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SHORT COMMUNICATION

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Genetic diversity of the Carpathian capercaillie in space and time

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Abstract

Knowledge about changes occurring in the genetic structure of populations is extremely important in the case of endangered species. Research studies conducted in space and time allow us to identify factors that are influencing gene flow. Especially in the case of species occurring in the form of dispersed, more or less isolated populations, this is crucial for developing effective conservation strategies. The Carpathians are one of the most important capercaillies stronghold in Central Europe. The population here is fragmented, and at least, some groups of birds are small and isolated. This study aimed to determine whether the genetic structure of the capercaillie in the Polish part of the Carpathians has changed over the last decade. The research was conducted in three Polish national parks: Babia Góra National Park (BAB), Gorce (GOR) and Tatra (TAT). A total of over 1,300 non-invasive samples were collected for genetic analyses in two periods: 2010–2013 and 2021–2022. Individuals were identified using microsatellite markers. It was found that during the research period, there were approximately 200 capercaillie individuals in the Parks. The level of genetic variability in individual strongholds has not changed significantly. However, traces of a new gene pool were found in BAB, which could have appeared from other areas of the Carpathians where the species is being reintroduced. GOR shows features of a sink population: low genetic variability, low number of individuals and gradual decline in emigration. During the period covered by the study, the genetic structure has changed little: the studied strongholds are partially genetically isolated, but gene flow between BAB and GOR appears to be increasingly limited. TAT is a hybrid and transition zone for gene flow between smaller strongholds. The results emphasize the importance of areas with stable, numerous populations for the survival of smaller, peripheral groups of the capercaillie in mountain areas.

Key words

capercaillie, *Tetrao urogallus*, microsatellites, non-invasive genetic sampling, population structure, the Carpathians

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Introduction

Mountain areas are one of the most important capercaillie (*Tetrao urogallus*) strongholds in central and western Europe. The species occur in the Cantabrian Mountains, the Pyrenees, the Alps, the Carpathians, the Jura and Rhodopes (Coppes et al. 2015). Mountain populations are usually more numerous and less fragmented than lowland once, which occur in highly dispersed forests. While mountain capercaillie populations are considered metapopulations connected by more or less intensive gene flow, lowland populations are often territorially limited to single forest complexes, isolated from other forest environments by vast forestless areas, impossible to overcome in the case of birds with limited dispersal ability (Mäki-Petäys et al. 2007). Moreover, genetic analysis suggested that at least some mountain areas could have been refugia for the capercaillie and other forest grouse during the last glaciation period (Bajc et al. 2011; Klinga et al. 2015; Pavlowska and Hoglund 2015; Rutkowski et al. 2012, 2016). This makes mountain populations an important source of genetic variability. Preserving this source may prove crucial for maintaining the evolutionary and adaptive potential of the capercaillie, which is extremely important from the point of view of species conservation. At the same time, anthropogenic changes are increasingly affecting mountain environments. This also applies to mountain forests and therefore also affects the species living there (Coppes et al. 2017; Sabatini et al. 2018). In the past, forest management in mountain areas was less intensive than in lowlands (Jespen et al. 2015; Kulakowski et al. 2017). This situation is currently changing. This is especially visible in the valleys separating individual mountain ranges (Więcław-Michniewska 2013) and, consequently, also groups of animals occurring in individual ranges. This often leads to fragmentation of mountain populations. Taking into account the overlapping effects of natural barriers, for example, high peaks, which can be an important factor limiting gene flow for grouse (Caizergues et al. 2003; Svobodova et al. 2011), and anthropogenic effects, the genetic structure of mountain populations can be very complex and significantly affect genetic homogeneity. At the same time, it is known that population fragmentation, disturbing gene flow and even leading to strong isolation of some patches, may have a negative impact on the species' survival prospects. In isolated populations, genetic variation is eliminated by a genetic drift and homozygosity increases because reproduction occurs within a limited gene pool (Schlaepfer et al. 2018).

One of the largest capercaillie populations in Europe inhabits the Carpathians. It is estimated that the size of the Carpathian population is approximately 11,000 individuals. However, over the last decades, the environment suitable for this species has become significantly limited and fragmented (Mikoláš et al. 2017). The causes are mainly changes of anthropogenic origin, leading to the disappearance of vast areas of mountain forests and human encroachment on the areas inhabited by the capercaillie (Mikoláš et al. 2017, 2019).

The Carpathian capercaillie population is intensively studied using genetic methods. These studies allowed us to characterize the distribution of genetic variability and the genetic relationships between birds from the Carpathians and other mountain or lowland populations. Analyses on a continental scale have shown genetic distinctiveness of Carpathian and boreal populations (Klinga et al. 2015; Rutkowski et al. 2017b) although this has not been conclusively confirmed by genomic studies (Escoda et al. 2024). At the local scale, a clear genetic differentiation has been suggested between the western and eastern Carpathians, which is probably related to recolonization from different glacial refugia after the Last Glacial Maximum (Klinga et al. 2015). Additionally, a genetic comparison of samples from the Carpathian population from two different demographic periods (1960–1990 – a stable, well-connected metapopulation; and 2011–2015 – a period of rapid population decline) suggested that the genetic population structure is becoming more and more pronounced in the Carpathian population (Klinga et al. 2020). However, this effect was not very clear, despite the relatively long time separating both study periods. This is in contradiction with the results obtained for the mountain capercaillie population from the Black Forest – a lower mountain range, functioning as a metapopulation (Kunz et al. 2021). A comparison of the genetic structure, estimated on the basis of samples from two periods, showed that the genetic differentiation among subpopulations has increased significantly over approximately 15 years. These two results may suggest that the Carpathian population functions better in terms of maintaining integrity and adequate gene flow than the

Black Forest population. Despite the decline in numbers and connectivity between their individual groups in both populations, an impact on the genetic structure was only demonstrated in the case of the Black Forest. The results obtained by these authors indicate that different mountain metapopulations cope differently with changes in the environment, such as increased anthropopressure and forest fragmentation.

The number of capercaillies in the Polish part of the Carpathians is estimated at about 300 individuals. The birds inhabit a fragmented area of over 700 km^2, where there are seven main strongholds, with from 10 to 70 birds living there (Zawadzka et al. 2019). Additionally, the Polish population is most likely connected to the Slovak once, although the existence and potential level of gene flow have not been estimated so far. It is generally estimated that the number of capercaillie in the Carpathians has increased over the last dozen or so years, mainly as a result of the introduction of breeding birds into the environment in the Silesian Beskids and the Jaworzyna Krynicka Range (Zawadzka et al. 2019). In most other regions, numbers remain relatively constant, although in some strongholds the number of birds observed is decreasing alarmingly, for example, at the Polica Range, which may constitute an important migratory link for capercaillie north of the main strongholds.

Little is known about the movement of capercaillies among strongholds in the Polish part of the Carpathians. The geographical distribution of forest areas may indicate that they are connected by gene flow, constituting a Carpathian metapopulation. The persistence of migration and gene flow is also suggested by genetic studies, which confirmed a low level of genetic differentiation between individual strongholds at the beginning of the second decade of the 21st century (Rutkowski et al. 2017b). At the same time, however, it has been suggested that in some areas, for example, in the Gorce Mountains, signs of genetic isolation are beginning to be visible, leading to a decrease in genetic diversity (Rutkowski et al. 2017b). Moreover, data indicating a stable or even increasing population trend in the Polish part of the Carpathians may be illusory, as previous estimates were based on a methodology generating significant errors (Zawadzka et al. 2019). In addition, genetic research – identification of unique genotypes in non-invasive samples – suggests that at least in some Carpathian refuges, the number of identified individuals is gradually decreasing (Santorek et al. 2018, 2020; Szczepański et al. 2019). It is therefore generally unknown to what extent genetic changes are taking place in the Polish population of the capercaillie in the Carpathians, especially in terms of gene flow between strongholds. Since the Carpathian population is the largest capercaillie population in Poland, information about genetic variability and possible changes in gene flow is crucial for the protection of this species in Poland. Therefore, the aim of the study was to compare genetic differentiation and genetic population structure in three important Carpathian strongholds (hereafter: subpopulations) in two periods: 2010–2013 and 2021–2022.

Material and methods

Sampling area

The material for genetic analysis consisted of samples obtained in a non-invasive way – mainly faeces, but also occasionally feathers – biological traces left by the capercaillie and found while penetrating the area. The collection of biological material was carried out in three national parks in the Carpathians, which are the main strongholds of the capercaillie in Poland: the Gorce (GOR), Tatra (TAT) and Babia Góra (BAB) National Parks (Fig. 1).

The Gorce National Park covers the central Gorce Mountains range. In addition to the area located within the Park, the capercaillie also occurs in the adjacent forest districts. Spatially, the population from the Gorce Mountains is separated from other Carpathian subpopulations by a vast forestless area, dominated by the urbanized area of Nowy Targ. Capercaillie numbers in this area were very low in the 1970s and 1980s, before demographically stabilizing at the end of the 20th century – the presence of 25 to 30 individuals per year was detected there (Żurek and Armatys 2011; Zawadzka 2014). However, in the second decade of XXI century, using various methods, including genetic ones, 30–40 individuals were identified annually (Rutkowski et al. 2017b; Szczepański et al. 2019; Zawadzka et al. 2019).

The Tatra Mountains are the main stronghold of the capercaillie in the Carpathians. The birds occur in both Poland and Slovakia, creating a cross-border popula-

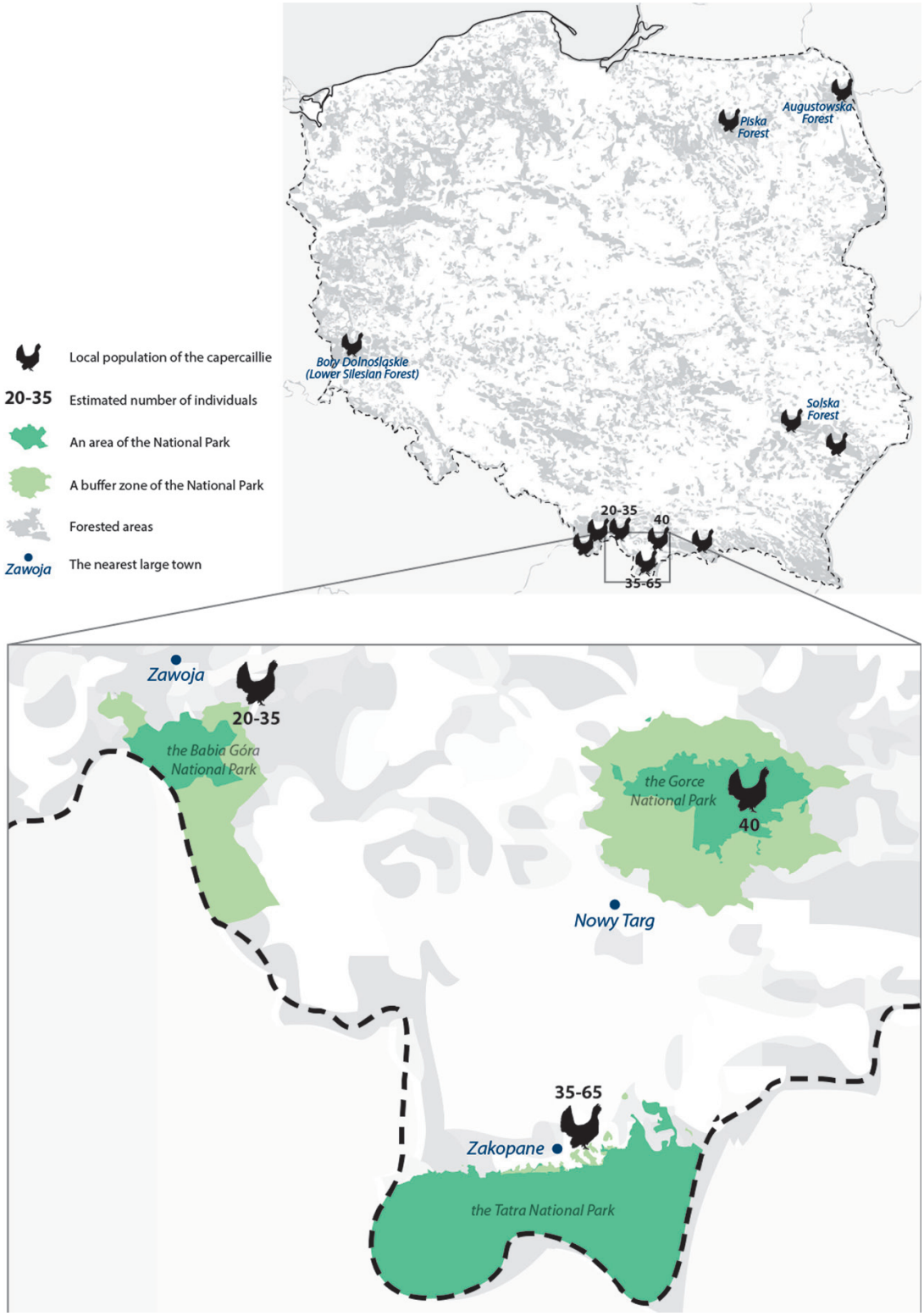


Figure 1. Map of the distribution of the capercaillie in Poland, with particular emphasis on the Carpathian subpopulations covered by this study. The estimated number of individuals was based on data from the work of Zawadzka et al. (2019)

tion. The number of capercaillies in the Tatra National Park has probably been stable over the last several decades. It is estimated that between 35 and 65 individuals live there (Rutkowski et al. 2017b; Zawadzka et al. 2019).

The subpopulation from the Babia Góra National Park is most likely connected by gene flow with the populations located in Slovakia (the southern slope of Babia Góra) and with the population located to the west in the Silesian and Żywiecki Beskids. In the first two decades of the 21st century, 20–35 individuals were identified in this area in individual seasons (Rutkowski et al. 2017b; Santorek et al. 2018 and works cited therein; Zawadzka et al. 2019).

Samples

Faecal samples were collected mainly from February to May during the snow period. After collection in the field, faeces were covered with hygroscopic silica gel to dehydrate the sample, then frozen and stored in a freezer at –72°C until extraction. Feathers were also collected throughout the season. They were stored in paper envelopes or plastic vials and placed in a freezer at –4°C upon arrival at the laboratory. A total of 789 samples were collected in 2010–2013 (hereafter: Old samples), while 535 samples were collected in 2021–2022 (hereafter: New samples) (Tab. 1).

Molecular analysis

All laboratory analyses performed on the Old samples were described in detail in Rutkowski et al. (2017b). The procedures for the New samples were very similar, with the difference that DNA from faeces was isolated using the NucleoSpin Soil Kits (MACHEREY-NAGEL, distributed in Poland by AQUA LAB).

As the material constituted non-invasive samples, several measures were taken (as described in Rutkowski et al. 2017b) in association with the DNA isolation process in order to minimize problems of contamination. Nine microsatellite loci were amplified in the obtained DNA extracts: TuT2, TuT3, TuT4, TuD4, TuD5, TTT1, Bg12, Bg16 and Bg18 (Segelbacher et al. 2000; Caizergues et al. 2001; Piertney and Höglund 2001). Microsatellites were amplified in two multiplex reactions, using reaction mixtures and conditions as described previously (Rutkowski et al. 2017b). The genotyping analyses

Table 1. Information about the collected biological material and basic measures of genetic diversity, determined on the basis of polymorphism of 9 microsatellite loci.

	GOR		TAT		BAB		GORT	TATT	BABT	Total
	GOld	GNew	TOld	TNew	BOld	BNew	Both periods			
*N*col	346	156	221	179	204	200	520	400	404	1324
*N*suc	227	102	117	99	127	117	329	216	244	789
*N*ind	44	21	28	39	35	29	64	66	64	194
*N*a	3.89	3.78	5.56	5.22	4.78	5.33	4.33	5.67	5.78	6.55
R	3.77	3.78	5.55	4.91	4.66	5.05	3.73	5.14	4.76	–
*P*a	0.111	0.111	1.111	0.444	0.333	0.333	0.111	0.565	0.565	–
*H*o	0.593	0.608	0.671	0.641	0.578	0.617	0.598	0.653	0.595	0.616
*H*e	0.583	0.591	0.669	0.629	0.616	0.660	0.590	0.653	0.649	0.654
HWE	0.009	0.052	0.445	0.068	0.132	0.303	<0.001	0.003	<0.001	<0.001
*F*is	–0.007	–0.005	0.015	–0.006	0.077	0.083	–0.005	0.007	0.090	0.060*

GOR – The Gorce National Park; TAT – The Tatra National Park; BAB – Babia Góra National Park; GORT – total data (for both study periods) about the Gorce National Park; TATT – total data (for both study periods) about the Tatra National Park; BABT – total data (for both study periods) about Babia Góra National Park; GOld – data for 2010–2013 about the Gorce National Park; GNew – data for 2021–2022 about the Gorce National Park; TOld – data for 2010–2013 about the Tatra National Park; TNew – data for 2021–2022 about the Tatra National Park; BOld – data for 2010–2013 about Babia Góra National Park; BNew – data for 2021–2022 about the Babia Góra National Park; *N*col – number of samples collected; *N*suc – number of samples successfully analysed (reliable genotype was obtained); *N*ind – number of unique genotypes identified, hence corresponding to the number of identified individuals; *N*a – number of alleles; *R* – allelic richness; *P*a – number of private alleles; *H*o – observed heterozygosity; *H*e – expected heterozygosity; *HWE* – the results of HWE exact test for heterozygote deficiency/excess (P-value was given); *F*is – inbreeding coefficient; * significant *F*is value after the Bonferroni correction.

were performed using a CEQ 8000 sequencer (BECKMAN COULTER). To obtain reliable genetic data, several measures were taken to avoid genotyping errors, as described in Szczepański et al. (2019).

Since the genotype readings of Old samples were performed approximately ten years earlier, analyses of ca. 10% of these successfully analysed samples ($n = 70$) were repeated to confirm the consistency of the readings. No differences were found, so it was assumed that the laboratory procedure scheme allows for a reliable comparison of microsatellite genotypes from both study periods.

Statistical analysis

All population analyses were based on unique genotypes found in the investigated subpopulations. For Old samples, the identification of unique genotypes has been extensively described in previous work (Rutkowski et al. 2017b). Exactly the same strategy was used for New samples – it was assumed that the presence of identical microsatellite genotypes in two or more independent samples attested to the samples belonging to the same individual. Comparisons of genotypes were performed using GenAlEx v. 6.501 (Peakall and Smouse 2012).

Based on unique genotypes, basic genetic measures were estimated: (i) for each subpopulation, including unique genotypes from both periods (total data – GORT, TATT, BABT), the deviation from the Hardy-Weinberg equilibrium was assessed using Fisher's exact test in Genepop v.4 (Raymond and Rousset 1995; Rousset 2008), with the following settings: 10,000 dememorization, 1000 batches and 10,000 iterations; (ii) mean values for basic genetic indices, i.e. number of alleles identified in analysed loci (*N*a); allelic richness – the number of alleles corrected for the sample size using the rarefaction method (*R*; Petit et al. 1998); number of private alleles (*P*a); observed (*H*o) and expected heterozygosity (*H*e; Nei 1978) and inbreeding coefficient (*F*is). These analyses were performed using GenAlEx and FSTAT version 2.9.3.2 (Goudet 2001). Basic indicators were also determined for each subpopulation divided into study periods. The statistical significance of differences between indicators of genetic variability in populations and seasons was estimated by a permutation test using the 'Comparison among groups of samples' option in the FSTAT (1000 permutations were applied).

Because 'null alleles' could significantly affect microsatellite data, we also analysed identified genotypes in the PopGenReport V. 3.0.0 package in the R environment (R Core Team 2017) to identify possible problems, interlinked with 'nulls'. We used a method after Brookfield (1996), as this performs better when all genotyped individuals have at least one allele detected (i.e. there are no missing data).

Potential changes in allele frequencies between two study periods (Old and New) and among investigated subpopulations (GOR, TAT, BAB) were evaluated using *F*st (Weir and Cockerham 1984), as based on the Infinite Allele Model of mutation. Pairwise *F*st value and its significances were calculated in FSTAT.

Genetic population structure for both periods, as well as for total data set, was analysed using the Bayesian clustering approach implemented in STRUCTURE (Pritchard et al. 2000). Each analysis was run with 100 000 burn-in period and 500 000 MCMC iterations, with 15 iterations for each *K*. The value of *K* (number of genetic clusters) was set differentially depending of the level of analysis: (i) both periods independently in 3 subpopulations (GOld, GNew, TOld, TNew, BOld, BNew) analysed together – *K* from 1 to 7); (ii) total number of unique genotypes in each subpopulation (GORT, TATT, BABT, *K* from 1 to 4); (iii) independently for each period (GOld, TOld, BOld, *K* from 1 to 4 and GNew, TNew, BNew, *K* from 1 to 4). All analyses were performed with and without locprior option. The most likely number of clusters was estimated considering ΔK (Evanno et al. 2005) using STRUCTURE HARVESTER Web 0.6.94 (Earl and VonHoldt 2012). Post-processing of runs was done using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) to average the multiple runs given by STRUCTURE and correct for label switching. The output from CLUMPP was visualized with DISTRUCT v 1.1 (Rosenberg 2004) to display the results. Additionally, the population structure was analysed using multivariate Discriminant Analysis of Principal Components (DAPC, Jombart et al. 2010), implemented in R package adegenet 2.1.1 (Jombart 2008; Jombart and Ahmed 2011) in R 4.3.2.

Using GENECLASS 2.0 (Piry et al. 2004), first-generation migrants, i.e. individuals born in a subpopulation other than the one in which they were sampled (Bergl and Vigilant 2007), were detected. The Lh/Lmax likelihood test statistics to identify migrants was

used. We applied the Bayesian criterion of Rannala and Mountain (1997) in combination with the resampling method of Paetkau et al. (2004) to determine the critical value of Lh/Lmax beyond which individuals were assumed to be migrants. We selected an alpha level of 0.05 to determine critical values (Paetkau et al. 2004).

Results

Of the 1,324 samples, reliable genotypes were obtained in 59% of the samples. A reliable genotype was defined as a genotype confirmed in at least three out of five repeated readings, with no missing data. Hence, a total of 789 samples were successfully genotyped and 194 unique genotypes were found (Tab. 1). In 2010–2013, 107 unique genotypes were found, whereas in 2021–2022, 89 unique genotypes were found. The largest difference between periods in the number of identified genotypes was found for GOR (GOld: 44 vs GNew: 21). In the case of GOR and TAT, one genotype was repeated in both study periods. No identical genotypes were found in different subpopulations. In individual subpopulations, the total number of unique genotypes detected was very similar (approx. 65, Tab. 1). A high correlation (r = 0.701) was found between the number of samples collected (*N*col) and the number of identified individual genotypes (*N*ind) and between the number of samples successfully analysed (*N*suc) and the number of identified genotypes (*N*ind) (r = 0.668). The ratio of the number of collected samples to the number of identified genotypes in subpopulations (GORT: 502/65 = 7.7; GOld: 346/44 = 7.8; GNew: 156/21 = 7.4; TATT: 400/66 = 6.01; TOld: 221/28 = 7.8; TNew: 179/39 = 6.3; BABT: 404/64 = 6.3; BOld: 204/35 = 5.8; BNew: 200/29 = 6.89) indicates that one genotype is identified for seven to eight samples collected in GOR and approx. three attempts in TAT and BAB. To identify one unique genotype, the fewest samples had to be successfully analysed in TAT (approx. three) and the most in GOR (approx. five) (GORT: 329/65 = 5.0; GOld: 227/44 = 5.1; GNew: 102/21 = 4.86; TATT: 216/66 = 3.28; TOld: 117/28 = 4.17; TNew: 99/39 = 2.5; BABT: 244/64 = 3.8; BOld: 127/35 = 3.63; BNew: 117/29 = 4.03). This indicates that despite the clear relationship between the number of collected and effectively analysed samples and the number of identified genotypes, determining a unique genotype requires the greatest amount of work in the case of GOR.

All analysed microsatellite markers were polymorphic in the studied subpopulations. The Brookfiled (1996) method did not indicate the significant frequency (>2%) of null alleles at the examined loci, which is consistent with previous publications based on this panel of microsatellite markers (e.g. Rutkowski et al. 2017a; Szczepański et al. 2019).

Comparing the three subpopulations, it was found that *R* and *H*o were significantly higher in TAT than in GOR, *H*o was significantly higher in TAT than in BAB, and *F*is was significantly higher in BAB than in GOR. No significant differences were found in the examined parameters between the two periods. Overall genetic differentiation was low (θst = 0.042, 95%CI = 0.027–0.061). For both study periods, it was θst = 0.05 (95%CI = 0.023–0.081 and 95%CI = 0.032–0.070 for Old and New, respectively). Pairwise genetic differentiation was very low and statistically insignificant for the GOld /GNew and Told/ TNew comparisons, but significant, although still low, for the comparison of samples from two periods from the BAB subpopulation (Tab. 2). Significant genetic differentiation was also found for all other comparisons between subpopulations. The highest *F*st values were found for BAB and GOR comparisons (0.063–0.084) and the lowest for TAT and BAB comparisons (0.021–0.026). For individual subpopulation pairs, there were no clear differences between periods in the *F*st value, except for the comparison of TAT and

Table 2. Among-population genetic differentiation based on pairwise *F*st sensu Weir and Cockerham [1984]. All *F*st values are significant after Bonferroni correction, except of values shown in dark grey. Abbreviation of comparing groups of samples as in Table 1

	GOld	TNew	TOld	BNew	BOld
GNew	0.0013	0.0435	0.0557	0.0794	0.0843
GOld		0.0372	0.0515	0.0631	0.0672
TNew			0.0083	0.0415	0.0383
TOld				0.0258	0.0211
BNew					0.0229
		GORT	TATT		
	TATT	0.0426			
	BABT	0.0647	0.0251		

BAB, where the *F*st value increased from 0.021 (Old) to 0.041 (New) (Tab. 2). In the case of comparisons including all genotypes identified in the subpopulations, the differentiation between BABT and TATT was much lower than for comparisons of these subpopulations with GOR.

In general, analysis in STRUCTURE (Fig. 2) indicated the presence of at least two genetic groups. In all analysed genotype combinations: (i) 3 subpopulations: GORT, TATT, BABT (Fig. 2a); (ii) 6 groups: GOld, GNew, TOld, TNew, BOld, BNew (Fig. 2b); and (iii) separately for Old and New samples (Fig. 2c), Δ*K* indicated the most probable solution division into two genetic groups, both when the location of the sample was taken into account in the a priori analysis (locprior option) and without taking this information into account. In all cases, this division suggests the genetic distinctiveness of individuals from the GOR and the hybrid zone in the TAT area (Fig. 2a–c). The analysis of systems assuming a division into 3 groups (*K* = 3), i.e. corresponding to the locations where the samples were obtained, also suggests that the Carpathian capercaillie population is divided into three genetic groups, with the TAT region as the zone of the most intensive genetic admixture (Fig. 2a–c). Comparing the STRUCTURE results for the two study periods, it was found that the gene pool is gradually becoming more uniform.

The DAPC indicated clear genetic differentiation among investigated Carpathian subpopulations of the capercaillie (Fig. 3a–c). The structure was the most pro-

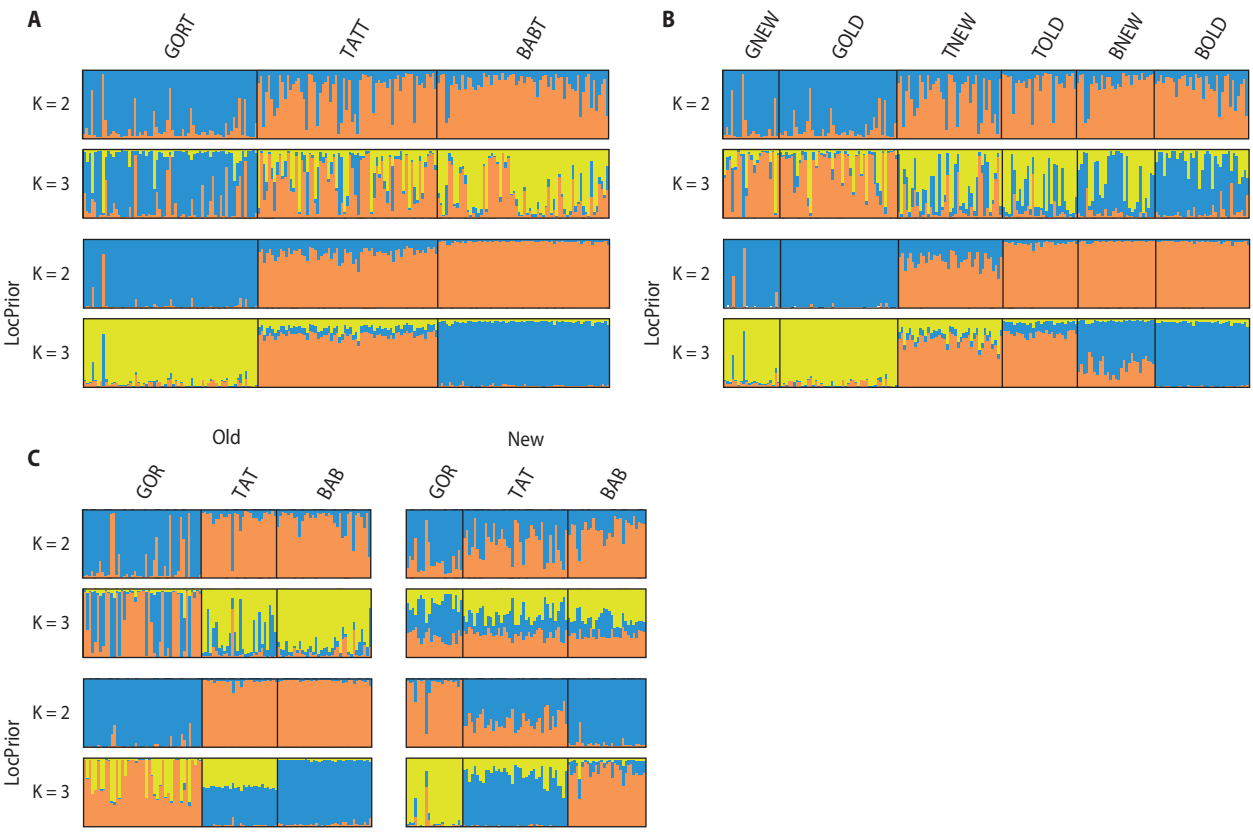


Figure 2. Results of analysis in STRUCTURE. In bar plots each individual is represented by a vertical bar partitioned into segments. The length of each segment describes the estimated membership proportions to each of the genetic clusters. Although ΔK suggested division into two clusters, the bar plot for K = 3 (corresponding to the number of geographical groups) was also presented. Abbreviations of groups (subpopulations) as in Table 1. A – results of analysis in STRUCTURE based on total (cumulative for both periods) number of unique genotypes in each subpopulation; B - results of analysis in STRUCTURE based on unique genotypes from both periods independently in 3 subpopulations; C – results of analysis in STRUCTURE based on unique genotypes identified independently for each period in each subpopulation

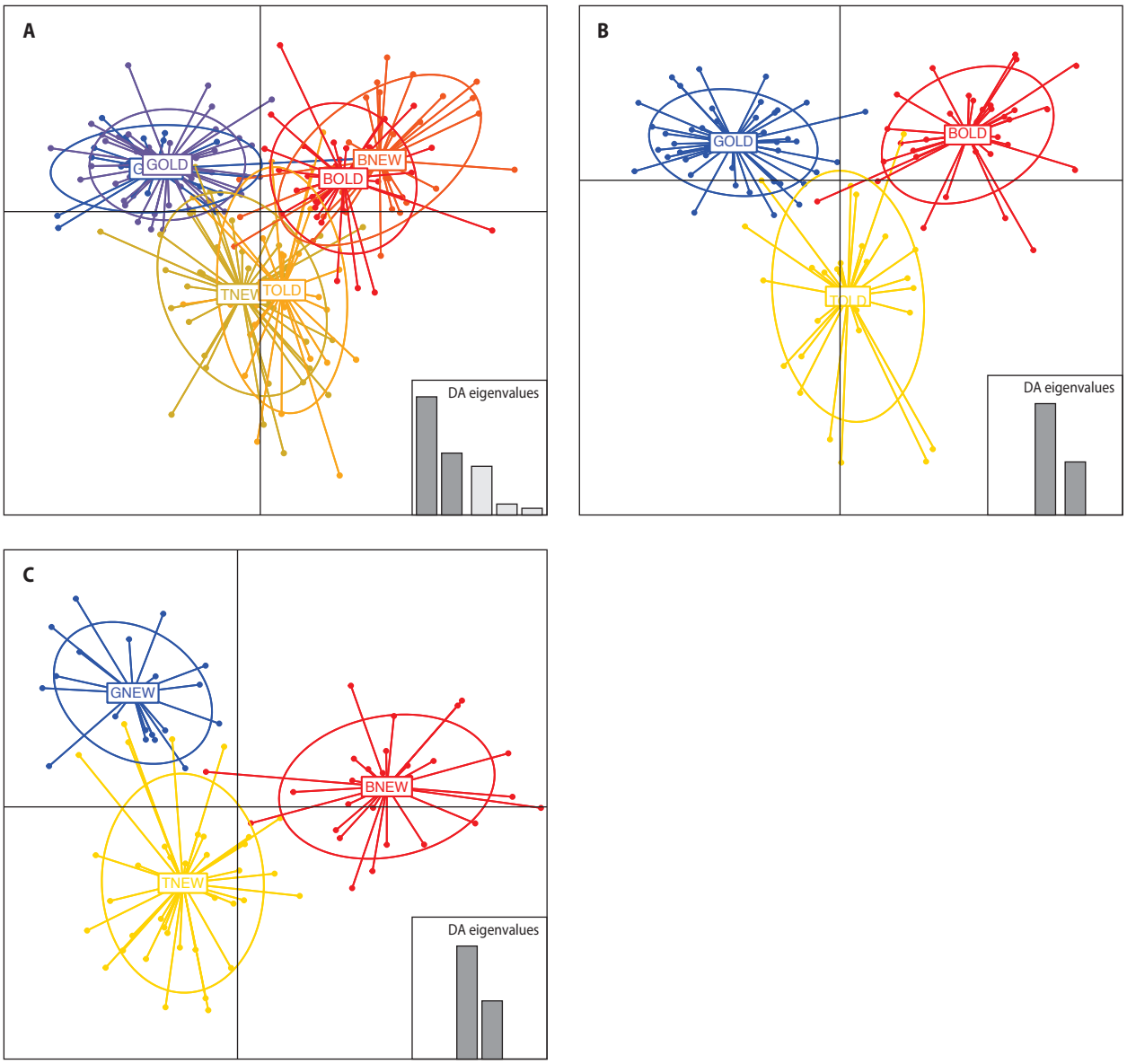


Figure 3. Results of DAPC analysis. DAPCs are shown for the first (x-axis) and the second (y-axis) discriminant function. Abbreviations of groups (subpopulations) as in Table 1. A – results of DAPC analysis based on unique genotypes in each subpopulation in each period; B – results of DAPC analysis based on unique genotypes identified in 2010–2013; C– results of DAPC analysis based on unique genotypes identified in 2021–2022

nounced in the analysis of the total data set (Fig. 3a). Again, position of TAT area as an admixture zone was confirmed. Comparison between Old and New samples showed no clear differences; however, isolation between GOR and BAB seems to increase.

Assignment of individuals in GeneClass indicated that genotypes were assigned to the subpopulation where they were sampled with 80% accuracy in both periods. The presence of 21 first-generation migrants (FGMs) in Old samples and 18 first-generation migrants in New samples was suggested. In Old samples, the number of FGM per subpopulation was very similar (6–8). In GOld, we found 4 FGMs from BOld and 3 from TOld; in TOld, we found 5 FGMs from BOld and only one

from GOld; and in BOld, 6 FGMs were detected from TOld and only 2 from GOld. These results suggested quite intensive dispersal of individuals in 2010–2013, but with asymmetric migration rate in the case of GOR – only sporadically birds emigrate from this area to TAT and BAB. In contrast, dispersal between TAT and BAB was rather symmetric – 5–6 exchanged migrants. In New samples, 18 FGMs were found. In GNew, 3 FGMs were detected (2 from BNew and 1 from TNew). Neither in TNew nor in BNew, FGMs from GNew were found. Again, symmetric dispersal between BAB and TAT was confirmed – 7–8 migrants exchanged in both directions in 2021–2022.

Discussion

Based on microsatellite data obtained from genotyping of non-invasive samples, we can conclude that individual strongholds in the Carpathian capercaillie population are isolated although gene flow still exists between them. The analysis of genetic data from two periods showed that the genetic structure in the Carpathian population has been changing over time.

Genotyping non-invasive samples, despite numerous controversies, is an extremely useful strategy for assessing the number of individuals of a given species occurring in the research area (Banks and Piggott 2022). Based on the compilation of various types of research and long-term data, the number of capercaillies occurring in the Polish part of the Carpathians is estimated at about 300 birds (Zawadzka et al. 2019). Intensive collection of non-invasive biological material allowed us to identify the presence of nearly 200 individuals over a period of approximately 10 years. The genetic data therefore appear to support the observational estimates. It should be noted that the collection of material in the field was very intensive. This indicates that intensive collection of non-invasive samples combined with stringent genotyping procedures may lead to the detection of a significant proportion of individuals. However, we confirmed the presence of only two individuals in both study periods: one in TAT and another in GOR. This supports previous observations that the survival of the capercaillie in natural populations extremely rarely exceed 9 years (Szczepański et al. 2019)

In the years 2010–2013, more unique genotypes were found, and therefore, a larger number of individuals were identified (N = 107 vs. N = 89 in 2021–2022), although much more samples were collected for analysis during this period. As our calculations have shown, the number of collected samples is, however, significantly related to the number of unique genotypes, i.e. individuals identified. Overall, the numbers of individuals for both periods were very similar, especially for TAT and BAB. However, there was a significant difference in the number of identified genotypes in the subpopulation from the Gorce Mountains. This subpopulation appears to be experiencing decline. In 2010–2013, over 40 individuals were identified (Rutkowski et al. 2017b and this study); in 2017–2018, 28 individuals were identified (Szczepański et al. 2019), and in 2021–2022, only 21 individuals were identified. Hence, the downward trend is clearly visible. Interestingly, the decline in the number of individuals seems to be very rapid.

In the case of the Babia Góra subpopulation, the differences are not so clear. It is true that previous studies also suggested a downward trend in the population size: in 2010–2013, 35 individuals were found, but in 2016–2017, only 20 unique genotypes were identified (Santorek et al. 2018); however, in the latter period, only 65 biological samples were analysed. In the years 2021–2022, the occurrence of 29 individuals was found with an almost identical number of analysed samples as in the period 2010–2013.

The most stable in terms of numbers – and even showing an increasing trend – seems to be the Tatra subpopulation. In the years 2010–2013, 28 individuals were found there (Rutkowski et al. 2017b and this study); in 2016, 34 individuals (Rutkowski et al. 2017a); and in the current study (2021–2022), nearly 40 unique genotypes.

To sum up, the genetic data – including the ratio of the number of samples collected to the number of identified individuals (unique genotypes) – suggest that the smallest subpopulation is currently in the Gorce Mountains.

Data on abundance, estimated on the basis of the identification of unique genotypes, are reflected in the level of genetic variability in the studied subpopulations. The lowest genetic variability was found in GOR, while the highest was found in TAT. This corresponds very well with previous studies (Rutkowski

et al. 2017b), proving that the patterns of distribution of genetic variation among Carpathian subpopulations are rather constant. The clearest difference was found in the case of the subpopulation from Babia Góra. Both the number of alleles and the level of heterozygosity in this subpopulation increased significantly during the period covered by the study. It is worth noting that BAB is also the only studied subpopulation in which a significant difference was found in the frequency of microsatellite alleles between the compared periods. This suggests that there may have been an influx of new genetic material into the Babia Góra stronghold. Perhaps, during the period covered by the research, individuals moved from Slovakia or, the most probably, from the Silesian and Żywiecki Beskids in the west, where intensive reintroduction activities are carried out (Zawadzka et al. 2019). This proves that active protection, consisting in reintroduction, not only leads to the restoration of local subpopulations in the Carpathians but also activates gene flow in this mountain metapopulation.

Undoubtedly, in Poland, the mountain capercaillie population is divided into subunits, between which the flow of genes is significantly limited. Previous studies, especially those conducted on the entire capercaillie population in Poland (Rutkowski et al. 2017b), have shown that, compared to lowland populations, birds from the Carpathians constitute a relatively uniform gene pool. However, more detailed analyses (e.g. this work) indicate that from a genetic point of view, the Polish Carpathian population should be perceived as a set of local genetic groups (subpopulations). The frequencies of microsatellite alleles between these groups are significantly different. Similar results were obtained for other capercaillie populations, for example, in Alps (Segelbacher et al. 2003), even for groups of individuals separated by only 10 km. This is the result of the fragmentation of the forest environment and, above all, the presence of vast agricultural and urban areas that are not willingly crossed by capercaillies. On the other hand, quite intensive movement of individuals has been confirmed (between 18 and 20 FGMs were identified per study period) in our study. This may have two explanations. Firstly, the isolation may be so recent that genetic analyses still do not reflect the actual assignment of individuals to subpopulations, or, secondly, the movements of individuals do occur quite often, but concern individual birds with a unique tendency to migrate far away, and do not significantly influence the unification of frequency microsatellite alleles in subpopulations. It was suggested that even one effective disperser per generation is theoretically necessary to prevent higher degrees of population differentiation (Wright 1943). This assumption, together with number of migrants calculated in our study, explains low overall genetic differentiation (calculated as *F*st) in Carpathian population of the capercaillie. Clearly, area of Tatra National Park should be considered as an admixture zone and a stepping stone for gene flow between GOR and BAB. A very similar pattern of differentiation was found between local populations of the capercaillie in other mountainous regions (Segelbacher et al. 2003), where the most numerous subpopulations constituted the admixture zone, but also a source of individuals emigrating to the areas with lower density of birds.

The analysis of the genetic structure of the Carpathian population (*F*st, STRUCTURE and DAPC) showed changes occurring over time, although the distance between the study periods was only 9–12 years. Firstly, it was found that the changes are small, which is most likely due to the short time between the compared periods. For example, no differences were found in the overall *F*st value. However, other studies on the mountain capercaillie metapopulation (Black Forest) showed much more visible changes in the genetic structure despite the similar length of the study period (15 years, Kunz et al. 2021). This indicates that if the factors influencing isolation between subpopulations are intense enough, a period of about 12–15 years may be sufficient to capture emerging changes, for example, increasing reduction in gene flow and increasingly pronounced genetic structure. In the case of the Polish population in the Carpathians, this could not be clearly confirmed. However, analysing changes over time, it was noticed that the differentiation between the BAB and TAT subpopulations, as well as between TAT and GOR, was decreasing, while between GOR and BAB was increasing. This is reflected in the results of STRUCTURE and DAPC. The identification of migrants suggests that the increasing genetic similarity between the GOR and TAT subpopulations is a consequence of the immigration of individuals to the Gorce area rather than opposite direction. Analysis of the New sample shows that no emigrants from the GOR subpopulation were found in TAT. Hence, we could state that most genetic analyses (including the low genetic diversi-

ty and probably the lowest subpopulation size) therefore suggest that the Gorce subpopulation should be viewed as a sink population (Gaggiotti 1993) also because of geographical separation of this area from other capercaillie groups. Indeed, it was shown that population of the capercaillie from the peripheral areas function as sink populations, for example, in the Alps (Segelbacher et al. 2003). Nonetheless, the subpopulations from the Gorce area are the most genetically different compared to the other studied Carpathian strongholds. This is seen in both *F*st and STRUCTURE. This is probably related to the peripheral location of this subpopulation. A similar pattern of genetic variation distribution was also found in mountain black grouse metapopulations (Sittenthaler et al. 2018).

Conclusions

This study documents the genetic diversity and genetic structure of the capercaillie in the Polish part of the Carpathians. A relationship was found between the level of genetic variability and the estimated size of the subpopulation. Birds from the Gorce area, located peripherally in relation to other Carpathian subpopulations, show the lowest genetic variability, and the subpopulation has the characteristics of a sink population. In the subpopulation from the Babia Góra National Park, an increase in genetic variability was noticed over a 10-year period, which may be related to reintroduction activities carried out for many years west of Babia Góra.

The Polish Carpathian population of the capercaillie is spatially divided, but genetically the differences are not yet clearly visible. The overall level of differentiation, although statistically significant, is low, and movements of individuals have been observed between individual subpopulations. The pattern of diversification was similar in both study periods, but in the case of the Gorce Mountains, there was a clear decline in emigration rate, which confirms the sink population type in this region. In general, genetic differentiation among Carpathian subpopulations seems to be decreasing, but this is related to more intensive emigration from the Tatra stronghold. The subpopulation from the Tatra Mountains constitutes the stepping stone for gene flow and admixture zone for clearly separated gene pools from Gorce and Babia Góra. The research results emphasize the great importance of this type of areas in the protection of mountain populations of the capercaillie and other species with limited migration ability, occurring in networks of small, isolated subpopulations.

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