



## Conservation genetics of the capercaillie in Poland – estimating the size of the Tatra National Park population by the genotyping of non-invasive samples

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**Abstract:** Knowledge about population size is of high importance for conservationists. We used non-invasively collected samples and microsatellite genotyping to estimate the size of the Tatra National Park population of the endangered capercaillie *Tetrao urogallus*. This population is one of the most important strongholds of the species in Poland. In 2016 over 150 samples (faeces and feathers) of the capercaillie were collected throughout area of the Tatra National Park. Then, DNA was extracted and genetic profiles were evaluated, using nine microsatellite markers. We obtained 81 reliable genotypes. Among them, 34 unique genotypes were found, corresponding to Minimum Number of individuals Alive in the investigated population. Application of capture-recapture models in the R package Capwire indicated, that the area was inhabited by approx. 54 birds, whereas regression model suggested presence of 36–64 individuals. Previous field surveys suggested that the number of birds in the Tatra National Park is about 50. Hence, we assumed that genetic tagging of non-invasive samples performs well in estimating the abundance of the capercaillie in the investigated population.

**Key words:** *Tetrao urogallus*, population size, genetic tagging, microsatellites, non-invasive samples

### INTRODUCTION

The field of conservation genetics investigates genetic processes, which affect populations of endangered species and identifies factors influencing the level of genetic diversity and distribution of genetic diversity among populations (Amos & Balmford 2001, Hedrick 2001, Sarre & Georges 2009, Kristensen et al. 2010). Molecular techniques are applied to broaden our knowledge about biology, ecology, phylogeography and the taxonomy of endangered species (Haig et al. 2011, Martinez-Cruz 2011). The results of such investigations may have important implications for the planning of conservation programmes and the management of the endangered populations of a wide range of species (e.g. DeSalle & Amato 2004, Morin et al. 2010, McCartney-Melstad & Shaffer 2015). Investigating individuals at the level of their genes also allows unique genetic profiles to be created. This, in turn, combined with polymorphic molecular markers, such as microsatellites and non-invasive sampling, could be used to track a particular specimen in a field without the necessity of direct observation or tagging (Taberlet et al. 1999, Selkoe & Toonen 2006). Hence, this strategy is often used in the case of endangered and/or timid species, increasing the important role molecular population genetics and molecular ecology now play in modern conservation planning.

The capercaillie (*Tetrao urogallus* L., 1758) is one of the most threatened woodland grouse in Europe (Storch 2007a, BirdLife International 2012). During the last few decades,

populations of the capercaillie have declined throughout their range (Storch 2007b). In Poland, the number of birds has dropped from 2,500 at the beginning of the 20th century to as little as 400–500 individuals. Currently, the Polish population is scattered in four isolated areas: the Polish part of the Western Carpathians, Solska Forest, Augustów Forest and Lower Silesian Forest, in which population is of the reintroduced status (Rutkowski et al. 2017 and references therein). The need for the active protection and management of existing populations has stimulated several genetic studies of these birds, applying molecular techniques (Rutkowski et al. 2007). The characterisation of species-specific microsatellite markers and the development of laboratory methods allowing for the use of non-invasive samples as a source of genetic material (Segelbacher et al. 2000, Segelbacher 2002, Regnaut et al. 2006b) provided insight into the ecology of the species (Mäki-Petäys et al. 2007, Regnaut et al. 2006a, Segelbacher et al. 2007, 2008) as well as large body of research work focused on its population genetics (e. g. Segelbacher et al. 2003, Liukkonen-Anttila et al. 2004, Bajc et al. 2011, Rutkowski et al. 2005, 2017). Indeed, molecular techniques and non-invasive sampling were used to estimate the population size of some populations (Jacob et al. 2010, Mollet et al. 2015, Rutkowski et al. 2015).

The Western Carpathians are the most important stronghold of the capercaillie in Poland. The size of this population is estimated at 300 individuals, and most importantly, it is probably connected with the capercaillie population in Slovakia (Žurek & Armatys 2011). Nonetheless, reliable information about the actual population size is still lacking. Field surveys are interlaced with some errors, as rarely every member of the population is detected, leading to underestimates of the population size (Mollet et al. 2015). In this study, we used microsatellite genotyping of non-invasive samples to estimate the Minimum Number of individuals Alive (MNA) of the capercaillie population from the Tatra National Park in the Western Carpathians, and used this data to estimate the size of the population based on genetic tagging (analogous to the conventional Capture-Mark-Recapture approach).

## MATERIAL AND METHODS

### Sample collection

Non-invasive samples – faeces and feathers of the capercaillie – were collected during field surveys in 2016. Surroundings of the known leks were surveyed, as well as regions of the Park, where the capercaillie has been frequently observed. Searching for faeces samples were conducted between February and May, during snow retention. Additionally, some feather samples were collected between June and September 2016. After being collected in the field, the faeces were immediately froze and stored in a freezer at  $-22^{\circ}\text{C}$  until extraction. The feathers were stored in paper envelopes or plastic vials, while after delivery to the laboratory, they were kept in a freezer at  $-4^{\circ}\text{C}$ . In total, 156 samples were collected, including 150 faeces and 6 feathers. The distribution of the sampling sites is presented in Fig. 1.

### Laboratory procedures

DNA from faeces was extracted using the NucleoSpin Soil Kits (MACHEREY-NAGEL, distributed in Poland by AQUA LAB), using the manufacturers' protocol, except for the fact that a double volume of lysate was used for each sample. DNA extractions from feathers were performed using a NucleoSpin Tissue Kits (MACHEREY-NAGEL) in line with the standard protocol. As the material constituted non-invasive samples, several measures were taken, as it was described in the paper by Rutkowski et al. (2017), in association with the DNA isolation process in order to minimise problems of contamination.

All the extracts were made subject to PCR. We amplified 9 microsatellite loci, i.e. TuT1, TuT2, TuT3, TuT4, TTT1, Bg12, Bg16 and Bg18 (tetranucleotide repeats) and TuD4

(dinucleotide repeat) (Segelbacher et al. 2000, Caizergues et al. 2001, Piertney & Höglund 2001). Microsatellites were amplified in two multiplex reactions, i.e. MIX A containing primers for amplification of Bg16, TTT1, TuT2 and Bg12 loci, and MIX B: TuD4, TuT1, TuT4, TuT3 and Bg18. Each forward primer was labelled with one of the fluorescent dyes Dye2, Dye3 and Dye4 (from WellRead Dyes, distributed in Poland by Sigma-Aldrich Poland). The reaction mixture contained 1.5  $\mu$ l of the mixture of primers ('forward' and 'reverse' for each locus, 2 pmol/ $\mu$ l), 7.5  $\mu$ l PCR MasterMix (QIAGEN, distributed in Poland by Syngen Biotech), and 2–3  $\mu$ l of DNA extract, depending on the source of the DNA: 2  $\mu$ l of extract was used in the case of feathers and 3  $\mu$ l in the case of DNA obtained from faeces. Additionally, in the latter case we also added 0.3  $\mu$ l of PCR anti-inhibitor (DNA GDAŃSK, distribution in Poland by Blirt). The reaction mix was made up to 15  $\mu$ l of the final volume with water for PCR (SIGMA-ALDRICH, distribution in Poland by Sigma-Aldrich Poland). The reactions were performed in the following conditions: 15 min at 95°C, 40 cycles of 30 s at 94°C, 90 s 57°C, 90 sec 72°C 1 cycle: 30 s at 94°C, 90 s 57°C, 10 min at 72°C. Controlling for contamination during the course of PCR was performed as described previously (Rutkowski et al. 2017). The genotyping analyses were performed using a CEQ 8000 sequencer (BECKMAN COULTER, distributed in Poland by Comesa-Polska).

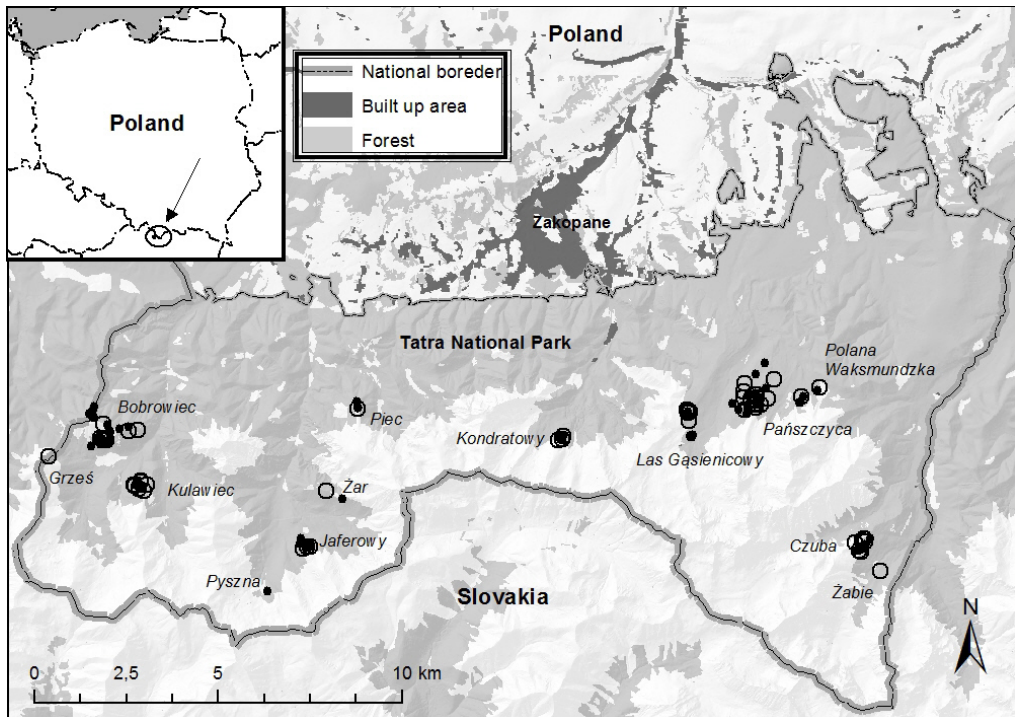


Fig 1. Distribution of sampling sites throughout area of the Polish Tatra National Park; (black dots – genotyped samples, empty circles – undetermined samples).

To obtain reliable genetic data, several measures were taken to avoid genotyping errors (Rutkowski et al. 2017). Briefly, all PCR reactions were repeated at least twice. All extracts lacking the PCR product in the two reactions were excluded from further analysis. Equally, extracts with two identical genotypes in both independent PCRs were classified as successfully

genotyped. All extracts showing signs of contamination (more than two microsatellite alleles at particular loci) were excluded from further analysis. Two additional PCRs were performed in the case of differences between genotypes obtained in the two first PCRs, which could be explained by typical technical problems observed frequently during the microsatellite genotyping of non-invasive samples (i.e. 'allelic drop-out' or 'false alleles'). Consensus genotypes were then created on the basis of the genotypes obtained in all four reactions. The extracts showing evidently different genotypes in successive PCR reactions were excluded from further analysis.

### Statistical analysis

We 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 GenAEx v. 6.501 (Peakall & Smouse 2006, 2012). We assumed that the number of identified unique genotypes reflects the Minimum Number of individuals Alive (MNA) in the investigated population from the Tatra National Park. Additionally, we attempted to estimate the size of the whole population. Two methods were used: (i) probabilistic estimation of the number of birds inhabiting the study area, based on the algorithms of a R package Capwire (Miller et al. 2005). Capwire applies capture-recapture models, which allow individuals to be sampled multiple times per session to estimate population size from non-invasive samples (Pennell et al. 2013). We used the Two-Innate Rates Model (TRIM, different probability of detecting individuals). This method, compared to other capture-recapture methods, yields better results when samples are collected continuously and the population is relatively small. Also, the method does not need to divide the sampling period into sessions and results in a relatively narrow confidence interval (Miller et al. 2005). The TRIM model was used because we assumed that in the case of the capercaillie, the probability of detecting males and females during the display period will vary widely, due to the frequency of residence and behavior of the birds during mating at a lek. (ii) Based on the assumption that more and more new individuals are being detected over time, a regression model of the relationship between the number of genotypes detected in the next days of the study season was created:  $POPULATION\ SIZE = 6.9521 (95\% CL 2.5166 - 11.388) + 0.3214 (95\% CL 0.2306 - 0.4121) \cdot DAY$  Based on the model parameters, an upper 95% prediction interval was calculated for the max. population size estimation on the last days of the study, when we found the last genotype.

Based on unique genotypes, basic genetic measures were estimated: (i) for each locus the deviation from the Hardy-Weinberg Equilibrium and linkage disequilibrium were assessed using Fisher's exact test in Genepop v.4 (Raymond & Rousset 1995, Rousset 2008), with the following settings: 10,000 dememorisation, 1,000 batches and 10,000 iterations; (ii) for each locus and for a combination of 9 loci, the Probability of Identity (the average probability that two unrelated individuals, randomly sampled from a population, will have the same genotype,  $P(ID)$ ) was calculated using GenAEx v. 6.501. Additionally, we also calculated the Probability of Identity with taking into account the genetic similarity among siblings ( $P(ID-Sibs)$ ); (iii) mean values for basic genetic indices, i.e. the number of alleles ( $A$ ), observed ( $H_0$ ) and expected heterozygosity ( $H_E$ ) (Nei 1978) and inbreeding coefficient ( $F_{IS}$ ). These analyses were performed using GenAEx and FSTAT version 2.9.3.2 (Goudet 2001).

### RESULTS

Among the 156 samples, we successfully genotyped 92 samples (60%). Among them, 9 samples were identified, based on their genotypes, as belonging to other species (the black grouse *Lyrurus tetrix* L. and the hazel grouse *Tetrastes bonasia* (L.)). Hence, we finally obtained 81 genotypes of the capercaillie. Within these data 34 unique genotypes were found

(G1–G34, Table 1). Among them, 17 genotypes were found in more than one sample. The most frequent genotype (G6) was found in 18 samples. The distribution of genotypes throughout the study area was presented in Table 1. In general, the genotypes were distributed around a single sampling site, instead of G6, which was found in two distant areas: Kulawiec and Piec; and G11 which was found in two nearby locations: Pańszczyca and Polana Waksmundzka. Based on these data, the MNA was 34 individuals.

Table 1. Distribution of microsatellite genotypes (G1–G34) throughout study area. Number of samples with particular genotype is given. Names of sampling sites as in Fig. 1, except of: BOB – Bobrowiec, KUL – Kulawiec, PYSZ – Pyszna, JAF – Jaferowy, ZAR – Żar, PIEC – Piec, KON – Kondratowy, LG – Las Gąsienicowy, PAN – Pańszczyca, PW – Polana Waksmundzka, CZUB – Czuba, ZAB – Żabie.  $N_C$  – number of samples collected,  $N_S$  – number of samples successfully genotyped,  $N_G$  – number of identified genotypes.

	Grześ	BOB	KUL	PYSZ	JAF	ZAR	PIEC	KON	LG	PAN	PW	CZUB	ZAB
$N_C$	1	26	19	1	10	2	16	10	4	30	2	13	1
$N_S$	0	15	12	1	7	1	15	3	4	15	1	5	0
$N_G$	0	6	3	1	2	1	3	2	3	9	1	5	0
G1		5	.	.	.	.	.	.	.	.	.	.	.
G2		2	.	.	.	.	.	.	.	.	.	.	.
G3		2	.	.	.	.	.	.	.	.	.	.	.
G4		2	.	.	.	.	.	.	.	.	.	.	.
G5		.	.	.	4	.	.	.	.	.	.	.	.
G6		.	5	.	.	.	13	.	.	.	.	.	.
G7		.	6	.	.	.	.	.	.	.	.	.	.
G8		.	.	.	.	.	.	.	.	2	.	.	.
G9		.	.	.	.	.	.	.	.	2	.	.	.
G10		.	.	.	3	.	.	.	.	.	1	.	.
G11		.	.	.	.	.	.	.	.	2	1	.	.
G12		2	.	.	.	.	.	.	.	.	.	.	.
G13		.	.	.	.	.	.	.	2	.	.	.	.
G14		.	.	.	.	.	.	2	.	.	.	.	.
G15		.	.	.	.	.	.	.	.	2	.	.	.
G16		.	.	.	.	.	.	.	.	2	.	.	.
G17		.	.	.	.	.	.	.	.	2	.	.	.
G18		.	.	.	.	.	.	.	.	.	.	1	.
G19		1	.	.	.	.	.	.	.	.	.	1	.
G20		.	.	.	.	.	.	.	.	.	.	1	.
G21		.	.	.	.	.	.	.	.	.	.	1	.
G22		.	.	.	.	.	.	.	.	.	.	1	.
G23		.	.	.	.	.	.	.	.	1	.	.	.
G24		.	.	.	.	.	.	.	.	1	.	.	.
G25		.	.	1	.	.	.	.	.	.	.	.	.
G26		.	.	.	.	.	.	1	.	.	.	.	.
G27		.	.	.	.	.	.	.	1	.	.	.	.
G28		.	.	.	.	.	.	.	1	.	.	.	.
G29		.	.	.	.	1	.	.	.	.	.	.	.
G30		.	1	.	.	.	.	.	.	.	.	.	.
G31		.	.	.	.	.	.	.	.	.	.	.	.
G32		.	.	.	.	.	.	.	.	1	.	.	.
G33		.	.	.	.	.	1	.	.	.	.	.	.
G34		.	.	.	.	.	1	.	.	.	.	.	.

The analysis in Capwire indicated that the area was inhabited by 54 birds (95% C.I. 49–81 ind.). Among them 4 birds belonged to the category of individuals with a high probability of detection and 50 birds to the category of individuals with a low probability of detection. The regression model suggested 36–64 individuals in the population (Fig. 2).

The indicators of genetic diversity, estimated based on the microsatellite polymorphisms of 34 individuals, are shown in Table 2. All loci were polymorphic, with 3–6 alleles per locus. In two cases (locus TuT1 and Bg12), we found significant heterozygote deficiency. Accordingly,

the population was not in Hardy-Weinberg Equilibrium. We did not find significant linkage disequilibrium among the investigated loci.

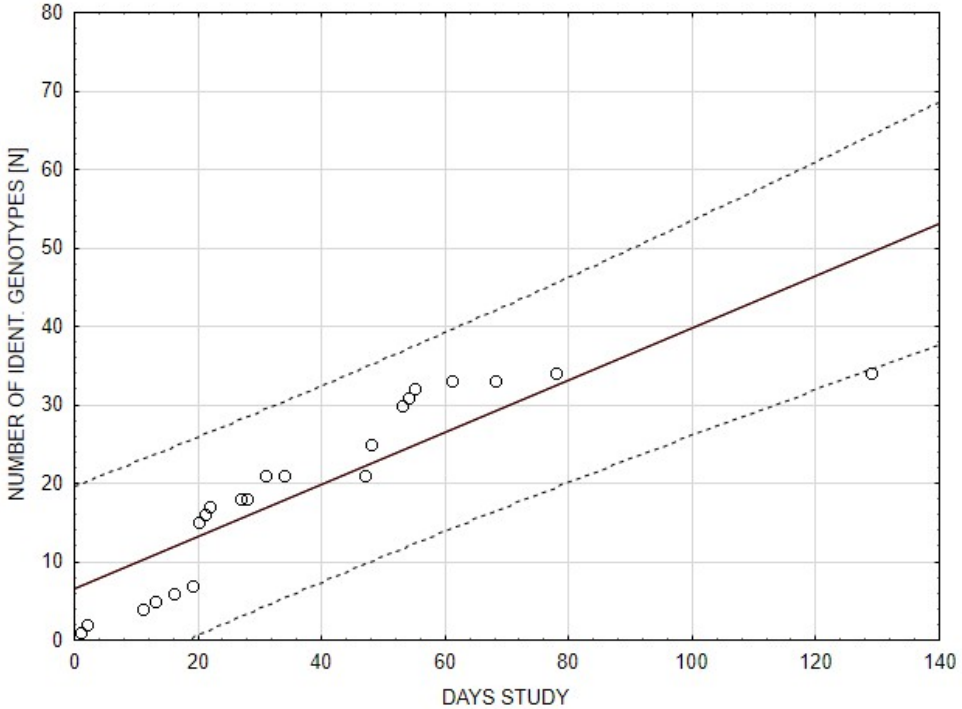


Fig. 2. Regression model of the relationships between the number of detected genotypes and numbers days of study; dotted line – 95% prediction intervals

Per locus, the Probability of Identity ( $P(ID)$ ) ranged from 0.36 (locus TTT1) to as low as 0.08 (TuT4). For the combination of 9 loci, both  $P(ID)$  and  $P(ID-Sibs)$  were  $7.38 \cdot 10^{-7}$  and 0.002, respectively. Hence, according to our data, the expected number of different individuals with the same genotype was very low.

Table 2. Indices of genetic diversity in the population of the capercaillie from the Tatra National Park, estimated based on microsatellite polymorphisms of 34 individuals.  $A$  – number of alleles per locus,  $H_o$  – heterozygosity observed,  $H_e$  – heterozygosity expected,  $HWE$  –  $P$ -values for  $HWE$  exact test for heterozygote deficiency/excess (ns – non-significant [ $P > 0.05$ ]),  $F_{IS}$  – fixation index (\* –  $F_{IS}$  value significant after Bonferroni correction, 180 randomization, adjusted  $P$ -value = 0.0056).

Locus	$A$	$H_o$	$H_e$	$HWE$	$F_{IS}$
BG16	5.00	0.636	0.659	ns	-0.007
TTT1	5.00	0.412	0.419	ns	0.084
TuT2	5.00	0.529	0.602	ns	0.078
BG12	5.00	0.471	0.652	0.005	0.217
TuT1	3.00	0.176	0.564	<0.001	0.683*
TuD4	4.00	0.485	0.638	ns	0.040
TuT4	6.00	0.765	0.784	ns	0.020
TuT3	5.00	0.353	0.376	ns	0.037
BG18	6.00	0.735	0.656	ns	-0.107
Mean/overall	4.89	0.507	0.594	<0.001	0.111*

## DISCUSSION

Reliable estimates of population size have become important in monitoring and management for conservation (Williams et al. 2002, Royle & Dorazio 2008). Invasive methods are often inappropriate in the case of endangered species and require multisession sampling, for example during subsequent seasons (Capture-Mark-Recapture) – the strategy difficult to achieve in the case of elusive and rare species. On the contrary, genetic tagging allows estimating population size based on samples collected during single session (Petit & Valiere 2006). Accordingly, genotyping of non-invasively collected samples is an issue of increasing importance (Miller et al. 2005, Kéry et al. 2011, Mollet et al. 2015). Nevertheless, the strategy has its pitfalls. For example distinguishing among individuals in declining and small populations based on their genotypes can be challenging, because of the genetic similarity of individuals. Moreover, DNA obtained from non-invasive samples is prone to genotyping errors (Jacob et al. 2010). These problems may lead to over- or underestimating population size, resulting in inappropriate management decisions.

Field surveys suggested that the number of birds in the Tatra National Park is approximately 45 (Zwijacz-Kozica & Zięba 2017). Our analysis, based on genetic data, indicated similar results: the Minimum Number of individuals Alive (MNA) was 34, the population size estimated based on genetic tagging was 54, and regression model suggested 36–64 individuals in the population. These indicate that our genotyping procedure performs well in estimating the abundance of the capercaillie in the investigated population. Indeed, genetic analysis of non-invasively collected samples provided insight into the demography and ecology of different capercaillie populations (Jacob et al. 2010, Morán-Luis et al. 2014, Mollet et al. 2015).

In a previous study (Rutkowski et al. 2015), that analysed more than 200 non-invasive samples, collected during three subsequent seasons (2012–2014), 57 unique genotypes were found in the population from the Tatra National Park. Combining these estimates with current results, we can assume that the size of this population contains a range of 34–60 individuals. Other local populations are probably smaller, with 25–30 individuals reported for Gorce and 20 individuals for Babia Góra, however within Polica region there are an estimated 60 individuals (Żurek & Armatys 2011). This constitutes the Tatra National Park as the main stronghold of the capercaillie in the Polish Carpathians.

The genetic diversity of the population from the Tatra National Park, estimated based on samples from 2016, was slightly lower than the values reported previously for 2012–2014 (Rutkowski et al. 2015, 2017), either in terms of the number of alleles or the level of heterozygosity. This could indicate that genetic diversity has been decreasing in the investigated population. It was shown that some local populations in the Carpathians have recently disappeared, while others are still decreasing in size (Saniga 2003, Mikoláš et al. 2015). The Slovakian population of the capercaillie in the Western Carpathians has been rapidly decreasing during the last thirty years, both in terms of number of individuals and leks (Saniga 2011). Hence, it is possible that a negative demographic trend and accompanied isolation and decrease of genetic diversity have also affected the capercaillie population in the Polish Carpathians. This study analysed genetic diversity in a sample of individuals from a single season, whereas higher values were found for pooled data from three different seasons (Rutkowski et al. 2017). Clearly, including samples from a longer period allowed more genetic polymorphism to be detected. Accordingly, multi-season analysis should effect in more precise estimation of population size, as more individuals would be detected. This could be especially important for species with different detection probability among individuals, for example due to behavioural differences. Such a detection probability bias was indeed suggested for the capercaillie (Mollet et al. 2015). On the other hand, non-invasive genetic

tagging was shown to provide reliable estimates of population size, even based on data obtained from just one sampling occasion (Petit & Valiere 2006, Mollet et al. 2015).

We found significant heterozygote deficiency in the investigated capercaillie population. Such a deviation from the Hardy-Weinberg Equilibrium could be caused by an internal genetic structure within a population, for example, by a large distance between leks, scattered around an extensive area. Indeed, genetic data suggested that the birds gather and stay within limited area, as we found only two cases of movement during the study period (genotypes G6 and G11). However, we rather suggest that the observed deficiency of heterozygotes results mainly from a deviation from the Hardy-Weinberg Equilibrium in locus TuT1. It was shown that this locus bears null alleles in the capercaillie (Jacob et al. 2010). The presence of null alleles is one of the factors decreasing heterozygosity in populations.

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#### STRESZCZENIE

#### [Genetyka konserwatorska głuszca w Polsce – ocena wielkości populacji z Tatrzańskiego Parku Narodowego na podstawie genotypowania prób nieinwazyjnych]

Wiedza o wielkości populacji ma istotne znaczenie dla ochrony gatunków zagrożonych. Genotypowanie mikrosatelitarne prób nieinwazyjnych jest często wykorzystywane do oceny liczby osobników występujących na danym obszarze. W odróżnieniu od tradycyjnych metod obserwacyjnych lub odłowu i znakowania, strategia oparta na profilowaniu genetycznym pozwala śledzić poszczególne osobniki bez bezpośredniego kontaktu z nimi, wyznaczyć minimalną liczbę osobników żyjących na terenie badań (MNA), a także szacować wielkość populacji na podstawie jednej sesji gromadzenia danych. Głuszc ( *Tetrao urogallus* ) jest kurakiem leśnym zagrożonym wyginięciem w wielu krajach Europy. W Polsce występuje w czterech izolowanych populacjach, wśród których populacja karpacka uważana jest za najliczniejszą. Tatrzański Park Narodowy stanowi prawdopodobnie główną ostoję głuszca w polskiej części Karpat, ale dokładna liczba żyjących tam osobników nie jest znana. Dane obserwacyjne sugerują występowanie około 45 głuszców. Celem badań było więc oszacowanie liczby osobników głuszca, występujących na terenie Tatrzańskiego Parku Narodowego na podstawie genotypowania prób nieinwazyjnych. Materiał genetyczny (DNA) był izolowany z piór i odchodów (n = 150) zebranych na terenie Parku w 2016 roku. Profile genetyczne określano w 9 loci mikrosatelitarnych. Wiarygodne wyniki uzyskano w przypadku 81 prób, wśród których stwierdzono 34 genotypy unikatowe (MNA = 34). Zastosowanie modelu odpowiadającego badaniu typu odłów-znakowanie-odłów (CMR) w programie Capwire wykazało, że w badanej populacji występuje ok. 50 osobników, natomiast regresja pomiędzy liczbą dni badań i liczbą stwierdzonych genotypów sugerowała występowanie maksimum 36–64 osobników. Są to wartości bardzo zbliżone do wielkości populacji z Tatrzańskiego Parku Narodowego, szacowanej na podstawie obserwacji. Wskazuje to, że genotypowanie prób nieinwazyjnych stanowi przydatną metodę oceny liczebności głuszca.