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GENETIC DIVERSITY AND STRUCTURE OF THE BLACK GROUSE *LYRURUS TETRIX* LINNAEUS, 1758 POPULATION IN BELARUS

Abstract. It is known that black grouse is a valuable resource species of the wild fauna of Belarus. The Belarusian population went through the stages of population decline and redistribution into new agrarian landscape territories – extensive anthropogenic involvement transformed significant parts of the species' habitat in the course of large-scale land reclamation campaigns, which originated in 1950s. In order to rationally use the preserved black grouse subpopulations, an assessment of the level of their genetic diversity and degree of differentiation was made. For the latter purpose, microsatellite analysis was utilized. It was found that at the present stage the black grouse population has a sufficient level of adaptability (based on indicators of genetic diversity and effective population size) necessary to maintain viability in the foreseeable future.

Keywords: *Lyrurus tetrix*, black grouse, genetic diversity, genetic differentiation, microsatellities, Belarus

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ГЕНЕТИЧЕСКОЕ РАЗНООБРАЗИЕ И ПОПУЛЯЦИОННО-ГЕНЕТИЧЕСКАЯ СТРУКТУРА ТЕТЕРЕВА *LYRURUS TETRIX* LINNAEUS, 1758 В БЕЛАРУСИ

Аннотация. Тетерев – ценный ресурсный вид дикой фауны Беларуси. В результате широкомасштабной мелиорации, начатой в 1950-е годы, популяция прошла через этапы снижения численности и перераспределения на новые территории – экстенсивно эксплуатируемые антропогенно трансформированные угодья. В целях рационального использования сохранившихся субпопуляций тетерева была проведена оценка уровня их генетического разнообразия и степени дифференциации. В качестве генетических маркеров использовались микросателлиты. В результате проведенной работы установлено, что на современном этапе популяция тетерева обладает достаточным уровнем адаптивности (на основании показателей генетического разнообразия и эффективного размера популяции), необходимым для сохранения ее жизнеспособности.

Ключевые слова: *Lyrurus tetrix*, тетерев, генетическое разнообразие, генетическая дифференциация, микросателлиты, Беларусь

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Introduction. The black grouse range covers the entire forest zone of Northern Eurasia from Scandinavia to southeastern Siberia, as well as part of the steppe zone. There has been a significant reduction in range and a decrease in numbers in the southern and western parts of the range over the course of the XX century. At the present time, isolated local black grouse populations inhabit mainly mountainous territories, and on plains they are confined to such habitats as peat bogs and moorlands. A particularly sharp decline was observed in 1970–1990s [1–5] and, to date, the size of most local isolated populations in western and central Europe does not exceed 100–200 individuals [1, 6–14].

Regarding Belarus, it can be stated that declining population numbers and receding range of black grouse has been noted in Belarus as a general trend over the last decades of the XX century, in line with the similar processes observed in Europe over the past century.

One of the preferred habitats of this species in Belarus – swampland – suffered a significant reduction in its total area in the course of large-scale drainage reclamation efforts, starting in 1950s. About 700 thousand hectares of bogs were drained for agricultural needs in Belarusian Polesie alone, of which more than 80 % were in the Pripyat basin [15]. The density of black grouse in the country almost halved in 1970s as compared to 1950s as a result of intensive land reclamation and agricultural development of natural lands with structural indicators optimal for black grouse (a combination of open spaces with a certain type of tree and shrub vegetation) [16–18].

In that twenty-year period, due to the reduction in the area of natural habitats, the black grouse began to inhabit local agrolandscape. The black grouse is well suited to live in conditions of extensive farming. In this connection, by the middle, and especially towards the end of 1980s, stabilization of the number and increase in the density of the black grouse population was noted in Belarus. The number of black grouse in that period counted in the range of 45–54 thousand individuals [19]. The local population maintained those approximate numbers until the late 1990s.

A steady downward trend in population numbers for black grouse emerged in Belarus in 2000s. 2008 saw a reduction of Belarussian black grouse population numbers from their 2001 values by 21 %, and 2014 – a reduction of 30.4 % [20–22].

The decline in the number of black grouse that began in the last decade is generally tied to farming intensification. A statistically significant negative correlation was found between the density of black grouse and the area of arable land in the Grodno region ($r = -0.70$; $p < 0.05$). An increase in the predators' numbers such as the fox and the northern goshawk is an additional factor contributing to the decline in the numbers of black grouse. Until recently, the increased number of wild boar, which is dangerous for all land-nesting birds, was a very significant threat factor, but in recent years, due to mass shooting (the fight against ASF, since 2013), this factor's role has decreased. The number of wild boars in Belarus fell from 80.4 thousand in 2013 to 7.8 thousand in April 2014. At the end of 2014, the number of wild boar counted approximately 8.6 thousand individuals, and in 2015 – 8.0 thousand [23]. In 2016–2018 the number of wild boars ranged between 2.6 thousand and 2.8 thousand individuals [24]. Thus, at present, the number of wild boars has decreased approximately by a factor of 30 compared to 2013, and by now it should not pose a significant threat to the black grouse. The latter is considered one of the major reasons of the increasing number of black grouse in 2014–2018. According to the Ministry of Forestry of The Republic of Belarus for 2012–2014 years, the numbers of the species' population counted approximately 34.6–39.9 thousand individuals according to spring surveys. The current population in 2018 reached 43.2 thousand individuals [24].

By the end of 2018, the fox population was also 1.8 times lower than in 2006. However, the local numbers of predatory invasive alien species such as a raccoon dog continues to grow. By 2015, the raccoon dog count in Belarus doubled compared to 2005.

A decrease in the number of black grouse in a short period of time were also noted in the regions neighboring Belarus. In the 1970s in Poland the number of black grouse counted approximately about 40–45 thousand individuals, and in the next 7 years, it decreased by 68 % [25].

An inventory of black grouse leks in Belarus showed that over the past decades there has been a significant change in their biotopic distribution. The decrease in the area of natural habitats of black grouse that occurred over the past 40–50 years as a result of large-scale drainage reclamation led to a redistribution of black grouse populations towards extensively exploited anthropogenically transformed lands. However, in the case of land-use change towards further intensification of agriculture a rapid decline in the black grouse number can be predicted. Considering the changes in land use that have occurred in the country and the trends of a drastic decline in the numbers of black grouse in the past, it is necessary to assess the stability of the species' population at the current stage. The importance of studying the genetic diversity and the genetic structure of animal populations is that these indicators have a direct impact on the continued success of their existence. This has been shown by an example of some black grouse populations in Europe [26]. The authors demonstrated that genetic diversity (observed heterozygosity, gene diversity (Hs)) is lower and inbreeding is higher in isolated populations (populations from southeastern Austria, England and Germany) compared with extended populations (from Scandinavia) and populations that are classified as adjacent (from the Alps and the Scottish

highlands). The role of fragmentation in the genetic differentiation of populations has been shown for the capercaillie when studying the metapopulation structure in the Alps [27]. The significant differentiation between all populations by allele frequency was demonstrated. The total differentiation based on all loci was 0.046 ($p < 0.001$). Similar results were obtained in the study of capercaillie in the Bavarian Alps. The authors found a reliable genetic differentiation between pairs of populations separated by a distance of less than 10 km [28]. In another work, the genetic consequences of fragmentation on the capercaillie population were also studied using microsatellite markers in the European part of the range at various levels along the spatial gradient from high population connectedness in the forests of the boreal zone (Russia (Arkhangelsk, Yaroslavl, Karelia), Norway) to the metapopulation system in the Alps, as well as in the context of recent (Central Europe) and historical (Pyrenees) isolation [29]. As it could be expected, the genetic differentiation was the least pronounced within the continuous range of boreal forests. Based on the data received, the authors conclude that anthropogenic disturbance of habitats and fragmentation can lead to significant genetic and evolutionary consequences for the survival of the species.

Taking into account the significant fluctuations of the black grouse population in Belarus, we considered it relevant to assess the level of genetic diversity of the species in order to clarify the possible negative consequences of a decrease in numbers as a result of landscape transformation.

Materials and methods. A panel of microsatellite markers, originally developed for black grouse and capercaillie [30, 31], was selected for studying the intraspecific genetic diversity and structure of the black grouse populations (Tab. 1).

T a b l e 1. **Microsatellite markers used for black grouse genotyping**

No.	Locus	Primer sequence (5′–3′)	Annealing temperature, ◦C
1	BG15_F BG15_R	AAATATGTTTGCTAGGGCTTAC TACATTTTTCATTGTGGACTTC	54
2	BG16_F BG16_R	GTCATTAGTGCTGTCTGTCTATCT TGCTAGGTAGGGTAAAAATGG	54
3	BG18_F BG18_R	CCATAACTTAACTTGCACTTTC CTGATACAAAGATGCCTACAA	53
4	TUD1_F TUD1_R	ATTTGCCAGGAAACTTGCTC AACTACCTGCTTGTTGCTTGG	59
5	TUT1_F TUT1_R	GGTCTACATTTGGCTCTGACC ATATGGCATCCCAGCTATGG	60
6	TUT2_F TUT2_R	CCGTGTCAAGTTCTCCAAAC TTCAAAGCTGTGTTTCATTAGTTG	60
7	TUT3_F TUT3_R	CAGGAGGCCTCAACTAATCACC CGATGCTGGACAGAAGTGAC	60
8	TUT4_F TUT4_R	GAGCATCTCCCAGAGTCAGC TGTGAACCAGCAATCTGAGC	60

Forward primers were labeled on 5′ end with fluorescent dyes (PRIMETECH ALC) – Cy5 (BG) and Cy5.5 (TUD/TUT). PCR protocols for microsatellite loci TUT/TUD and BG are presented in Tab. 2, 3, respectively.

T a b l e 2. **PCR protocol for TUT/TUD loci**

Phase	Temperature, number of cycles		Time
Initial denaturation	94 °C		3 min
Denaturation	94 °C	35 cycles	30 s
Annealing: TUT/TUD	59 °C (TUD) 60 °C (TUT)		30 s
Extension	72 °C		45 s
Final extension	72 °C		5 min
Hold	4 °C		∞

T a b l e 3. **PCR protocol for BG loci**

Phase	Temperature, number of cycles		Time
Initial denaturation	92 °C		2 min
Denaturation	92 °C	30 cycles	30 s
Annealing: BG	Annealing at the specified temperature, °C		30 s
Extension	72 °C		30 s
Final extension	72 °C		5 min
Hold	4 °C		∞

Black grouse muscle tissues were provided by independent hunters from hunted birds. DNA extraction from black grouse muscle tissues was carried out with commercial genomic DNA purification kits (Fermentas).

We sampled genetic material from 42 individuals from 7 collection regions across Belarus (Tab. 4).

T a b l e 4. **Black grouse samples included in the study**

No.	Sample code	Site collection	No.	Sample code	Site collection
1	AV00250	Borisov District, Minsk Region	22	AV00659	Polotsk District, Vitebsk Region
2	AV00640		23	AV01242 (Tet_4)	Gantsevichi District, Brest Region
3	AV00641		24	AV01243 (Tet_5)	
4	AV00673		25	AV01244 (Tet_6)	
5	AV00675		26	AV01245 (Tet_7)	
6	AV00677		27	AV01246 (Tet_8)	
7	AV00678		28	AV01247 (Tet_9)	
8	AV00688		29	AV01248 (Tet_10)	Lelchitsy District, Gomel Region
9	AV00721		30	AV01249 (Tet_11)	
10	AV00410	Krupki District, Minsk Region	31	AV01239 (Tet_1)	Gomel Region, PSRER*
11	AV00637		32	AV01240 (Tet_2)	
12	AV00638		33	AV01241 (Tet_3)	
13	AV00639		34	AV01250 (68-14)	
14	AV00671		35	AV01251 (71-14)	
15	AV00672		36	AV01252 (70-14)	
16	AV00717		37	AV01253 (67-14)	
17	AV00663	Myadel District, Minsk Region	38	AV01254 (78-14)	
18	AV00666		39	AV01255 (77-14)	
19	AV00655	Polotsk District, Vitebsk Region	40	AV01257 (55-14)	
20	AV00656		41	AV01258 (56-14)	
21	AV00657		42	AV01259 (60-14)	

*State Environmental Research Institution “Polesye State Radiation-Ecological Reserve”.

The distribution of black grouse samples are presented in Fig. 1.

PCR products were genotyped using commercial protocols, reagents and software for the GenomeLab GeXP genetic analysis system (Beckman Coulter, USA). Software Tandem v 1.09 [32] was used for allele binning.

Fragment analysis data was evaluated for genotyping errors (null alleles, stuttering, large allele dropout) using software Micro-Checker version 2.2.3 [33, 34]. An additional estimate of the frequency of null alleles was carried out in Genepop version 4.3 [35, 36].

Analysis of the genetic structure of black grouse was carried out for 4 subpopulations (Fig. 1, *1–4*) and two groups of subpopulations (Fig. 1, *A*, *B*).

Analysis of the genotypes matching was done using GenAlEx v. 6.501 [37–39]. Samples with absolute genotype similarity were excluded from further analysis.

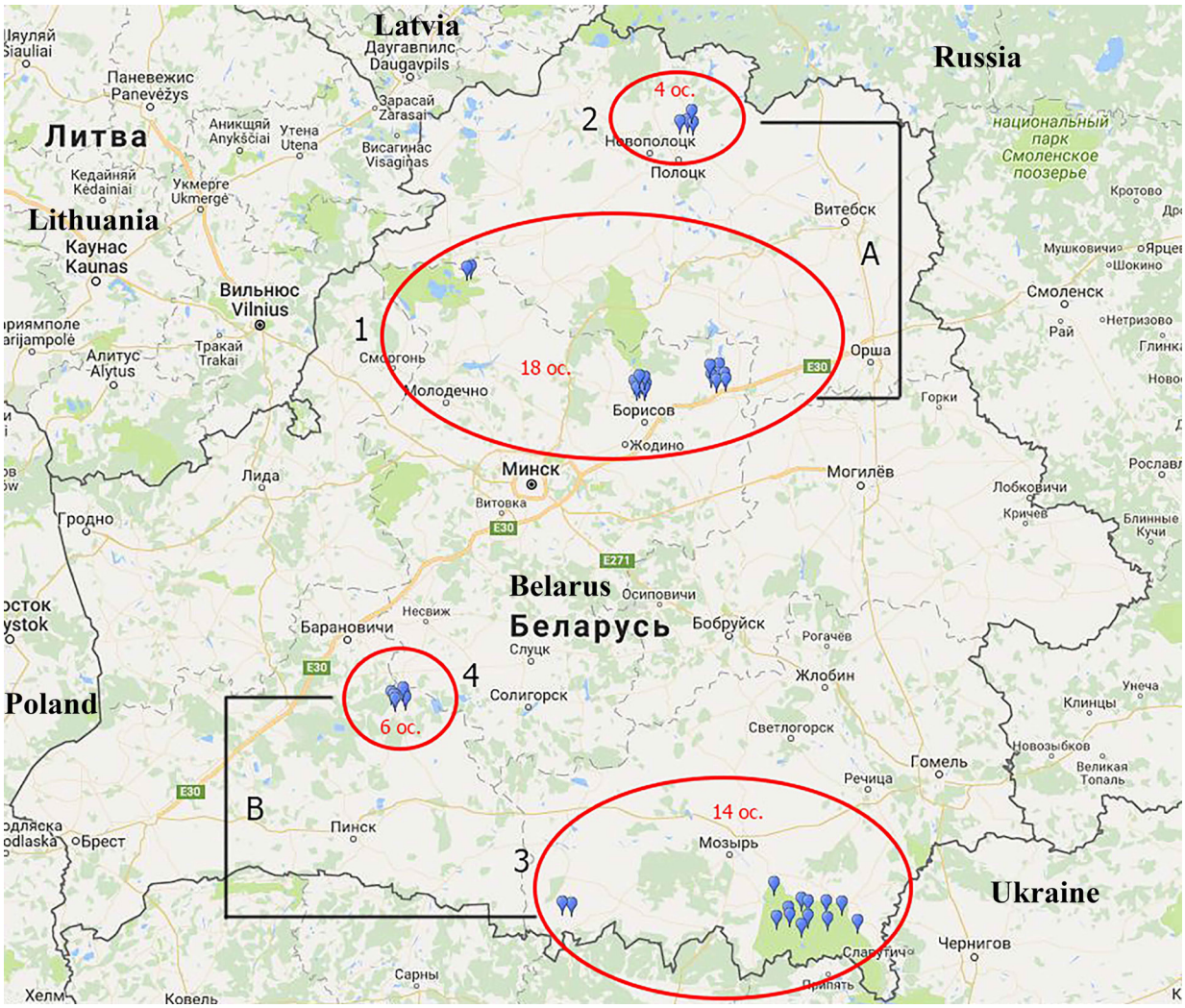

Fig. 1. Map of the black grouse samples distribution (“oc.” – specimens)

Linkage disequilibrium between loci was carried out in the Arlequin version 3.5.2.2 [40]. Parameters used: 10,000 permutations, confidence level at $p < 0.05$. The deviation of the studied loci from the Hardy-Weinberg equilibrium (HWE) was also evaluated in the Arlequin with default settings.

A test for the past decline of black grouse population was carried out in the Bottleneck 1.2.02 [41]. In this analysis, the TPM (two phase model) was used with the following parameters: proportion of SMM (stepwise mutational model) in TPM = 95 %, variance = 12 % (in accordance with [42]). In addition, I.A.M. (infinite allele model) and S.M.M. models were used. Significance of heterozygote excess was assessed using the sign test, standardized differences test and Wilcoxon’s sign-rank test.

Number of alleles per population, allelic richness (AR), Weir and Cockerham’s inbreeding coefficient estimator (Fis, Weir & Cockerham, 1984), observed (Ho) and expected (He) heterozygosity were calculated using R package diveRsity v1.9.90 [43].

Bayesian inference of population structure was performed using the software STRUCTURE [44, 45]. STRUCTURE runs were performed under admixture model, correlated allele frequencies among populations and using sampling locations as prior information to assist the clustering (only for 4 subpopulations), length of burning period = 50 000, number of MCMC (Markov chain Monte Carlo) = 100 000. STRUCTURE analyses were conducted for 1–6 putative genetic clusters (K) with 15 runs for each value of K for 4 subpopulations and for 1–5 putative genetic clusters (K) with 20 runs for each value of K for 2 groups of black grouse subpopulations. To visualize the STRUCTURE results we used STRUCTURE HARVESTER [46]. An alternative way to find genetic structure was Principal

Coordinates Analysis (PCoA) in GenAlEx. Visualization of PCoA data was carried out in the PAST [47]. The population genetic structure was checked by pairwise comparing the fixation index (Fst) between the selected subpopulations of black grouse in diveRsity and conducting a hierarchical analyses of molecular variance (AMOVA) in Arlequin. Additional calculating index of the population differentiation D_{est} (Jost, 2008) was performed in diveRsity. The index D_{est} was carried out due to the fact that Fst can be unreliable when the genetic diversity of the studied populations is very high (Jost, 2008 cited from [48]).

The calculation of the effective population size of the black grouse, as a measure to estimate the rate of loss of genetic variation due to genetic drift and inbreeding, was made according to the formulas given in Braude, 2010 [49].

The effective population size was calculated taking into account inbreeding (*inbreeding effective size*, *Nef*) – this is the size of an ideal population that would allow the same accumulation of pedigree inbreeding as the actual population of interest; this effective population size indicates the loss of heterozygosity across all alleles in population of interest; calculated as a harmonic average population size over time from the founding generation to the penultimate generation.

Additionally, *variance effective size* (*Nev*) was calculated (2). The variance effective population size is the size of an ideal population that would accumulate the same amount of variance in allele frequencies as the population of interest; this effective population size indicates how rapidly allele frequencies are likely to change.

Results and discussion. Testing in Micro-Checker indicated the presence of null alleles among the microsatellite results only for the singular locus TUT1. This was consistent with analysis for null alleles in Genepop. Therefore, TUT1 was excluded from further analysis. Concerning the rest of the loci, there was no indication of additional genotyping errors. Two pairs of samples – AV00677/AV00673 and AV00671/AV00672 had similar genotypes. The samples AV00673 and AV00672 were excluded from analysis.

Linkage disequilibrium analysis did not show any stable linkage between loci as it was seen from tests for 4 subpopulations and 2 groups of subpopulations of black grouse. This can be explained by the characteristic of the analyzed sample and is unlikely to have any connection with real linkage. All loci except TUT1 were in accordance with the HWE. We didn't find any convincing signs of rapid black grouse population decline in the past.

The indicators of genetic diversity (allelic richness, mean number of alleles) of black grouse subpopulations (Tab. 5) show nearly the same mid-level of diversity. This outcome remains unchanged whether the sample is considered are 4 subpopulations or 2 groups of subpopulations in the analysis. The lowest value of allelic richness is found in Pop2 (the northern region of Belarus) and corresponds to its low sample size. The level of both observed and expected heterozygosity is consistent with the inbreeding coefficient value – there are no signs of significant close related mating or genetic drift.

T a b l e 5. **Summary of the genetic diversity indices at 7 microsatellite loci among the black grouse subpopulations**

Population	N	Na	AR (95 % CI)	Ho	He	Fis (95 % CI)
Pop1	16	5.86	3.58 (2.71–4.43)	0.73	0.69	–0.05 (–0.15…0.05)
Pop2	4	3.43	2.85 (2.00–3.43)	0.62	0.55	–0.12 (–0.40…0.06)
Pop3	14	5.0	3.26 (2.57–3.86)	0.67	0.63	–0.08 (–0.17…0.02)
Pop4	6	4.14	3.31 (2.57–3.86)	0.76	0.62	–0.24 (–0.41…–0.13)
PopA	20	6.14	5.58 (4.86–6.00)	0.71	0.68	–0.03 (–0.12…0.06)
PopB	20	5.86	5.21 (4.57–5.71)	0.70	0.65	–0.07 (–0.15…–0.004)

N o t e. N – sample size, Na – mean number of alleles, AR – allelic richness, Ho – observed heterozygosity, He – expected heterozygosity, Fis – inbreeding coefficient, 95 % CI – 95 % confidence intervals, Pop1 – the central region of Belarus, Pop2 – the northern region of Belarus, Pop3 – the southeast region of Belarus, Pop4 – the southwest region of Belarus, PopA – the northern region of Belarus, PopB – the southern region of Belarus.

We didn't observe significant genetic differentiation among most but for one pair of the investigated subpopulations neither through Fst nor through D_{est} (Tab. 6). The pair Pop3–Pop4 had low significant genetic differentiation for Fst (0.0487), but not for D_{est}. The last results are also consistent with AMOVA. The only one fixation index – Fsc (differentiation among subpopulations within groups) was significant (0.03, $p < 0.05$), which most likely connected with genetic differentiation between Pop3 vs Pop4. Whereas there was no apparent genetic structure for pairwise black grouse subpopulations groups (A and B) comparison (Fct = –0.002, $p > 0.05$).

T a b l e 6. **Genetic differentiation among the black grouse 4 subpopulations and 2 groups of subpopulations**

Pairwise comparison	Fst (95 % CI)	Dest (95 % CI)
Pop1 vs Pop2	–0.0008 (–0.06...0.09)	–0.0040 (–0.07…0.18)
Pop1 vs Pop3	0.0266 (–0.01...0.07)	0.0239 (–0.04…0.11)
Pop1 vs Pop4	0.0195 (–0.02...0.09)	0.0004 (–0.06…0.14)
Pop2 vs Pop3	–0.0005 (–0.07...0.09)	–0.0032 (–0.04…0.14)
Pop2 vs Pop4	0.0770 (–0.01...0.20)	0.0217 (–0.09…0.20)
Pop3 vs Pop4	0.0487 (0.005...0.11)	0.0159 (–0.05…0.11)
PopA vs PopB	0.004 ($p > 0.05$)*	0.03 (–0.01…0.09)**

N o t e. * – estimation was made in Arlequin, ** – estimation was made in GenAIEx, CI – 95 % confidence intervals.

Results from STRUCTURE indicated that there is no apparent population genetic partitioning (Fig. 2). Despite K = 2 having the highest ΔK value this estimation isn't distinctly different from other K values. Individuals from all putative populations have nearly equal membership proportions to each of the genetic clusters. Moreover, K = 1 has the highest logarithmic probability among the K values.

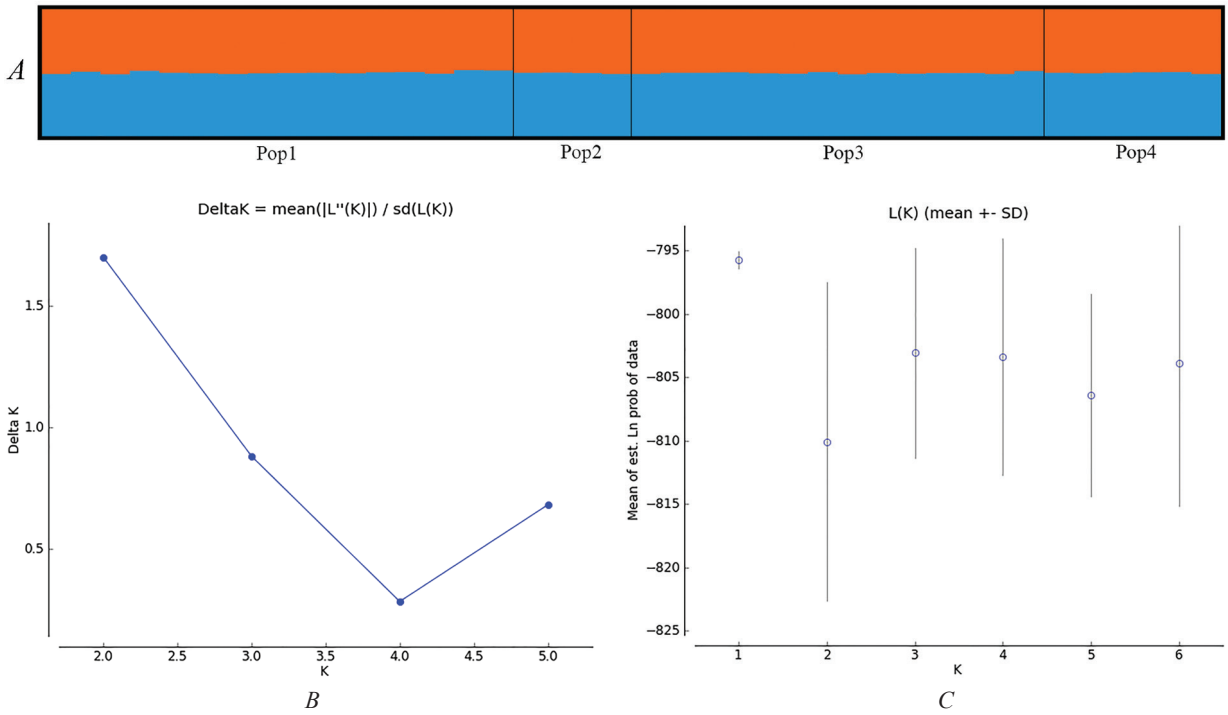

Fig. 2. Results of STRUCTURE analysis for 4 black grouse subpopulations: *A* – bar plot for K = 2 (Mean LnP(K) = –810.0733), each individual is represented by bar, the length of each segment of bar plot describes the estimated membership proportions to each of the genetic clusters; *B* – ΔK; *C* – estimated mean likelihoods of each number of genetic clusters

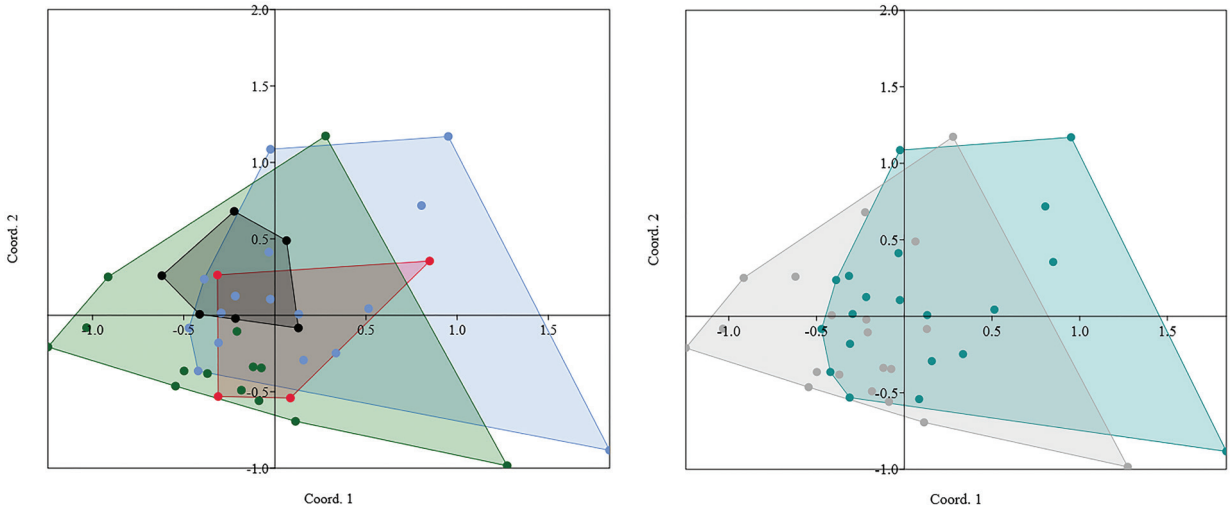

Fig. 3. Principal coordinate analysis of the black grouse 4 subpopulations and 2 groups of subpopulations: *A* – 4 subpopulations of black grouse (Pop1 – blue color, Pop2 – red color, Pop3 – green color, Pop4 – black color); *B* – 2 groups of black grouse subpopulations (PopA – turquoise, PopB – gray)

Principal coordinate analysis also did not indicate any significant genetic differentiation for either the 4 subpopulations of black grouse (Fig. 3) or for the 2 groups of black grouse subpopulations. The first 2 axes explained 31.14 % of the total variation.

The microsatellite analysis is consistent with the estimates of the effective population size of the black grouse in Belarus based on long-term census (Tab. 7).

The both inbreeding effective size (Nef) and variance effective population size (Nev) had very high values ≈ 42 669 and 41 940 respectively.

T a b l e 7. **The number of black grouse in Belarus**

Year	Population size	Year	Population size
2001	52 000	2009	37 900
2002	51 900	2010	37 442
2003	49 930	2011	37 868
2004	48 204	2012	36 108
2005	47 464	2013	37 000
2006	45 730	2014	42 800
2007	41 631	2015	40 100
2008	41 168		

Conclusion. Taking into account the data obtained on the genetic diversity and population genetic differentiation of black grouse subpopulations in Belarus, the following conclusions can be drawn:

– the black grouse population has sufficient connectivity, which is expressed in the absence of a pronounced subpopulation subdivision (data from Bayesian analysis, Principal coordinate analysis, analysis of molecular variance and values of the indices of population separation Fst and D_{est});

– the black grouse population can be characterized as stable and viable on the basis of genetic diversity estimation – the rates of both observed and expected heterozygosity are moderate, the population has not experienced a significant decline in numbers and there is no sign of inbreeding.

Thus, despite the strong change in the main black grouse habitat (swampland area reduction), the species has a good adaptive potential. In addition, the maintenance of high abundance of black grouse population in Belarus, as one of the main source of genetic variation, was facilitated by the ecological flexibility of the species, that is the ability to move into the new habitat – agricultural landscape. Of course, this situation will not be observed ubiquitously and will be determined by the quality of the habitat.

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