dispersal distance is essential to provide gene flow through highly fragmented landscapes. Considering the small size of suitable capercaillie habitat patches in the Bavarian Alps, many local populations only persist due to their connectivity with other populations. The amount of suitable habitat has to be maintained or restored in edge populations as well as in source populations to maintain the carrying capacity of the whole metapopulation system.

My study confirmed that microsatellites have the potential to detect differences among populations and source-sink dynamics even at a local scale. Results of such studies help to develop improved, effective management and conservation plans for endangered species such as the capercaillie.

Zusammenfassung

Der Einfluss der Lebensraumfragmentierung auf die genetische Populationsstruktur ist ein zentrales Problem des Naturschutzes und Wildtiermanagements. Eine zunehmende Zerstückelung der Landschaft durch menschliche Aktivitäten führt zur Bildung von Metapopulationen und schließlich zur Isolierung einzelner Populationen. Am Beispiel des Auerhuhns (*Tetrao urogallus*), einer wichtigen Zielart für den Naturschutz in Mitteleuropa, untersuchte ich auf verschiedenen räumlichen Ebenen die Auswirkung der Habitatfragmentierung auf die genetische Diversität von Populationen mittels nicht-invasiver Sammelmethoden.

Dazu entwickelte ich spezifische Mikrosatelliten (Kapitel 3.1) und zeigte die Eignung dieser genetischen Marker für Populationsuntersuchungen an Vögeln. Als Ausgangsmaterial dienten in der vorliegenden Studie hauptsächlich im Gelände gesammelte Mauserfedern und Kotproben. Für die Extraktion und Analyse dieser Proben entwickelte ich spezielle Methoden (Kapitel 3.2) und demonstrierte die Zuverlässigkeit der nicht-invasiven Probennahme für populationsgenetische Untersuchungen an Vögeln (Kapitel 3.3). Ein wesentliches Problem bei der Analyse von Feder- und Kotproben aus dem Gelände stellt die Degradierung der DNA und die damit verbundenen Probleme der korrekten Identifizierung des Genotyps dar. Eine verlässliche genetische Analyse ließ sich bei ~50% aller gesammelten Mauserfedern durchführen. Einzelne Federn, die nach der Mauser für mehrere Monate im Feld lagen, konnten noch erfolgreich für eine genetische Analyse verwendet werden. Federn, die von einem Riss, einer Rupfung oder einem geschossenen Vogel stammten, wiesen größere Mengen an DNA auf, und die Amplifikation mittels PCR war zuverlässiger als bei Mauserfedern. Grosse Federn (Arm- und Handschwingen, Steuerfedern) lieferten eine höhere Ausbeute an DNA als kleine Federn (Körpergefieder). In dieser Untersuchung zeigte ich zum ersten Mal, dass mittels im Gelände gesammelter Mauserfedern und Kotproben populationsgenetische Untersuchungen an Vögeln durchführbar sind. Dies eröffnet eine neue Perspektive für Populationsstudien, insbesondere bei störungsempfindlichen und bedrohten Vogelarten.

Der Lebensraum des Auerhuhns ist auf mehreren räumlichen Ebenen fragmentiert. Innerhalb des europäischen Verbreitungsgebietes kommen Auerhühner in isolierten Vorkommen unterschiedlicher Größe vor. Diese sind voneinander geographisch getrennt und die Distanzen zwischen den einzelnen Verbreitungsgebieten können bis zu mehrere hundert Kilometer betragen. Zwischen den Vögeln dieser Gebiete konnte ich signifikante genetische Unterschiede nachweisen, die zeigten, dass die Populationen voneinander isoliert sind (Kapitel 4.1). Die genetische Variabilität war in den Pyrenäen und isolierten Populationen in Mitteleuropa, wie

Schwarzwald oder Vogesen, geringer als zwischen den alpinen Populationen oder den Vorkommen in Nordeuropa. Diese Ergebnisse weisen darauf hin, dass eine Verkleinerung des Verbreitungsareals zu einer Isolierung der Pyrenäen und erst in den letzten Jahrzehnten zu einem Zusammenbruch der Metapopulationsstruktur in Mitteleuropa geführt hat. Damit sind ehemals verbundene Populationen, wie Fichtelgebirge und Thüringer Wald oder Schwarzwald und Vogesen heute isoliert. Eine fortschreitende Verkleinerung des Lebensraums wird zum Entstehen weiterer isolierter Vorkommen führen. Die genetische Vielfalt kann jedoch nur erhalten werden, wenn die Verbindungen zwischen den noch bestehenden einzelnen Vorkommen erhalten und verbessert werden.

In den Alpen kommen Auerhühner in Waldgebieten einzelner Bergstöcke vor. Die räumliche Anordnung dieser Populationen lässt sich als eine Metapopulation beschreiben. Ein Ziel meiner Arbeit war es, die genetischen Auswirkungen dieses Verbreitungsmusters zu beurteilen (Kapitel 4.2). Ich untersuchte den relativen Einfluss von Genfluss und genetischer Drift auf die genetische Struktur in zwei Gruppen von Populationen. Die erste Gruppe am Alpennordrand bestand aus Populationen, die aufgrund ihrer Lage am äußersten Rand des Verbreitungsgebiets begrenztem Austausch mit Nachbarpopulationen hatten. In der zweiten Gruppe von Populationen fasste ich Populationen aus dem zentralen alpinen Verbreitungsgebiet zusammen, die allseitig von benachbarten Populationen umgeben waren. Innerhalb aller alpinen Populationen entdeckte ich eine hohe genetische Variabilität, die sich zwischen den beiden untersuchten Gruppen nicht unterschied. Die geographische Distanz hatte einen signifikanten Einfluss auf die genetische Distanz in den Populationen der Nordgruppe, jedoch nicht in den zentralen Populationen. Dieser Zusammenhang zwischen Genfluss und räumlicher Isolierung in den Nordalpen konnte am besten mit einem Trittstein-Modell erklärt werden: Jungvögel immigrieren in die jeweils benachbarten Populationen. Gene werden somit von Population zu Population weitergegeben, wobei die einzelnen Bergstöcke als Trittsteine fungieren. In den zentralen Populationen jedoch war eine genetische Differenzierung der Populationen durch Drift die wahrscheinlichste Erklärung: hier dürften die hohen Bergkämme Barrierewirkung haben, so dass der Genfluss immer wieder unterbrochen wird.

Zwischen einzelnen Bergstöcken der bayerischen Alpen fand ich große Unterschiede im Genfluss zwischen einzelnen Populationen (Kapitel 4.3). Diese ließen sich durch ein „source-sink“ Modell beschreiben: Einzelne Populationen (sink) existieren nur, weil Vögel aus benachbarten Quellpopulationen (source) einwandern. Auerhühner zwischen einzelnen Bergstöcken unterschieden sich in ihrer genetischen Diversität nicht, aber die einzelnen Vorkommen ließen sich eindeutig voneinander abgrenzen. Diese Differenzierung konnte ich sogar auf kurzer räumlicher Distanz (~10 km) feststellen. Die effektive Populationsgröße war in einer Population mit ausgeprägter Randlage zum Agrarland der Voralpen am geringsten. Dies unterstützt die „source-sink“ Hypothese. Meine Ergebnisse zeigten deutlich, dass ein Netzwerk geeigneter Auerhuhnlebensräume notwendig ist, um den Genfluss in einem hoch

fragmentierten Lebensraum sicherzustellen. Die Zerstörung des Lebensraums von Quellpopulationen kann das Aussterben zahlreicher kleiner Populationen nach sich ziehen. Wenn man die vergleichsweise kleinen Auerhuhnlebensräume in den nördlichen Alpen berücksichtigt, wird klar, dass viele dieser kleinen Populationen nur noch existieren, weil sie mit anderen Populationen in Verbindung stehen. Für den Schutz des Auerhuhns ist es demnach zwingend erforderlich, dass die vorhandenen Lebensräume erhalten und verbessert werden, um den Erhalt des gesamten Metapopulationssystems sicher zu stellen. Dies gilt ganz besonders für die Populationen der Randlagen, die auf zuwandernde Vögel angewiesen sind.
In der vorliegenden Studie zeigte ich, wie mit genetischen Markern wichtige Erkenntnisse für den Naturschutz gewonnen werden können. Selbst auf kleinräumiger Ebene, zwischen Bergstöcken, lässt sich eine genetische Differenzierung zwischen Populationen feststellen und die Dynamik von Migration und Genfluss beschreiben. Damit können genetische Studien dazu beitragen, verbesserte, effektive Managementpläne und Schutzmaßnahmen für Auerhühner zu entwickeln.

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8 Acknowledgments

This work could not have been done without the help of many people, who shared their knowledge, helped with practical things and contributed to this thesis by keeping up my enthusiasm for science.
I thank my supervisor, Ilse Storch, for investing large amounts of time and correcting various drafts of papers. From Ilse, I got fast and sensible comments on anything from my introduction to the last paper. If we hadn´ t walked that mountain together, this thesis would not have existed.

To start with the official heads,
I thank Wolfgang Schröder for providing logistic support at the Unit of Wildlife Research and Management, Freising.
Ralph Kühn, Christian Ludt, Berhard Gum, Tatjana Käser and Ute Simon shared the lab in Freising, Christine Gerschlager and Uda Wester solved all the administrative secrets, with Miriam Hansbauer, Aurelia Munck and Christiane Averbeck I had many stimulating discussions.
Gerhard Müller-Starck and Roland Schubert supported this project in the beginning. Eliane Röschter and Holger Paetsch helped by solving technical problems.

Dieter Ammermann provided lab and working space in Tübingen.
Martha Schneck, Gabi Dürr, Anne Jürgens, Gabi Laufer, Sigrid Hojak, Peter Trontelj, Karl-Heinz Hellmer created a good working atmosphere.
Günther Steinbrück introduced me to molecular techniques, improved my manuscripts and provided invaluable advice in many details.
Robert Paxton introduced me to the secrets of microsatellite cloning and spent considerable amounts of time improving my manuscripts. Although his study animals are social and small (and no birds at all) – I learned much!
Jürgen Tomiuk was always there to discuss population genetic problems and gave helpful comments on the data analysis and the interpretation of the results.
Diether Sperlich provided helpful comments on my manuscript and Birgitt Schönfisch helped with the data analysis of the permutation tests.
Dominik Begerow and Michael Weiss introduced me to sequencing techniques and let me do that part of my thesis in their lab.
Stuart Piertney helped with the enrichment protocol.

8 Acknowledgments

I thank Jacob Höglund at the Evolutionary Biology Centre in Uppsala for inviting me to his lab, for encouraging me to carry on with this project, for numerous hours in very inspiring discussions and for deep insights in these crazy lekking birds and freezing together in Dovre Fjell.
Lisa Shorey was a good company in discussing matters of science and life and she offered a place to stay - even when I came from the fjell (smelling like a snipes´ bottom) and helped to improve my English.
Ursula and Beat shared their apartment with me and were a pleasant company.

Monika Kern provided the maps and Jonathan Pritchard sent the C program for the assignment test.
Peter Burger was a great help when my computer crashed a few days before submitting the thesis.

A great number of people provided feather samples during the years: particularly S. Brunner, H-J. Fünfstück, H. Gruber, R. Kilzer (Birdlife Vorarlberg), S. Klaus, J. Kurhinen, H. Kuske, E. Menoni, R. Mohr, R. Ploner, F. Preiss (GTV), B. Scherer, R. Suchant (FVA), P. Trontelj, P. Wegge, H. Zeiler and A. Zeitler.
I am indebted to the University of Munich for helping me with a graduate scholarship in the beginning and the DFG provided financial support later on.
Thanks to my family for giving me financial support during my studies and letting me enjoy the freedom of this sometimes strange bird business not only in my childhood but through to adulthood. With Moni I discussed some English (and other) problems, my father kindly draw the feather on the front page.
Sebastién Sachot was as pleasant company in the field as at conferences and especially in the finish of our theses.
Albin Zeitler, his family and the best dogs (and craziest lop ears) of the world Gitti and Ares, for laying the foundation of this thesis in bringing me to grouse.
I have to thank especially Sabine for many inspiring discussions about our theses and life in general. Julia, Birgit, Sigi, Hans, Rainer, Maren, Arp, Simone, Christine, Sünne, Michael, Christoph and many others helped to show me that there is more to life than science (although everything is science…).
Finally I thank Cornelia for all her patience (and many feathers) during the time of this thesis, although I could never convince her that one should search for feathers in dense bushes, better to pick bilberries then.

Last but not least, I do not wish to forget the horses of the forest, which were finally co-operative and stopped hiding from me after more than twenty years of birdwatching.

9 Appendix

Description of microsatellite loci for *Tetrao urogallus;* sequences and repeat type, PCR fragment length (based on the clone sequence and Genbank accession numbers of the clone sequences.

Locus	repeat type	PCR product (bp)	Accession number
TUD 1	$(CA)_{14}$	209	AF254644
TUD 2	$(CA)_{13}TA(CA)_9(CAAA)_4$	200	AF254645
TUD 3	$(GT)_{11}$	192	AF254646
TUD 4	$(CA)_{21}$	167	AF254647
TUD 5	$(GT)_{23}$	193	AF254648
TUD 6	$(CA)_{21}$	210	AF254649
TUD 7	$(GA)_2(CA)_{11}$	200	AF254650
TUD 8	$(GT)_{15}$	187	AF254651
TUD 9	$(CA)_5CG(CA)_{10}$	149	AF254652
TUT 1	$(CTAC)(CTAT)_2AT(CTAT)_{12}$	217	AF254653
TUT 2	$(GATA)(GACA)(GATA)_{12}(GACA)(GATA)_7$	160	AF254654
TUT 3	$(TATC)_{11}$	154	AF254655
TUT 4	$(TATC)_2TGTT(TATC)_4TGTT(TATC)8$	179	AF254656
TUT 5	$(GAAA)_{42}GAAAA(GAAA)_6$	358	AF254657
TUT 6	$[(GAAA)_2(GAAAA)_5]_2(GAAAGAAAA)_4(GAAA)_{22}$	221	AF254658
TUT 7	$(CTTT)_5CTTTT(CTTT)_{46}$	329	AF254659
TUT 8	$(GTTT)_4CTTTCCTTTTT(CTTT)_{12}(CTTT)_{25}$ CTTC$(CTTT)_9(CTTTT)_{12}$	395	AF254660
TUT 9	$(GAAAGAAAA)_4(GAAAA)_{21}GAGAA(GAAA)_{39}$	468	AF254661
TUT 10	$(CTAT)(CCAT)(CTAT)(CCAT)(CTAT)_8$	200	AY036082