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Ukrainian Black grouse (*Tetrao tetrix*)

Genetic diversity and population structure

Mariia Pavlovska

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Biology Education Centre and Department of Population Biology and Conservation Biology , Uppsala University
Supervisor: Jacob Höglund
External opponent: Biao Wang

Abstract

Standardized, unbiased and full data on the population diversity and structure of endangered species is one of the most essential needs of conservation biology, as it gives the possibility to effectively manage the species across its distribution range. The present study is, so far, the first one focusing on the neutral and adaptive genetic diversity of Ukrainian populations of Black grouse *(Tetrao tetix)*. It is aimed to fill in the gap in the data on the diversity of European populations of this species. The specific goals of this study are: (1) to estimate both neutral (microsatellite) and adaptive (MHC) diversity of the Northern and the Carpathian Black grouse populations, (2) to infer the population structure of Ukrainian Black grouse, (3) to study the population history of this species, (4) to compare Ukrainian populations with the European ones and estimate the rate of differentiation between them, (5) to draw conclusions about the possible management efforts of the Black grouse on the territory of Ukraine and make suggestions about the subsequent research of this species.

The results illustrate that Ukrainian populations are more diverse than the European ones and highly differentiated from them. Both studied populations did not show any signs of a recent bottleneck event. Population structure of Ukrainian Black grouse was more pronounced for the neutral variation than for the adaptive one, suggesting that balancing selection is shaping the MHC diversity. Yet, the MHC differentiation between the two studied populations was still high (Dest = 0.454), which could be a sign of local adaptations. Thus, it is suggested that the Northern and the Carpathian Black grouse populations should be treated as separate Management Units (MU).

Black grouse population in the Carpathian Mountains appeared to be more diverse than the one in the North in terms of neutral and adaptive genetic variation. Therefore, it was suggested that the Carpathian Mountains could have been a refuge for the Black grouse during the last glaciations period. This hypothesis should be tested in the subsequent study involving more extensive sampling in both regions and inferring phylogeographical analysis of Ukrainian Black grouse population.

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Introduction

It is highly important to have full, unbiased and standardized data on the distribution, dynamics and diversity of endangered species as it contributes to the efficient allocation of management efforts towards their protection. However, the challenge in getting this type of data from the Eastern European non-EU countries (Ukraine, Belarus, Georgia and European part of Russia) still exists, because of the lack of capacities in this region. Taking into account the fact that the mentioned countries belong to the same zoogeographical region as the rest of Europe, and, moreover, share some continuous populations, it is necessary to fill the gap in the species data. This will facilitate comparative analysis of the European species distribution and diversity.

It is recognized that one of the most valuable and productive tools of conservation biology is genetics. It allows receiving highly precise data on population size, structure, genetic variation, origin and hybridization (DeSalle & Amato 2004). Therefore, genetic studies of populations are of high concern.

So far, more conservation genetic studies have been focused on neutral variation with the use of microsatellite markers. These markers are codominant and non-coding, are characterized by high mutation rate and studies on them require relatively little amount of material, which can be sampled non-invasively (Höglund 2009). All this makes them an ideal instrument for conservation biology. However, microsatellite studies can provide the information on the neutral diversity only. They are used to infer the population structure, to study the demographic history and to assess the effects of genetic drift and subsequent inbreeding on population genetic variability.

On the contrary, studying Major Histocompatibility Complex (MHC) genes reflects the functional and expressed adaptive variation that is shaped by the selection processes. In vertebrates MHC genes are responsible for coding the cell surface glycoproteins, which launch adaptive immune response against pathogens. MHC molecules present the antigens on the cell surface, which is followed by antibody production or by destruction of antigen-presenting cell (reviewed in Ekblom *et al.* 2007).

The selection on MHC genes is believed to be driven by the interaction between an individual and its environment, and to be responsible for its evolutionary potential. Spatial and temporal pattern of functional variation reflects the possibility of the population adaptation to any novel conditions (Höglund 2009). In addition, it has recently been shown that MHC variation is more sensitive to a bottleneck event, as the loss of MHC polymorphism is 15 % greater than that of neutral genetic diversity in case of the rapid reduction of population size (Sutton *et al.* 2011). Therefore, it is highly important to study both the neutral and the adaptive genetic variation to get the full insight into species diversity pattern.

The following study is focused on neutral and adaptive genetic diversity of Black Grouse *(Tetrao tetrix)* in Ukraine. Black Grouse is characterized by a wide range of distribution - from Britain to Eastern Siberia with a northern limit in Norway (70° N) and a south limit in Kyrgyzstan and North Korea (40° N) (Figure 1). It is considered as the Least Concern according to the IUCN Red List of Species. Though, the populations of this species are fragmented and shrinking in size in the densely-populated Western and Central Europe and are listed in the national Red-data book (Storch 2007).

Figure 1. The distribution of Black Grouse in Eurasia, shaded in grey (from Storch 2007)

There are three putative populations of Black Grouse in Ukraine – in the North, in the West (Rostochia) and in the Carpathian Mountains (Fesenko & Bokotei 2002) (Figure 2). The Northern one is distributed through the whole Polissia region (from Volyn' to Chernigiv region). It is considered to be continuous and expanding in Central Polissia (Zhytomyr region) (Panov *et al.* 2002), yet there is evidence that the species density is shrinking in the North-Western part of Ukraine (Gorban' & Mateichyk 2009). The Carpathian population is believed to be isolated and characterized by the negative population trend. The total size of Ukrainian population of the Black grouse was estimated at roughly 13.000 individuals in 2007 (according to the Red-data book of Ukraine).

Figure 2. The distribution of Black grouse in Ukraine (reprinted with permission from the Red-data book of Ukraine)

One of the main aims of the following research was to estimate both neutral and adaptive genetic variation in two Ukrainian populations of the Black Grouse – the Northern one and the Carpathian one. On the basis of this data I planned to compare genetic diversity pattern in continuous (Northern) population and the isolated (Carpathian) one. This will give the possibility to draw conclusions and make suggestions on the allocation of management efforts towards the

conservation of Black grouse in Ukraine. I also aimed to infer the population structure of Ukrainian Black Grouse and to study its history by comparing the present field data with the museum samples. Another goal was to compare Ukrainian and the European populations of *Tetrao tetrix* in terms of neutral and adaptive genetic diversity.

Methods

Materials. Sampling and DNA extraction

Sampling of moulted feathers was conducted in the two geographic regions in Ukraine – in the Carpathian Mountains and in the northern Polissia – during the lekking period in spring 2011 (Figure 3). The feather samples were collected non-invasively, as the Black grouse hunting is illegal in most regions in Ukraine. In total 125 moulted feathers were collected in the North (N) and 37 in the Carpathian Mountains (C). All the samples were put individually in envelopes, labeled for place and date of collection and stored dry for 3-6 months.

Figure 3. Sampling areas of Black grouse in Ukraine: a) the Northern population; b) the Carpathian population (adapted from Wikimedia Commons under the Attribution-ShareAlike 3.0 Unported (CC BY-SA 3.0))

For the purpose of comparative analysis of the past and present genetic diversity of Ukrainian Black grouse populations, 24 stuffed Black grouse, collected between 1852 and 1974 and stored in Ukrainian museums (National Karazin University of Kharkiv, Zoological Museum of the National University of Uzhgorod, Zoological Museum of Taras Shevchenko University of Kyiv, Zoological Museum of the National Fedkovych University of Chernivzi, Natural History Museum of the Academy of Sciences of Ukraine (Lviv)), were sampled for feathers (Table 1). On average 4 feathers per individual were collected. Additionally, tissue samples were taken from 3 out of all stuffed birds sampled. All the material was put individually in envelopes, labeled for sex, place and date of collection and stored dry for the period of 1 month, until being processed.

Table 1. Museum samples from 3 regions in Ukraine

Region	**Number of samples**
Polissia (North)	11
Rostochia (West)	11
Carpathian mountains	2

DNA was extracted using QIAGEN DNeasy tissue kit (QIAGEN, Valencia, California). The standard manufacturer's protocol was optimized in order to receive higher yield and better quality of the DNA. The modifications to the extraction procedure included: 1) extended lysis time (24 hours) (Corrales & Höglund 2011) 2) double volume of K-proteinase (40µl) and ATL-buffer (360 µl), 3) double volume of AL-buffer and ethanol (400 µl of each), 4) adding cold ethanol. When dealing with the museum samples another protocol was used, this is dictated by their suspected lower quality. The changes applied to the standard manufacture's protocol, when processing the museum samples, were: 1) adding DTT solution to the digestion mix, 2) extended lysis time (24 hours) (De Volo *et al.* 2008), 3) additional incubation step after adding buffer AL (10 min. at 70°C), 4) warming up buffer AE at 55°C prior to using it (for the purpose of increasing the DNA yield from the membrane). The DNA yield and quality was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.) and varied between 0.1 ng/µl and 230.2 ng/µl. However, the DNA quantity and quality from moulted feathers is usually low and is believed to be hard to measure, therefore I have drawn conclusions about the DNA quality based on the amplification success (Gebhardt *et al.* 2009).

Neutral variation assessment

Genotyping

The QIAGEN Multiplex PCR Kit was used for the amplification of 11 microsatellite loci - ADL230, ADL 257, ADL 142, ADL 184, BG 15, BG 16, BG 18, TUT1, TUT2, TUT3, TUT4 (Piertney & Höglund 2001, Segelbacher *et al.* 2002). Microsatellites were organized in 3 multiplexes according to their annealing temperatures (48°C, 54°C and 60°C). The reaction mix was prepared in 10 µl volume following the standard manufacturer's protocol (Appendix 1 a). PCR amplifications were performed in BIOER thermal cycler. Negative control was included for each sample to test for the false amplifications. In order to avoid contamination DNA extraction, pre-PCR and post-PCR pipetting were done in the separate rooms.

In order to minimize scoring errors (due to allelic dropout, stuttering and the presence of null alleles) each sample was amplified in 3 separate PCR reactions.

PCR products were run on a Megabace^{TM} 1000 Automatic Sequencer (Amersham Biosciences, Buckinghamshire, UK) and then a Megabace Genetic Profiler v.1.2 was used to score the allele sizes for each locus (Amersham Biosciences 2003).

Data analysis

Prior to conducting the initial analysis the data set was examined for scoring errors. The rates of allelic dropout and null allele frequencies for each locus were estimated with the help of Gimlet (Valie`re 2002) and Genepop 1.2 software (Raymond & Rousset 1995) respectively. The ADL 257 locus was excluded from the further analysis due to high frequency of null alleles in the Northern population (Table 2). Some of the other loci showed signs of the presence of null alleles as well, but they were still included in the analysis, as the frequency of the mentioned scoring error was comparatively low and it was present in one of the two populations only. BG 16 was not used in the following study as almost no samples amplified at this locus.

Table 2. Frequency of null alleles calculated for each locus for the two Ukrainian Black Grouse populations – the Northern (N) and the Carpathian (C) ones.

Locus	Population	
	N	C
ADL230	0.0000	0.0000
ADL257	**0.6203**	0.0000
ADL184	0.0000	0.1374
ADL142	0.0000	0.0000
BG15	0.2211	0.0374
BG18	0,0038	0.0381
TUT1	0.1861	0.0825
TUT2	0.1210	0.0874
TUT3	0.0812	0.2934
TUT4	0.0000	0.0215

The next step was cleaning the data set from repeated genotypes. As all the samples were collected non-invasively, some of them could belong to the same individual and, therefore, the same genotype could have been typed several times. The Gimlet software was applied to find the matching pairs of samples. The samples were considered to be identical if there was at most one mismatch for one allele at one locus (Straka *et al.* 2011).

The data set was analyzed from the point of the amplification success of the samples. The samples that did not amplify at more than a half of all loci used were excluded from further analysis as they did not meet the quality criteria.

As a result of all checking procedures, the data set used in the following study, included 106 samples (75 – from the Northern population and 31 – from the Carpathian mountains), which were genotyped for 9 loci.

Prior to calculating the actual diversity measures, tests for linkage disequilibrium and for deviation from Hardy-Weinberg equilibrium were conducted in Genepop 1.2 (Raymond & Rousset 1995).

The two populations were examined for any recent bottleneck events with the help of the Bottleneck 1.2.02 software (Cornuet & Luikart 1997). The main idea of that program is that alleles are lost more rapidly than the heterozygosity, when the population experiences reduction in the number of individuals. Therefore, when the expected heterozygosity at Hardy-Weinberg equilibrium (He) is compared to the expected heterozygosity at mutation-drift equilibrium (Heq), the He will be higher in the previously reduced populations. In this study the TPM model with 88% step-wise mutations (Ps = 0.88) and the variance of 10% was used to test for the recent bottlenecks, as recommended by Straka *et al.* (2011).

There is evidence that the approach described above can fail to predict the bottleneck event if the effective size of studied population is too small and the new mutation-drift equilibrium is set rapidly (Höglund 2009). Therefore, the MRatio approach is used additionally to detect possible recent reduction of the population size in this study. The main idea of this method is that the total number of alleles (*k*) is reduced faster than the range in allele size (*r*) when a population experiences a bottleneck event (Garza & Williamson 2001). It is a consequence of the fact that *k* is decreased by the loss of any allele, while r is dropped down only in case of the largest or the smallest allele. Thus, the MRatio

$$M = k/r \qquad \text{(Garza \& Williamson 2001)} \qquad (1)$$

is expected to be lower in recently reduced populations than in those that are in equilibrium. In general, it is claimed that the MRatio is greater than 0.82 in the populations that have not experienced a recent bottleneck event, and that it is lower than 0.7 in those groups that suffered a rapid reduction in size (Garza & Williamson 2001).The main advantages of the method are that it gives an accurate resolution even for the small samples (25 diploid individuals is believed to be enough) and that M value is preserved during comparatively large time scale.

In order to test for the recent bottleneck in the Northern and in the Carpathian populations of Black grouse, MRatio (M) was calculated in M_Pval program (Garza & Williamson 2001). Then an equilibrium MRatio (Meq) and the Critical MRatio (Mc) were estimated in CriticalM program with the proportion of one-step mutations Ps= 0.88, the average size of non one-step mutations Δg=2.8, and with 10 000 times simulation (Garza & Williamson 2001). The $\theta = 4Ne\mu$ was varied over three values – 0.1, 1 and 10 as recommended by Straka *et al.* (2011) in order to account for the various possible effective population size prior to the bottleneck event. The M value was then compared to the threshold values and the Meq was aligned to the Mc to draw conclusions about the recent reduction in the population´ sizes.

The measures of genetic diversity were computed in ARLEQUIN (Excoffier *et al.* 2005). I have calculated observed and expected heterozygosities (He and Ho) and the number of alleles per each locus using Microsatellite Toolkit (Park 2001). Heterozygosity expected at Hardy-Weinberg equilibrium (He) was calculated as:

$$He = 1 - \sum_{i=1}^{\#alleles} p_i^2 \qquad \text{(Nei 1972)} \qquad (2)$$

Additionally, allelic richness (AR) and Wright's inbreeding coefficient (FIS) (Wright 1943) were calculated for each locus using FSTAT 2.9.3.2 (Goudet 1995) software. AR is a measure of the number of alleles in the population, which is weighted by sample size. Fis measures the effects of either assortative mating or random mating of close genetic relatives. The latter results in inbreeding and, therefore, is of great interest for conservation. The inbreeding coefficient is defined as:

$$Fis = \frac{H_e - H_o}{H_e} \qquad \text{(Wright 1943)} \qquad (3)$$

ANOVA was performed in the R software (R Foundation for Statistical Computing) in order to estimate the significance of the differences in diversity measures between the two populations.

The structure and differentiation of the populations was examined in ARLEQUIN by calculating the global Fst and conducting multi-locus hierarchical analysis of molecular variance (AMOVA). Fst estimates the extent of population subdivision (Höglund 2009) and is expressed as:

$$Fst = \frac{H_T - H_S}{H_T} \qquad \text{(Weir \& Cockerham 1984)} \qquad (4),$$

where H_T is expected heterozygosity within the total population and H_S is the average expected heterozygosity within subpopulations. The Fst itself is believed to be biased in estimation of demographic parameters. Therefore, linearized Fst was used for the purpose of further analysis (Slatkin 1995).

It is argued that Fst and Gst indexes of differentiation can be unreliable and imprecise, when the diversity of the studied populations (the number of unique alleles) is high (Jost 2008). This is the result of their dependence on mean subpopulation heterozygosity Hs. In order to control for such an effect, the Dest index was also used (Jost 2008). Dest was calculated in the SPADE software (Chao & Shen 2010).

Population structure was visualized in Genetix 4.05.2 (Belkir *et al.* 2000) by performing factorial correspondence analysis (FCA).

Another approach to study the population structure of Ukrainian Black Grouse was the Bayesian clustering implemented in STRUCTURE software. The program finds population structure under the assumptions of Hardy-Weinberg or linkage disequilibrium and searches for the groups of individuals that are not in disequilibrium (Pritchard *et al.* 2000). Both admixture and non-admixture models were tested and then the admixture model was chosen as the more realistic one. The parameters used were: undefined population structure, correlated allele frequencies among populations, length of burning period = 50 000, number of MCMC replicates after burning = 10 000 (Evanno *et al.* 2005). I have tested 10 possible numbers of groups (K = 1-10), each trial was run for 20 times. Structure Harvester was used to visualize the STRUCTURE output and to find the most probable ΔK. ΔK was used instead of K value, as it is more reliable and accurate according to Evanno *et al.* (2005). Finally, the results for the most probable ΔK were generalized in CLUMPP (Jakobsson & Rosenberg 2007) and visualized in DISTRUCT software (Rosenberg 2004).

Adaptive genetic diversity assessment. RSCA.

Reference strand-mediated conformational analysis (RSCA) was applied for the purpose of genotyping MHC genes in Ukrainian Black Grouse population. The main idea of the RSCA is that the sequences of interest are amplified and hybridized to a given, fluorescently labeled reference strand (FLR). As a result of hybridization heteroduplexes with mismatches for each sequence variant are formed. Particular sequence variants are then detected, depending on the mobility of heteroduplexes in non-denaturating environment (Lenz *et al.* 2009).

In order to save time and resources, 56 samples (36 from the Northern population and 20 from the Carpathian Mountains) were chosen out of the whole data set to be processed in RSCA. The main criterion for this choice was the amplification success of the samples during the microsatellite genotyping - most samples included amplified in at least 7 loci out of 9.

The protocol, originally developed for the Black Grouse by Strand & Höglund (2011) was used in the analysis of Ukrainian Black Grouse populations (Appendix 1 b). First, 4 different FLRs from the known clones of grouse were amplified, using FAM-fluorescently labeled RNA F1a and the non-labeled RNA R1a primers. It should be mentioned that the reference alleles were chosen from both the Black Grouse (1 FLR) and the Hazel Grouse (3 FLRs) species. Using the FLRs from the Hazel grouse minimizes the chance of omitting the alleles of interest that could be identical to the sequence variants in the reference strand (Strand & Höglund 2011).

The second step was the amplification of the alleles from the test samples, using the primer pair (RNA F1a (5'-GACAGCGAAGTGGGGAAATA-3') and RNA R1a (5'CGCTCCTCTGCACCGTGA-3'). All the PCR products were confirmed on a polyacrylamide gel.

The original protocol was only modified for the hybridization step. The dilution factor for the FLR was chosen to be twice as low as in the protocol in order to get higher reference peak on the electrophorerogram for the more accurate measurement of the test allele size.

The resulting heteroduplexes were run on a Megabace^{TM} 1000 Automatic Sequencer (Amersham Biosciences, Buckinghamshire, UK) and afterwards alleles were scored in the Megabace Fragment Profiler v.1.2.(Amersham Biosciences 2003).

Prior to the initial analysis of the RSCA results one should take into account that the RSCA does not provide information about each locus separately. So, the data on adaptive genetic diversity in this study is based on the frequency estimates of the alleles and not on the entire diploid haplotypes (unlike the data on microsatellite diversity). This is a consequence of the fact that both MHC BLB loci in Black grouse are subjected to duplication and homogenizing concerted evolution through the inter-genetic exchange between the BLB1 and the BLB2 (Strand *et al.* 2011). The separate amplification of the two BLB loci is possible with the use of the long-range anchored PCR method, which is, however, much more time- and resource consuming than the RSCA. Yet, for the purpose of species conservation study the simpler – RSCA genotyping – is reasonable and yields sufficient resolution on the population scale (Strand *et al.* 2011).

Data analysis and sequencing

The resulting MHC alleles were compared to those listed in the RSCA library constructed by Strand & Höglund (2011) and the scoring rules of the authors were followed. Individual allele was confirmed if it was detected in 3 out of 4 FLRs (for the strong single peaks) or in 4 out of 4 FLRs (for the weaker peaks). These rules were applied for all alleles from the library, except BLB1, as it was identical to the FLR1 and, therefore, couldn't have been detected. All samples were genotyped twice to receive higher resolution and precision.

The diversity measures calculated for the MHC data were – the total number of alleles per population, the number of alleles per sample size, the number of private alleles in each population, theta k – the allelic richness, Pi – nucleotide diversity and $\hat{H}$ – gene diversity (Nei 1987). Theta k and Pi were calculated in ARLEQUIN (Excoffier *et al.* 2005). $\hat{H}$ was computed in the Microsoft Excel using the following formula:

$$\hat{H} = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right), \qquad \text{(Nei 1987)}$$

where n is the total number of gene copies in the sample, k is the number of haplotypes and p_i is the frequency of the ith haplotype. Since no haplotype can be identified in the RSCA k is considered to be equal to 1.

The differentiation in adaptive diversity of the Carpathian and the Northern population was estimated using Fst, calculated in ARLEQUIN (Excoffier *et al.* 2005) and Dest, calculated in SPADE (Chao & Shen 2010).

New alleles that have not been previously included in the RSCA library were detected. In order to confirm the presence of the unique variants, the new alleles were cloned and sequenced (Appendix 1 c).

I picked 4 individuals (3 from the Northern population and one from the Carpathian), which had the highest amplification success with both microsatellites and the RSCA markers and showed the presence of the unique alleles in two separate PCR reactions. First, the PCR reactions, as in the RSCA protocol, were performed for all four samples. The PCR products obtained were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, California) and next the A-tailing was done to improve the subsequent ligation success. The resulting PCR-products were ligated into

the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). The competent *E.coli* cells were added to the TOPO cloning reaction mix and then incubated at 37°C for 12-14 hours following the original manufacturer's protocol.

20 positive colonies were picked for each of the two samples from the North and 9 colonies were chosen for the remaining sample from the North and for the one from the Carpathian population. The difference in the number of colonies analyzed is due to the weaker ligation success of the two samples, which can be caused by the lower DNA quality. The PCR with the universal M13F and M13R primers (supplied in TOPO TA cloning kit) was performed for all the colonies and the resulting PCR products were cleaned with Exo – Fast AP (Exonuclease I-Thermosensitive Alkaline Phosphatase) (Fermentas, St. Leon-Rot, Germany) and send to Macrogen for sequencing (Macrogen, the Netherlands).

The sequences were analyzed in Codon Code Aligner version 3.7.1 (LI-COR, Inc, USA) and compared to those of the alleles listed in the RSCA library. The reference sequences were downloaded from the GenBank (Benson *et al.* 2005). If the analyzed sequence included one or more unique motifs and was present in at least 3 clones, it was scored as the new one. Hereon, the FLR-scores of the new alleles (calculated in the previously conducted RSCA) were assigned to their sequences and the speculations about the frequency of the detected variants were made. However, the following results should be treated with cautiousness, as the FLR-scores of the new alleles should have been calculated in an additional RSCA on them. Yet, this could not have been done in the present study due to the time limit. In order to avoid subjectiveness the diversity measures and the differentiation indexes for the data set both with and without the new alleles will be reported.

Comparative analysis of genetic diversity in Ukrainian and European populations

Comparative analysis of the pattern of neutral and adaptive genetic diversity of Ukrainian Black grouse populations in relation to the other European populations was conducted. First, the appropriate source of the data on the other European Black grouse populations was chosen. I have decided on using the data of Strand *et al.* (2011) and Segelbacher *et al.* (2011) because of the high consistency of methods and protocols between the study cited and mine. The first study mentioned is useful for comparison, as it allows identifying the types of Ukrainian populations of Black grouse according to the diversity pattern that they exhibit. The authors divided the European populations into 3 groups based on their size and the isolation pattern: 1) continuous (N=>1000); 2) isolated (N<300); 3) small isolated (N<=30). The first group included the populations from Finland (Jyvaskyla), Norway (Kristiansand), Sweden (Jämtland), Latvia and Switzerland (Alps); the second one – from the UK (Northern Pennines), Germany (Luneburger Heide) and Poland (Sudety Mountains); and the third one – from the Netherlands (Sallandse Heuvelrug), Austria (Waldviertel) and Germany (Rhön). This subdivision scheme allows identifying the types of Ukrainian populations based on the comparison of their diversity parameters with those of the appropriate European populations. Analysis of the differentiation between the Ukrainian and other European populations of Black grouse and inferring Fst and Dest indexes is not done based on the described study, as the individual locus-by-locus data is not available.

The data from the study of Segelbacher *et al.* (2011) gives the insight of the diversity of both contemporary and historical European Black grouse populations and allows analyzing it in relation to the genetic variation of the Ukrainian ones. There are 5 populations studied by the authors: Danish historical, German historical, Dutch historical, German present and Dutch present. The locus-by-locus data is available here, so the pairwise Fst (ARLEQUIN) and the Dest (SPADE) indexes are calculated for all the populations.

The other European Black grouse populations were compared to the Ukrainian ones for the following parameters: expected heterozygosity (He), allelic richness (AR) – for the microsatellite diversity; and the number of the MHC alleles in the population/sample size (MHC/Pop), the nucleotide diversity (Pi) and allelic richness (theta k) – for the adaptive genetic diversity. ANOVA and Welch Two-Sample t-test were performed in R to check for the significance of any differences between the Ukrainian population and the ones studied by Segelbacher et al.

It should be mentioned that the entire analysis of European Black grouse diversity and differentiation is based on the molecular data from 5 loci (TUT1, TUT3, TUT4, BG15, BG18), as only those were used both in the present study and in the referenced ones.

Results

Museum samples

One of the goals of this study was to compare the present and the past genetic diversity of the two Ukrainian populations. Yet, the quality of the museum material appeared to be quiet low and the subsequent amplification was not sufficient to include the museum samples in the analysis. The frequency of null alleles was too high in ADL257, BG15 and TUT1 for the Northern population and in ADL 257, ADL 142, TUT1 and TUT2 for the Rostochia one. The two samples from the Carpathian Mountains had even lower amplification success (amplified in less than half loci). The tissue samples amplified efficiently, yet their quantity was too low to include them in the analysis. For the purpose of future investigations, it is highly desirable to sample tissue instead of feathers from the museum material, as it significantly increases the chance of getting sufficient amplification of the samples.

Microsatellite genetic diversity and differentiation

Signs of linkage disequilibrium (LD) were present among ADL 230 and ADL 142. In order to test, whether it could influence the estimation of the standard diversity measures and population differentiation parameters, separate tests were conducted, excluding these loci from the analysis. The results did not differ significantly from those, received with ADL 230 and ADL 142 included. Therefore, LD between those loci did not influence the estimation of the population neutral diversity measures.

Both populations showed signs of the deviation from Hardy-Weinberg equilibrium at certain loci. Yet, globally, the Northern population did not deviate, whereas the Carpathian did (Table 3). The two populations did not deviate from the expected L-shaped distribution and had high probability for heterozygosity deficiency (P=0.00293 for the Northern population and P=0.00488 for the Carpathian one), which illustrates that they did not experience any severe bottlenecks in the past.

Table 3. Fis inbreeding coefficient calculated for each locus for the Northern and the Carpathian population of Black Grouse.

Locus	Northern	Carpathian
ADL230	-0.292	-0.123
ADL184	-0.553	0.247
ADL142	-0.173	-0.073
BG15	0.535	0.058
BG18	-0.435	0.040
TUT1	0.393	0.102
TUT2	0.339	0.101
TUT3	0.105	0.675
TUT4	-0.421	-0.077
All	-0.045	0.111

Additional conclusions about the possibility of a recent bottleneck event were made from a MRatio analysis. The MRatio estimated from the number of alleles and their size range was 1.47 for the Northern Black grouse population and 1.03 for the Carpathian one. Both values are much higher than the threshold 0.7 mentioned in Garza and Williamson (2001). The simulations' results for the data are presented in Figure 4. The Meq was higher than the Mc in both populations, in all the replicates, regardless of the different θ. This supports the results of the Bottleneck and indicates that both the Northern and Carpathian populations are unlikely to have experienced the recent reduction in population size.

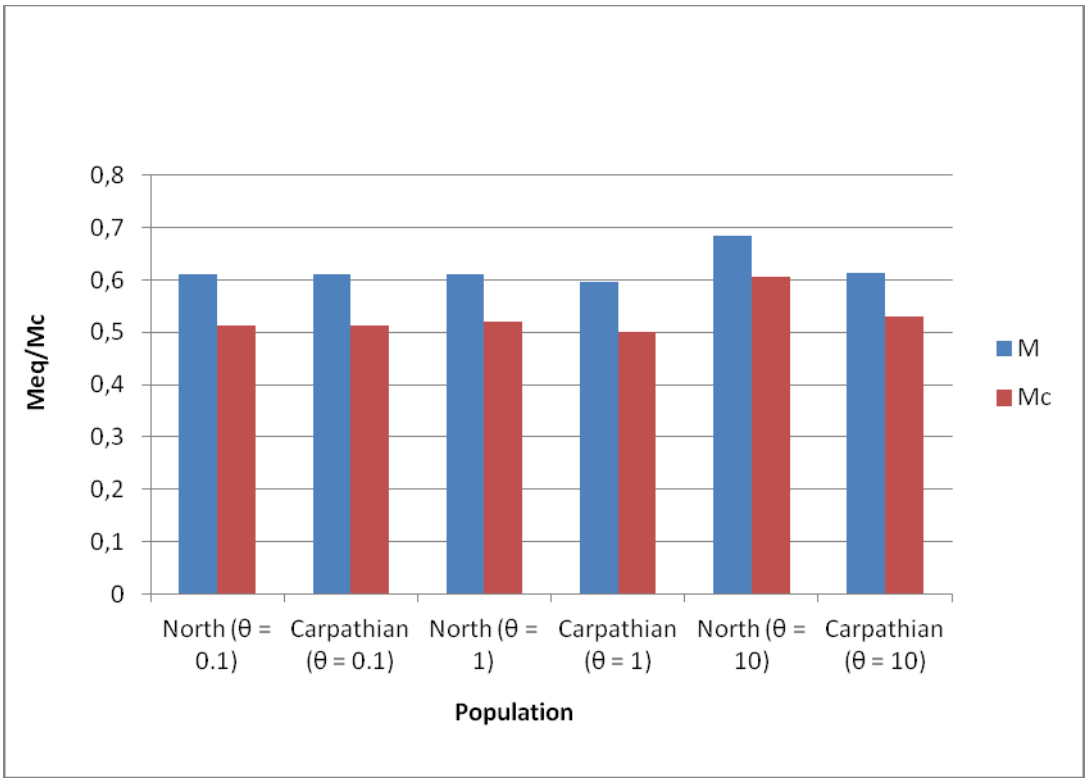

Figure 4. Equillibrium MRatio (Meq) and Critical M (Mc) calculated for the Northern and the Carpathian population with 3 different θ (0.1, 1 and 10)

Both observed and expected heterozygosities were higher for the Carpathian population than for the Northern one (0.74/0.71 and 0.87/0.78 respectively). Allelic richness appeared to be higher for the Carpathian population as well (9.18 compared to 6.44) (Table 4). Yet, ANOVA illustrated that the only significant difference in population diversity measures between the two populations was for the allelic richness (F value = 9.81, Pr(>F) = 0.0064) (Figure 5).

Table 4. Microsatellite genetic diversity indexes calculated for the Northern and the Carpathian Black Grouse populations. (Ho – observed heterozygosity, He – expected heterozygosity and AR – allelic richness)

Population	He	Ho	AR
Northern	0.71	0.75	6.44
Carpathian	0.87	0.78	9.18

a) b)

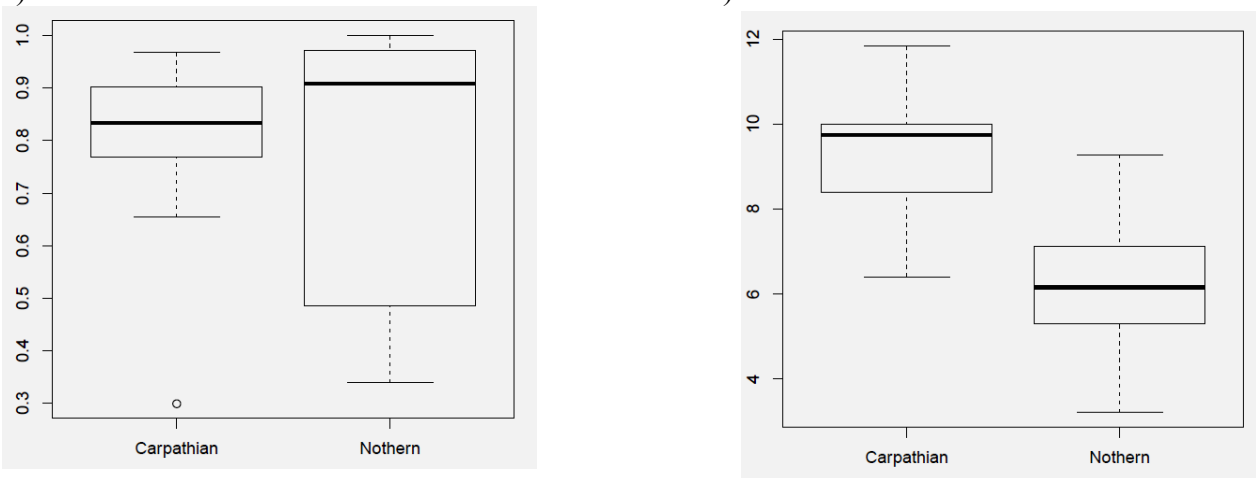

Figure 5. Genetic variation comparing the Northern and the Carpathian populations for the a) expected heterozygosity (He) and b) allelic richness (AR)

AMOVA analysis illustrated that the most significant variation was within populations (84.6%). The amount of variation among the Northern and the Carpathian populations was still significant – 15.4% (Table 5).

Table 5. AMOVA results (average for 9 loci)

Source of variation	Percentage variation
Among population	15.4
Within populations	84.6

The significant variation among the populations was supported by the global microsatellite fixation index Fst, which was estimated at 0.148 and was highly significant ($p<0.001$ after 110 permutations). Dest was calculated as 0.662 with the standard error of 0.024 after 1000 bootstraps.

The FCA plot for two factors illustrated that Ukrainian Black Grouse population is clearly differentiated into 3 separate clusters – the Northern one and the two groups from the Carpathian Mountains. The first axis on the plot explains 4.84 % of the total variation and the second axes – 3.84 % (Figure 6).

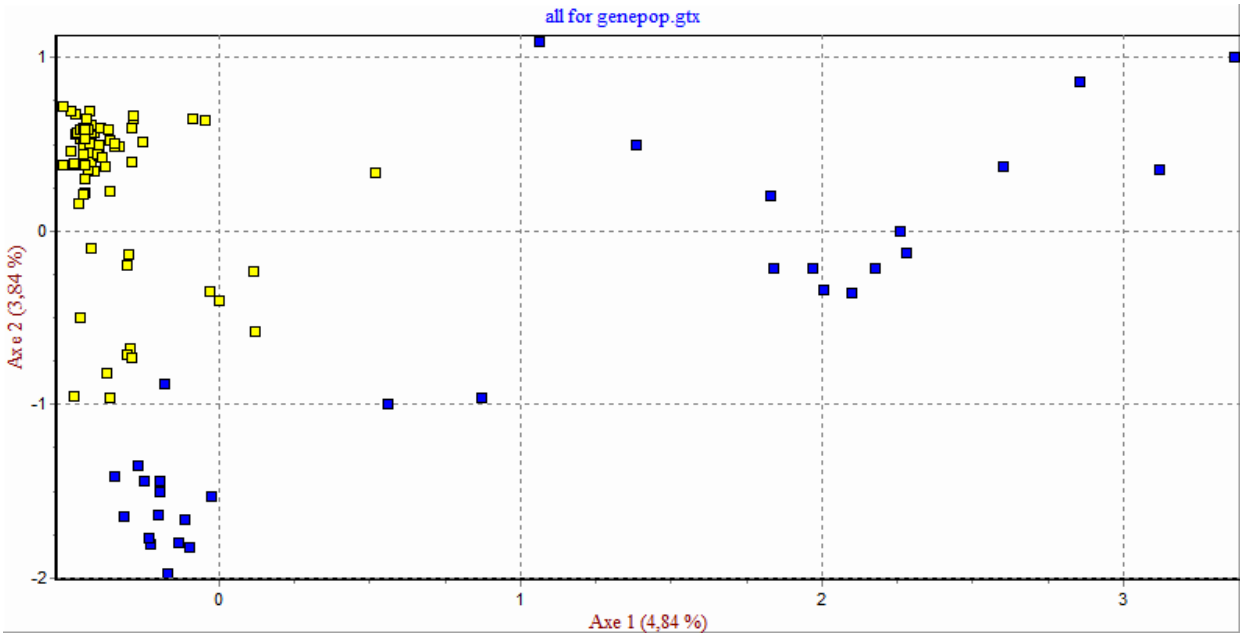

Figure 6. Two-factorial analysis for all genotyped microsatellites in two populations of Black Grouse in Ukraine (the Northern one - yellow squares and the Carpathian one – blue squares).

Further analysis with STRUCTURE illustrated the presence of both global and regional differentiation in two populations (Figure 7). The highest likelihood was for the presence of 4 clusters (ΔK=35.745), whereas 2 clusters had high probability as well (ΔK= 30.423) (Appendix 3). Therefore, here both variants are presented. When compared to the geographical origin of the samples, the 2 clusters coincided with the data from the Carpathian and the Northern region; and the 4 clusters corresponded to the different leks in each region. The 2 clusters approach illustrated that there were some individuals from the Northern population assigned to the Carpathian one. However, the higher resolution trial with 4 clusters indicated that these individuals belong to one of the leks in the North, not in the Carpathian region. The 2 leks in the Carpathian Mountains were highly differentiated from one another and, in general, there were no admixed genotypes in that region. The structure of the Northern population was different in a way, that there were some admixed individuals and the barriers between the two leks were weaker.

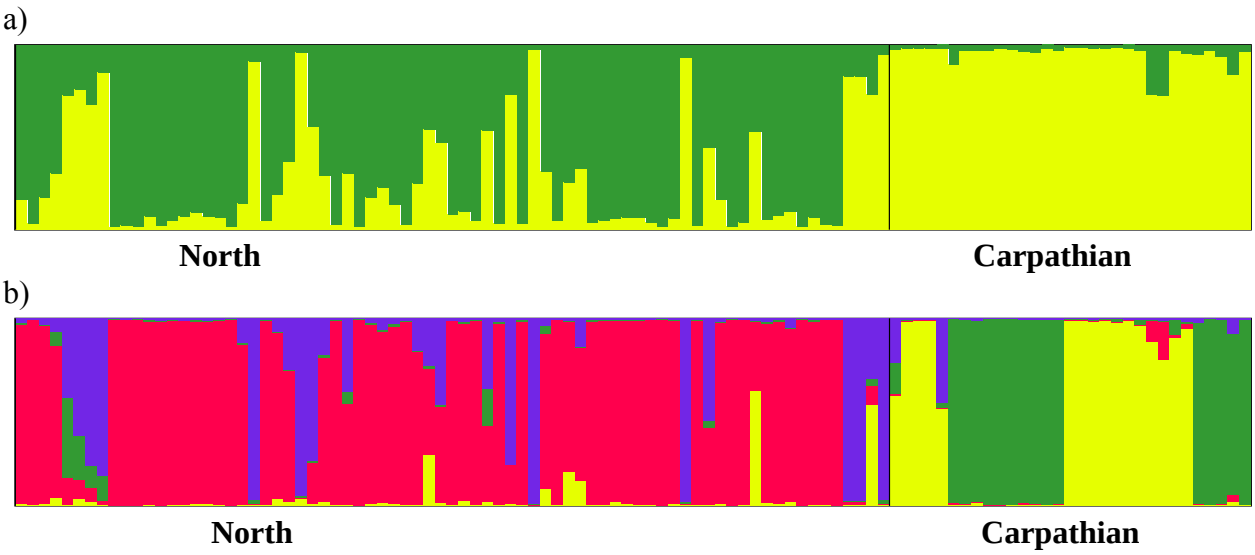

Figure 7. Bayesian clustering for the Northern and the Carpathian populations of Black Grouse a) 2 clusters; b) 4 clusters

Adaptive genetic diversity and differentiation

MHC allele frequencies were calculated for each individual and for the two populations. The total number of alleles for the Northern population was estimated as 13 and for the Carpathian one – as 7 (Table 6). The Northern population carried seven private alleles – BLB3, BLB4, BLB6, BLB9, BLB11, BLB21 and BLB22, whereas the Carpathian one had one private allele – BLB25. The gene diversity was higher for the Northern population ($\hat{H}$ = 0.81), but was high for the Carpathian population as well ($\hat{H}$ = 0.77). The index of allelic richness (theta k) was estimated as 4.16 for the Northern population, which is again higher than that for the Carpathian one (3.04). However, the significance of this difference can be questioned, because of the wide 95% confidence interval of these estimations for the both populations (Table 6). The nucleotide diversity for the Northern population was calculated as 12.69, whereas for the Carpathian one it was 10.44. Yet, it only allows making general conclusions about the MHC diversity in each population, without comparing the indexes among them, because the standard deviations of the measures are high.

Table 6. The MHC diversity calculated for the two Ukrainian populations of Black Grouse – the Northern and the Carpathian ones.

Population	Number of alleles	Number of allels/sample size	Number of private alleles	$\hat{H}$	Theta k	95 % confidence interval	Pi	SD
Northern	13	0,16	7	0.81	4.16	[2.21,7.50]	12.69	6.41
Carpathian	7	0,30	1	0.77	3.04	[1.24,7.09]	10.44	5.51

The distribution of the different alleles in two populations is illustrated by Figure 8. The most frequent in the Northern population are the BLB1, BLB5, BLB7 and BLB16. The Carpathian population, in turn, is characterized by the prevalence of BLB2 and BLB16.

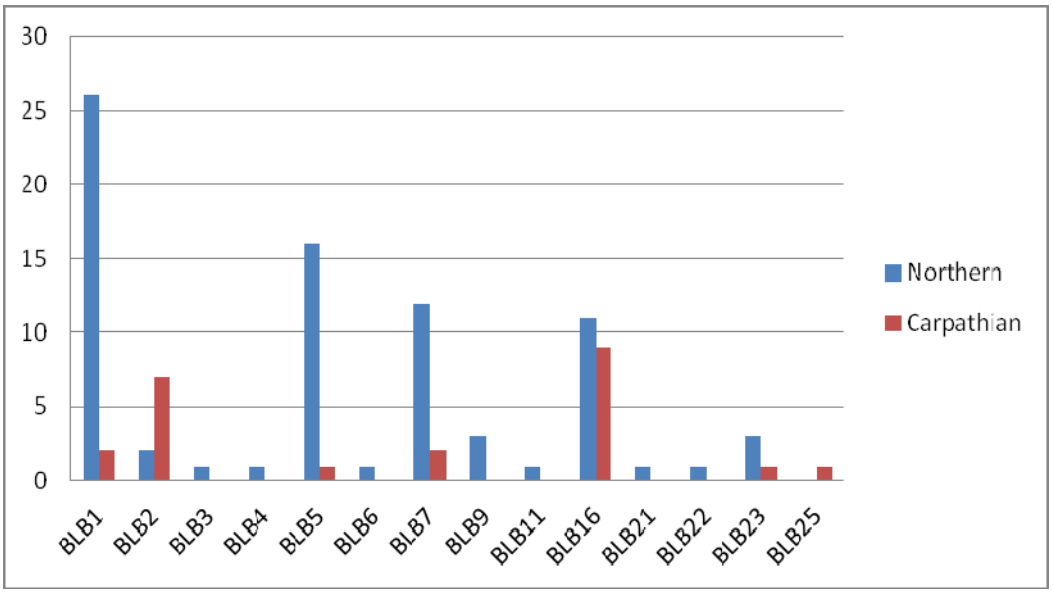

Figure 8. The distribution of the MHC alleles' frequencies in the Northern and the Carpathian populations of Black Grouse.

The global Fst value was estimated as 0.038, but wasn't significant after 1023 permutations. The Dest index revealed some differentiation between the two populations (Dest = 0.451). Yet it is hard to draw conclusions about the extent of this differentiation due to the high standard error (0.122) and wide 95% Confidence interval (0.160, 0.640).

Three new alleles were sequenced – BLB27, BLB28 and BLB29. The sequences are presented in the Appendix 2. Including these alleles in analysis of the two populations did not change the overall pattern of adaptive genetic diversity. BLB27 and BLB28 were shared among the two populations, but BLB29 was found in the Northern population only. Yet, the limited sampling size of the Carpathian population doesn't allow claiming that BLB29 is unique to the individuals from the North. All the measures of MHC diversity appeared to be respectively higher when the new alleles were included in calculations (Table 7). The Northern population was discovered to be more diverse than the Carpathian one, considering higher gene diversity ($\hat{H}$) and more alleles in total. However, as in the previous trial (without the new alleles) the population from the Carpathian Mountains had more alleles/sample size.

Table 7. The MHC diversity calculated for the two Ukrainian populations of Black Grouse – the Northern and the Carpathian ones, including the newly sequenced alleles.

Population	# of alleles	# of alleles/sample size	# of private alleles	$\hat{H}$	Theta k	95 % confidence interval	Pi	SD
Northern	16	0,18	8	0,85	5,35	[3,00, 9,18]	14,10	7,07
Carpathian	9	0,36	1	0,83	4,19	[1,88, 9,01]	12,51	6,48

The distribution of the frequencies of different alleles in studied populations changed slightly after the new alleles were added. Figure 9 illustrates that BLB28 has comparatively high frequencies in the Northern population and BLB27 is frequent in the Carpathian one (Figure 5).

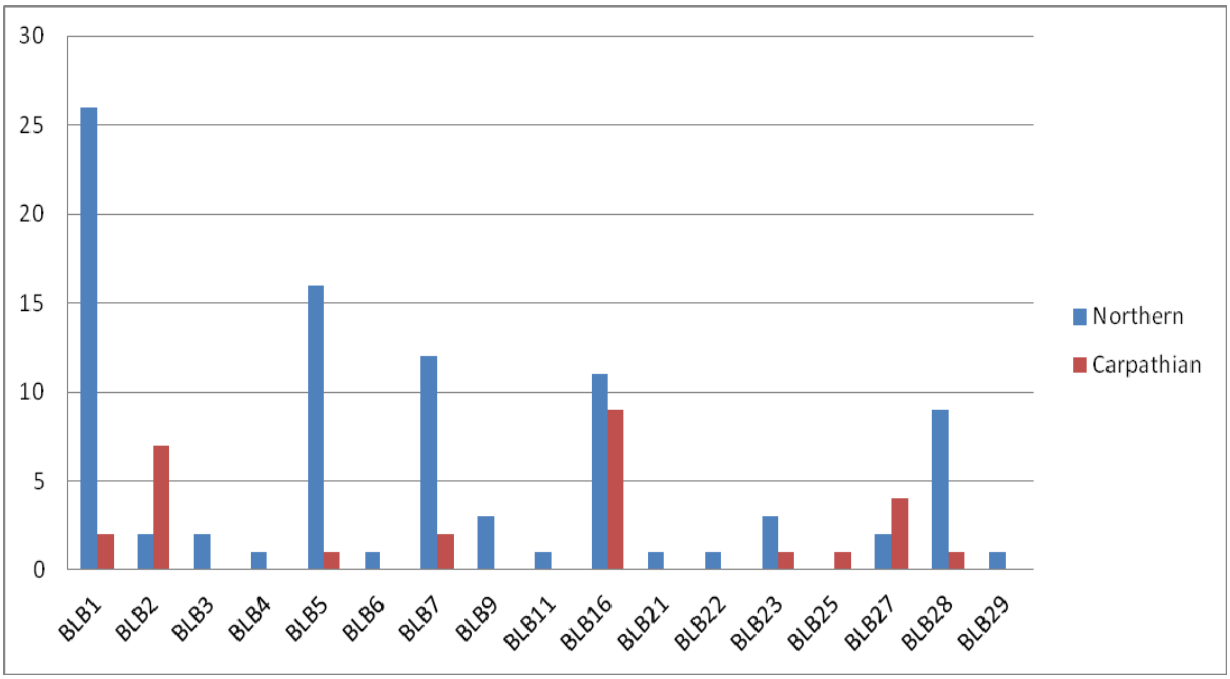

Figure 9. The distribution of the MHC alleles' frequencies in the Northern and the Carpathian populations of Black Grouse, including the newly sequenced alleles

The global Fst was 0.046 and not significant after 1023 permutations, as in the previous trial. The Dest wasn't influenced by the new alleles added and was calculated as 0.454 with the standard error of 0.132 and the 95% Confidence interval (0.183, 0.694).

Comparative analysis of genetic diversity and differentiation in Ukrainian and European populations

The levels of heterozygosity, allelic richness, nucleotide diversity and adaptive allelic richness in both Ukrainian populations of Black grouse correspond to those of Finland (Jyvaskyla), Norway (Kristiansand), Sweden (Jämtland), Latvia and Switzerland (Alps), which allows assigning them to the group of large continuous populations. Both studied populations have higher rates of allelic richness and the Carpathian Black grouse are characterized by higher heterozygosity compared to the other European populations (Appendix 4).

The results of Welch Two-Sample t-test revealed that the only significant differences between the European populations of Black grouse studied in Segelbacher *et al.* (2011) and the Ukrainian ones were in allelic richness AR. Therefore the results for this parameter only are presented here. AR was shown to be significantly different between the Northern and the Carpathian populations and all the European (Table 10).

Table 8. Results of Welch Two-sample t-test for the significance of allelic richness differences in European population of Black grouse

	Present Europe	Historical Europe	UA_North	UA_Carpathian
Present Europe		0.2477	0.02799	0.0003719
Historical Europe	0.2477		0.04614	0.0006857
UA_North	0.02799	0.04614		0.04238
UA_Carpathian	0.0003719	0.0006857	0.04238	

The similar pattern was identified by ANOVA – the Northern and the Carpathian populations were significantly different from the other European populations of Black grouse for the allelic richness, but not for the remaining parameters of neutral genetic diversity. F value = 31.655 (p = 2.607e-08), when two Ukrainian populations are pooled and compared to the other European populations, pooled into the "present" and the "historical" categories. F value = 19.703 (p = 7.219e-09), when all the populations are compared as separate units (Figure 10).

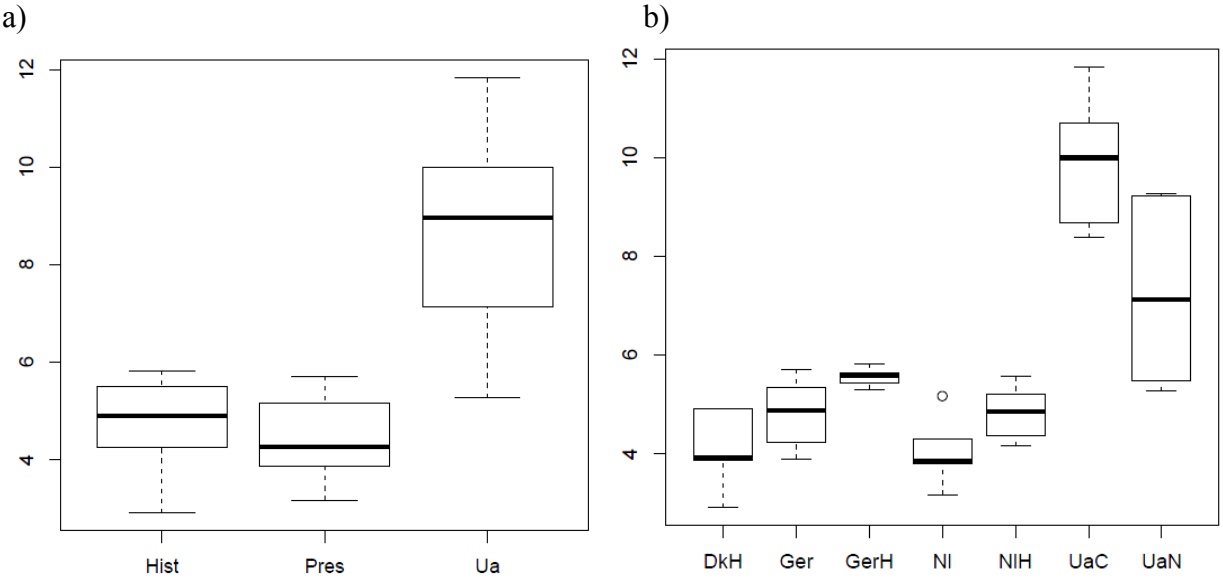

Figure 10. The differences in allelic richness of European populations of Black grouse: a) the populations pooled in 3 categories – the present European (Pres), the historic European (Hist) and Ukrainian (Ua): b) the populations considered as separate units: Danish historical (DkH), German present (Ger), German historical (GerH), Dutch present (Nl), Dutch historical (NlH), Carpathian Ukrainian (UaC) and Northern Ukrainian (UaN).

All pairwise Fst indexes were significant ($p<0.001$ after 110 permutations) and ranging from 0.027 between the historical German and Dutch populations and 0.492 between the Northern Ukrainian and the historical Danish populations (Table 9). The pattern of high genetic differentiation between the Black grouse populations in Ukraine and in the other European countries appeared to be even more pronounced, when pairwise Jost Dest was estimated. The Dest values between the Ukrainian populations and the European ones exceeded the Dest between both contemporary and historic European populations of Black grouse. The lowest differentiation was again between the two historical European populations (0.115) and the highest – between the historic German and Danish populations and the Northern Ukrainian population (0.985).

Table 9. Neutral genetic differentiation between European population of Black grouse (Fst values are given below the diagonal and Dest – above the diagonal; Fst and Dest for Ukrainian Black grouse populations are in bold)

	Dk_H	LH	Ger_H	SH	NI_H	N	C
Dk_H		0.299	0.244	0.545	0.391	**0.985**	**0.969**
LH	0.136		0.213	0.290	0.229	**0.967**	**0.947**
Ger_H	0.088	0.067		0.399	0.115	**0.985**	**0.955**
SH	0.309	0.135	0.158		0.353	**0.950**	**0.921**
NI_H	0.172	0.082	0.027	0.152		**0.983**	**0.968**
N	**0.492**	**0.377**	**0.328**	**0.411**	**0.353**		0.613
C	**0.295**	**0.192**	**0.142**	**0.231**	**0.167**	0.098	

Discussion

According to the microsatellite diversity pattern the Carpathian population of Black grouse carries more neutral genetic variation then the Northern one (Table 4). Taking into account the smaller sample size from the Carpathian region, the pattern shown can be considered even more pronounced. The two populations are not significantly different in terms of the standard measures of adaptive diversity (Pi, theta k), except the total number of alleles in each of them and the number of alleles adjusted by the sample size. I was able to detect twice as many alleles for the Northern population, than for the Carpathian one. Yet, adjusting this measure to the sample size yielded a different result, illustrating that the Black grouse from the Carpathian Mountains are, in fact, more diverse than those from the North. Therefore, the lower nucleotide diversity and MHC allelic richness of the Carpathian population can be attributed to the smaller sample size, which should be controlled for in the subsequent research.

One of the putative reasons for the pattern described above is that the Carpathian region, is more close to the south, which was free from the ice during the last glaciations period (20 000 YA), and could have been a refuge for the Black grouse. The Carpathian basin has been shown to be a refuge for the several species, for instance European viper (*Vipera berus)* and the Moor Frog *(Rana arvalis),* and it is listed as an example of the core area for the distribution of continental species (Schmitt 2007). Similarly, the Brown bear (*Ursus arctos)* is considered to have spread to the North from the Iberian and the Carpathian/ Caucasian refuge (Hewitt 2000). There is also a suspected underestimation of the role of the Carpathian basin as a source of the post-glaciation colonization of the Northern Europe due to the lack of reliable data on species phylogeny from that region (Hewitt 2000).

If the Carpathian population of Black grouse is the source for the populations further north, the comparatively low diversity in the latter can be determined by the expansion-contraction model of their dispersal (Taberlet *et al.* 1998). According to this model the colonization process is characterized by a number of extinction events in the northern populations due to decreased temperatures and a series of the northwards expansions of the species range during the warm periods. The colonization implies a bottleneck, which causes the reduction of genetic diversity in the northern populations (Taberlet *et al.* 1998). This process is especially significant in case of the species with specific habitat requirements (Schmitt 2007), including the Black grouse. In any case, applying this theory for the purpose of explaining the pattern of Ukrainian Black grouse genetic diversity requires conducting the study on the mitochondrial diversity of this species.

The higher rate of genetic diversity in the Carpathian population can also be determined by the topography of that region. As the subpopulations from the two leks in the Carpathian Mountains were more subjected to isolation by distance and were characterized by the lower probability of gene flow between them, they could be more divergent then the Northern ones. In this case pooling these two leks together in one Carpathian population could have yielded higher number of unique alleles than in the North.

The Carpathian and the Northern populations experienced no recent severe bottlenecks (according to the results of the Bottleneck, M_P_val and Citical M programs). This result is reasonable for the Northern population as it is consistent with the fact that it did not deviate from Hardy-Weinberg equilibrium. However, the Carpathian population appeared to deviate from Hardy-Weinberg equilibrium (Fis=0,111), which could be the sign of the recent reduction in the population size. In this case the Bottleneck program might have failed to indicate the recent bottleneck event, as the new mutation-drift equilibrium has already been set and, therefore, the heterozygosity excess cannot be detected and measured. The same outcome has been described for the Dutch black grouse population by Larsson *et al.* (2008). However, the fact that the M_P_val and Critical M programs

did not find any signs of a recent size reduction as well, indicates that the deviation from Hardy-Weinberg equilibrium can be caused by some other reason, such as scoring errors (the presence of null alleles, allelic dropout).

So far, the only possibility to check if the Carpathian population really experienced a recent bottleneck is to compare the present genetic diversity with the one in the past using the DNA from the museum samples (Höglund 2009). Yet, as has already been mentioned, the amplification success of the feathers from the museum samples was too low to include them in the analysis. So, whether the Carpathian population experienced a recent reduction in size still remains a question.

The analysis using the STRUCTURE software revealed both the regional and the local differentiation of Ukrainian populations of Black grouse. The total population was first separated into 2 big clusters – the Northern one and the Carpathian one, each of which was split into 2 groups. It should be mentioned that the Carpathian population was more clearly differentiated than the Northern one, as the genotypes from the two clusters did not show signs of admixture. This supports the above discussed idea about the higher differentiation and more significant isolation of Black grouse subpopulations in the Carpathian Mountains, and could have contributed to the diversity pattern observed. Moreover, such structure illustrates that the Northern and the Carpathian populations differ in their genetic composition and should both be the subject of conservation, if one wants to preserve the highest possible diversity of Ukrainian Black grouse population.

Both the Fst and the Dest indexes supported the idea of genetic subdivision in neutral diversity in the two populations studied. Yet, it is nowadays pointed out that investigating the pattern and the differentiation of adaptive diversity can be even more important for conservation than studying the neutral variation, as the latter does not necessarily reflect the local adaptation (Miller *et al.* 2010, Ekblom *et al.* 2007). The microsatellite diversity is claimed to be a poor surrogate for MHC variation, as the mutation mechanisms between them differ to a large extend (Miller *et al.* 2010). Therefore, here MHC differentiation between the two populations is also studied. The difference of neutral and adaptive differentiation is inferred via comparison of the Dest index for the microsatellites and the MHC.

The differentiation in MHC between the Northern and the Carpathian population was shown to be lower than for the neutral markers. This pattern supports the idea that the MHC variation is shaped by the balancing selection and thus should respond less rapidly to any differentiation. There are 3 mechanisms that could be behind this type of selection: 1) overdominance – based on the idea that heterozygosity is favored, as bearing different alleles allows exhibiting more variable immune response; 2) frequency-dependent selection – meaning that rare alleles are selected for in the population, because it takes some time for the pathogens to adapt to them; 3) spatial and temporal variation in parasite load, which can lead to local adaptation (reviewed in Ekblom *et al.* 2007). Yet, it is so far impossible to judge about the driving mechanism of a balancing selection in Ukrainian Black grouse populations, as it requires data on individual rates of heterozygosity, susceptibility to infections and local parasitic fauna.

The same pattern of little structure on MHC compared to the neutral markers has been shown for Red grouse *Lagopus lagopus scoticus* (Piertney 2003), Trinidadian guppy *Poecilia reticulate* (Fraser *et al.* 2009) and Chinook salmon *Oncorchynchus tshawytscha* (Evans *et al.* 2010). The results of the present study are in line with those reported by Strand *et al.* (2012) for the European Black grouse. As the author detected higher differentiation in neutral diversity than the one in MHC, it has been claimed that the selection is uniform for the Black grouse across Europe, which can be the outcome of the previous wider distribution of this species and a consequent higher gene flow between the populations (Strand *et al.* 2012).

Some studies detected the opposite pattern of the population structure. Higher differentiation in adaptive diversity was found in the studies of other species – Great snipe *Gallinago media* (Ekblom *et al.* 2007), Atlantic salmon *Salmo salar* (Landry & Bernatchez 2001) and *Tuatara sphenodon spp*. (Miller *et al.* 2010).

It should be mentioned that there is some structure in adaptive variation between the Northern and the Carpathian population, even though the MHC differentiation is less than the neutral one. It is supported by the high Dest value for the MHC and by the fact that there were private alleles discovered in both populations. This pattern can potentially indicate some local selection pressures in two regions (Piertney 2003). However, in order to claim that there are significant differences in the allelic composition of the MHC of the Northern and the Carpathian Black grouse populations, larger sample sizes should be examined. The differences in sampling size between the two populations could have resulted in the underestimation of allelic diversity in the Carpathian Black grouse.

Comparative analysis between Ukrainian and the other European populations of Black grouse illustrated that both the Carpathian and the Northern population resemble large continuous European populations. This outcome seems to be reasonable for the Northern population, but it is unexpected for the Carpathian one, as the topography of Carpathian Mountains should result in isolation and restricted gene flow. Yet, the fact that the population from Alps is also assigned to the continuous category, suggests that this can be the case for the other populations in the mountains. A putative reason for the Carpathian population to have the same (or even higher) rate of genetic diversity as large continuous European populations of Black grouse is that it can be considered as having been a possible refuge during the last glaciation period (discussed above).

However, the abovementioned outcome should be treated with caution, as it is based on the comparison of means, not on the raw data. An attempt to check this pattern was done by comparing the data on neutral genetic diversity of European populations from Segelbacher *et al.* (2011) with the present study.

The contemporary populations studied by Segelbacher *et al.* (2011) were previously assigned as the isolated (Germany – LH) and the small isolated one (Netherlands – SH). Both the Carpathian and the Northern population were shown to harbour significantly higher allelic richness than the two mentioned contemporary European populations and the historical ones. Higher results were received for the Black grouse from the Carpathian Mountains, which is consistent with the speculations made above.

It should also be mentioned that 3 new MHC alleles were sequenced. As these have not been previously noted in the studies of European Black grouse populations, it is possible that they are private for Ukrainian Black grouse, reflecting some local adaptations. Yet, the presence of the new alleles should be proven prior to making any conclusions about them, as they were detected in one PCR only.

It was shown that both Ukrainian populations are highly differentiated from the other European populations. According to the Fst values, historical populations from Germany and the Netherlands were closer to Ukrainian populations than were the contemporary ones. This is reasonable, as the historical populations, used for comparison, were characterized with higher genetic diversity than the present ones (Segelbacher *et al.* 2011), and, therefore, are expected to be more similar to the Northern and the Carpathian population. The Dest values didn't support this, yet the proportional differences in Dest between Ukrainian and European historical and Ukrainian and European contemporary populations were negligible. In any case, it is clear so far that there is high differentiation between the Ukrainian and the European populations of Black grouse.

The differentiation pattern, described above, refers to the neutral diversity only. The structure of interpopulation adaptive diversity is expected to be less pronounced; as the MHC in European populations of Black grouse is shaped by the balancing selection, driven by the uniform selection pressures (Strand *et al.* 2011). However, it is recommended that the differentiation pattern of adaptive diversity is checked empirically.

Another suggestion, which could contribute to the deeper understanding of the differentiation of European Black grouse populations, is the inclusion of the genetic diversity data on Slovakian, Polish and Czech populations in the comparative analysis. The gene flow between the Ukrainian and European Black grouse is expected to be exercised through the populations in the listed countries, and thus gradient pattern of differentiation is a possible outcome of such an analysis. This assumption should be checked in the future study, as it could contribute to identifying and illuminating the barriers for the gene flow between the European populations of Black grouse.

Conclusion

The pattern of both neutral and adaptive genetic variation of Ukrainian Black grouse suggests that the Carpathian population is more diverse than the northern one. This is an unexpected outcome, as the Carpathian population was previously claimed to be small and isolated (and, therefore, subjected to genetic drift), whereas the Northern one was classified as large and continuous. Yet, the putative reason for this diversity pattern is that the Carpathian Mountains could have served as a refuge for the Black grouse during the last glaciation period. This suggestion should be checked with the mitochondrial DNA data in a subsequent study.

The STRUCTURE analysis revealed the regional and the local subdivision of Ukrainian Black grouse populations. The individuals from the North were shown to be more admixed than those from the Carpathian Mountains, which is in line with the topography of the Carpathian region and illustrates the presence of the barriers for the gene flow in the mountains.

The rate of neutral differentiation between the Carpathian and the Northern population was higher than the rate of adaptive differentiation, which is in line with the conclusions of Strand *et al.* (2012) on the balancing selection shaping the MHC variation in European Black grouse. Yet, the index of adaptive differentiation is still considerably high, which could be a sign of some unique selection pressures in the North and in the Carpathian Mountains. This idea should be studied in the subsequent research including larger sample sizes from the Carpathian region and the samples from the Rostochia population, which exists in between the two groups studied here.

In total, the pattern of differentiation between the Carpathian and the Northern Black grouse populations, revealed by STRUCTURE and Fst and Dest indexes, shows that the two studied populations can be considered, as the separate Management Units (MU), when management efforts are allocated towards their conservation (Moritz 1994).

According to the results of comparative analysis of genetic diversity in Ukrainian and in the other European populations of Black grouse, both the Northern and the Carpathian ones resemble large continuous populations in Sweden, Norway, Latvia and Switzerland. Ukrainian populations are also characterized by the higher neutral allelic richness than both the contemporary and the historical European Black grouse populations, studied by Segelbacher *et al.* (2011). This is evidence that the Northern and the Carpathian populations of Black grouse have sufficient level of diversity and do not currently suffer from genetic drift.

Analysis of population structure of European Black grouse illustrated that the Northern and the Carpathian populations are highly differentiated from the other European ones. This pattern should be additionally checked, including the samples from the Slovakia, Poland and Czech Republic in order to investigate the potential barriers of gene flow between the Ukrainian and the other European populations of Black grouse.

So far, the present study is the first one on the genetic diversity of Black grouse in Ukraine. It aimed to give the first insight of both adaptive and neutral genetic diversity of the Northern and the Carpathian population of this species. It has also identified some issues that could potentially be the subject of subsequent research. The future research of the population of Black grouse in the North of Ukraine (Polissia region) should focus on more extensive sampling of that area, including its western and eastern boarders, as the diversity pattern can be different there. Carpathian population requires more sampling effort as well, and larger number of isolated leks should be included in the subsequent analysis to capture the whole diversity pattern of that region. What can be most important, is a phylogeny study using mitochondrial DNA designed to test the suggestion that the Carpathian Mountains were a refuge for the Northern populations of Black grouse.

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Appendix 1

Protocols

a) Microsatellite PCR protocol

Table 10. Primer multiplexes organized according to dye and annealing temperature

Locus	Dye	Multiplex	Annealing T	Cycles
ADL230	FAM	1	48 °C	40
ADL257	HEX	1	48 °C	40
ADL142	HEX	2	54 °C	40
ADL184	NED	2	54 °C	40
BG15	FAM	3	54 °C	40
BG16	HEX	3	54 °C	40
BG18	NED	3	54 °C	40
TUT1	NED	4	60 °C	40
TUT2	HEX	5	60 °C	40
TUT3	FAM	4	60 °C	40
TUT4	FAM	5	60 °C	40

Table 11. PCR reaction

Component	Volume
Reaction mix	5 µl
10x primer mix	1 µl
Rnase-free H_2O	1 µl
Q-solution	1 µl
DNA	2 µl

Table 12. PCR program

Step	Cycles	Temperature	Time
Initial activation	1	95 °C	15 min.
Annealing and extension	40	94 °C	30 sec.
		Primer annealing T	90 sec.
		72 °C	60 sec.
Final extension	1	60 °C	30 min.
Stan-by	-	8 °C	∞

b) RSCA protocol

Table 13. FLR PCR reaction

Component	Concentration	Volume (µl)
10x Buffer (-$MgCl_2$)		10
dNTP´s	10mM	6
RNA F flour 1a	10µM	5
RNA R 1a	10µM	0.5
$MgCl_2$	50mM	6
Biotaq	5µ/µl	0.6
DNA		1
ddH_2O		70.9
Total		100.0

Table 14. FLR PCR Program

Step	Cycles	Temperature	Time
Initial activation	1	94 °C	5 min.
Annealing and extension	30	94 °C	60 sec.
		64.9 °C	30 sec.
		72 °C	30 sec.
Final extension	1	72 °C	10 min.
Stan-by	-	4 °C	∞

Table 15. MHC PCR reaction

Component	Concentration	Volume (µl)
10x Buffer (-$MgCl_2$)		2.5
dNTP's	10mM	1.5
RNA F 1a	10µM	1.2
RNA R 1a	10µM	1.2
$MgCl_2$	50mM	1.5
Biotaq	5µ/µl	0.15
DNA		1
ddH_2O		15.95
Total		25.0

Table 16. MHC PCR Program

Step	Cycles	Temperature	Time
Initial activation	1	94 °C	5 min.
Annealing and extension	35	94 °C	60 sec.
		64.9 °C	30 sec.
		72 °C	30 sec.
Final extension	1	72 °C	10 min.
Stan-by	-	4 °C	∞

Table 17. Heteroduplexes

FLR	Dilution factor
FLR1	1:10
FLRJ10	1:5
FLRJ13	1:5
FLRJ19	1:5

Reaction: 3 µl MHC PCR product + 2 µl diluted FLR

Table 18. Hybridization Program

Temperature	Time
95 °C	10 min.
Down to 55 °C	1°/sec. or faster
55 °C	15 min.
4°C	15 min.

c) Cloning protocol

Table 19. Chemical Transformation from fresh pcr-product

Component	Volume
Topo vector	1 µl
Salt solution	1 µl
Fresh PCR product	4 µl
DH5α competent cells	50 µl

Table 20. PCR on clones

Component	Volume
ddH_2O	18.02 µl
PCR Buffer (-$MgCl_2$)	3 µl
$MgCl_2$	2.3 µl
dNTP's	1.5 µl
M13 F	0.8 µl
M13 R	0.8 µl
Biotaq	0.08 µl
Total	26.5 µl

Table 21. PCR program for clones

Step	Cycles	Temperature	Time
Initial activation	1	95 °C	2 min.
Annealing and extension	30	95 °C	60 sec.
		52 °C	30 sec.
		72 °C	45 sec.
Final extension	1	72 °C	10 min.
Stan-by	-	8 °C	∞

Table 22. Exo – Fast AP Cleaning

Component	Volume
Fast AP	2 µl
Exo	1 µl
PCR product	20 µl

Table 23. Exo – Fast AP Cleaning Program

Temperature	Time
37 °C	15 min.
80 °C	15 min.
8 °C	∞

Appendix 2

Newly sequenced alleles

Table 24. New alleles and their sequences

Allele	Sequence
BLB27	TGTGGCCGATACACCGCTGGGAGAGTACCCGGCTGAATACTTCAACAACAACACTGAGTATCT GGAGTATAGACGGGGTGAGGTGGACAGATACTGCCGGCACAACTACGGGGTGTTTGAGCCCT
BLB28	TGAGGCCGATACACCTCTGGGAGAGCTGCAAGCTGAATACTGGAACAACAACACCCAGATTAT TGAGTACAAACAGGGTCAGGTGGACAATTACTGCCGGCACAACTACGGGGTGTTTGAGCCCT
BLB29	TGTGGCCGATACACCGCTGGGAGAGCTGCCAGCTGAATACATGAACAACGACACTGAGTATCT AGAGTATAGACGGGGTGAGGTGGTCAGATACTGCCGGCACAACTACGGGGTTGGGGAGCCCT

Appendix 3

The number of clusters with the highest probability (Delta K) in Ukrainian population of Black grouse

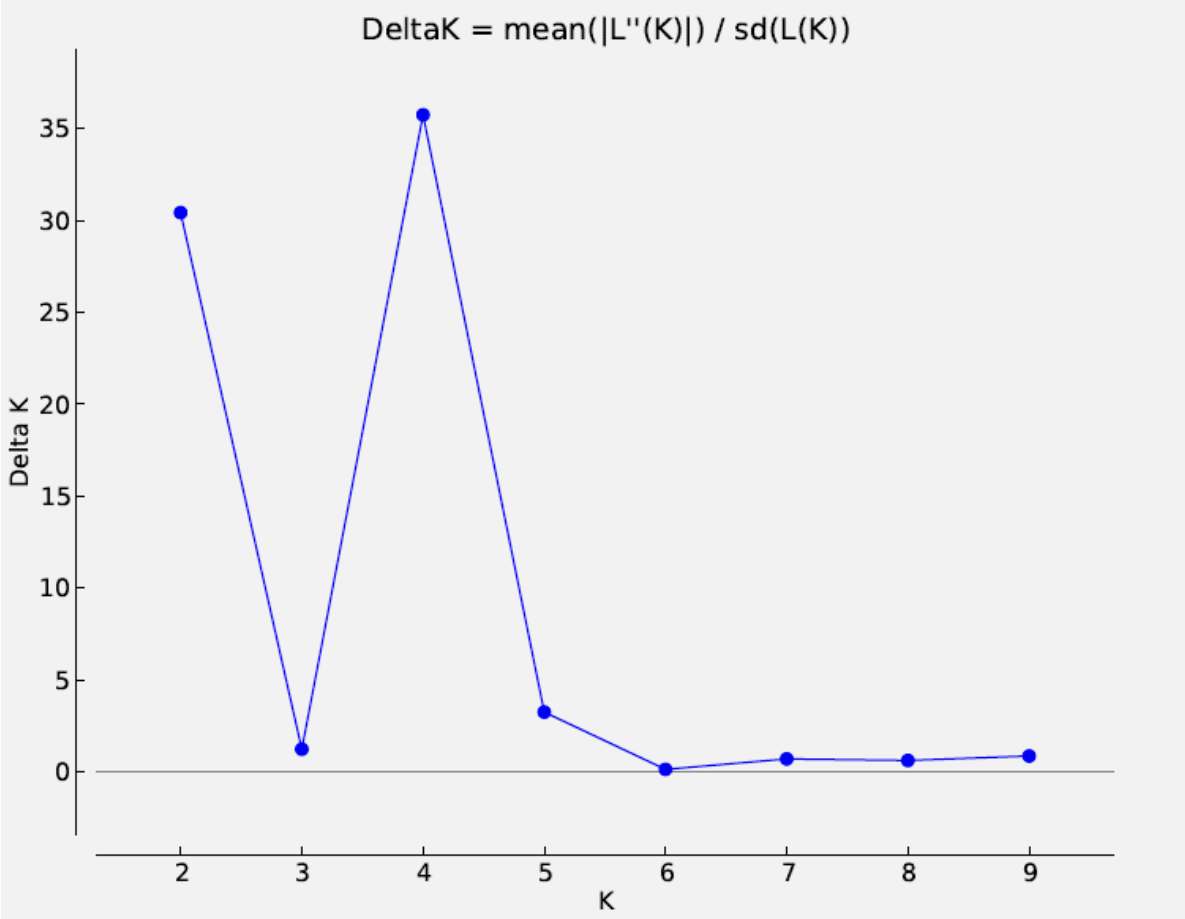

Figure 11. The number of clusters with the highest probability (Delta K) in Ukrainian population of Black grouse

Appendix 4

Microsatellite and MHC diversity of European Black Grouse populations

Table 25. Neutral and adaptive genetic diversity of European Black grouse: yellow – large continuous group, red – isolated group, green – small isolated group, white – Ukrainian populations (reprinted from Strand *et al.* 2011 and extended).

Population	N	He	AR	Nmhc	Alleles/sample size	Pi	theta k
Fin_C	57	0,78	5,76	29	0,48	13,96	4,5
Nor_C	31	0,74	5,32	11	0,91	12,68	6,18
SweJ_C	14	0,79	5,58	6	0,67	12,61	2,18
Lat_C	13	0,81	5,92	9	0,78	13,34	3,14
Alps_C	57	0,77	5,44	22	0,45	11,07	4,4
Eng_I	21	0,61	3,43	18	0,5	12,49	3
LH_I	24	0,64	4,83	6	1,17	8,83	6,15
Pol_I	23	0,68	4,31	14	0,36	7,59	1,86
Neth_SI	31	0,56	3,49	34	0,32	13,09	3,13
Aus_SI	14	0,62	4,09	8	0,5	13,1	1,57
Ger_SI	22	0,73	5,05	7	0,57	10,4	1,8
Ukr_N	75	0,71	6,44	36	0,18	14,1	5,35
Ukr_C	31	0,87	9,18	20	0,36	12,51	4,19