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Assessing the genetic structure of capercaillie (*Tetrao urogallus*) in Romania

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Abstract Romania holds the most extensive mountain range with old-growth forests, in which both habitat surface and capercaillie (*Tetrao urogallus*) numbers are ones of the highest in Central and Eastern Europe. While previous genetic studies have found that the individuals located in different European mountain ranges are isolated and have highlighted that the species is declining. Here, we are aiming to assess the genetic structure of capercaillie in Romania by genotyping 137 samples collected in the field with 9 STR markers. Expected heterozygosity was 0.586, whereas observed heterozygosity values were 0.859. Population structure analyses indicated weak population differentiation and suggested that sufficient gene flow exists among individuals sampled in different mountain regions. We did not find evidence for a past genetic bottleneck. Our findings contain important information to wildlife managers to focus conservation efforts in areas such as Curvature Carpathians, which serve as a connectivity corridor to avoid eroding the extent or quality of habitat and to prevent further fragmentation.

Keywords: capercaillie, genetics, gene flow, fragmentation, habitat

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Introduction

The risk of extinction on the long-term can be evaluated by studying a population's genetic diversity reduction, by the presence of inbreeding, or by the reduction of the response capacity for environmental changes (Keller & Waller 2002, Tallmon et al. 2004, Segelbacher et al. 2008, Cazacu et al. 2014). If different populations remain connected, e.g. due to the dispersal of individuals, and in some cases it has been shown that one migrant per generations may be enough (Mills & Allendorf 1996, Wang 2004), to maintain gene flow and thus keep genetic diversity preserved (Segelbacher & Storch 2002). The spatial distribution and genetic structure of some populations can be influenced by the dispersal of some individuals which is also the case of capercaillie showing no genetic evidence of sex-biased dispersal and could easily benefit from that rule of one migrant per generation (Mäki-Petäys et al. 2007).

In Central Europe, and not only, the capercaillie is also considered as an umbrella species regarding the conservation of mountain forest ecosystems (Storch 1993, Straupe et al. 2019). Capercaillie is a species which is spread across Europe, mainly in the northern countries and Russia (Regnaut 2004). The Carpathians are Europe's largest mountain range (Gurung et al. 2009), and most of the old-growth forests of Europe can be found there (Grodzińska et al. 2004, Mikolas et al. 2015). 52.9% of the total range of the Carpathians is located in Romania (Grodzińska et al. 2004). The status of the species in Europe is Least Concern (BirdLife International 2015) and in Romania is listed as a game species, but without a hunting period, which gives the specie a protection status. Thus, knowledge of the genetic substructure of the species is crucial for the species' management.

In Romania, both the habitat and the population numbers (between 2200 and 2400 calling/leking males) are one of the highest in Central and Eastern Europe (BirdLife International 2015). Even though there have been conducted numerous studies on this species in Europe, the information regarding the population from Romania is lacking still, and, only small subpopulations have been studied, or few samples have been used (Segelbacher & Piertney 2007, Klinga et al. 2015). Recent studies have suggested that the species is declining (Segelbacher et al. 2003, Bajc et al. 2011), and that individuals from different mountain ranges seem isolated from each other. However, a targeted study to study and evaluate the situation in Romania, has been so far lacking. Furthermore, a specifically targeted, genetic assessment combining size and fragmentation status of the habitat, to guide the management of this species can potentially predict if population numbers and connectivity may be stable on the long-them (Lencinas et al. 2018, Vallant et al. 2018). Assessing the population status of elusive species, like the capercaillie, can be challenging (Aleix-Mata et al. 2019), especially when the data set and sample numbers remain limited (Augustine et al. 2019).

The migration of individuals (dispersal = the movement of individual organisms from their natal area to other locations for breeding (Greenwood & Harvey 1982) or gene flow = the transfer of genes from one area to another by dispersal and successful reproduction (Slatkin 1985) is affected by the habitat and its change under the influence of the anthropic factors, with direct consequences for the long term conservation of the species. For a better understanding on the effect ofthese factors, it is needed to associate individual genetic information with individual geographic distances (i.e., sampling locations) (Peakall et al. 2003).

The goal of this study was to assess the genetic structure of capercaillie in Romania. We used non-invasive samples collected to identify the number of subpopulations located and to identify potential corridors of

connectivity and dispersal.

Materials and methods

Study area and sample collection

In the period 2013-2015, we collected noninvasively 138 capercaillie samples (faeces) from 7 different mountain ranges across Romania, after identification in the field, every sample was stored in a plastic vial, filled with alcohol, and kept at room temperature in the laboratory, until the analyses. Also, the location of the sample, using a GPS-unit was recorded. The samples were collected in the spring season and, however, we took only single faeces from a lek, during the collection process to avoid doubling the samples. In 2013 we collected samples from Suceava (6), Mureș (11) and Harghita (11), in 2014 from Neamț (34) and Curvature Carpathians (7) and in 2015 from Făgăraș (21) and Retezat (47) (Figure 1).

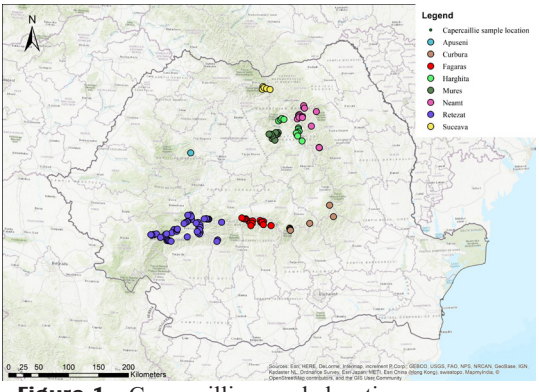


Figure 1 Capercaillie sample location

Genetic analyses

DNA was isolated in our laboratory, using an automated DNA extractor (Maxwell 16 from Promega); and we used 1 ml of the ethanol from the vial where the faeces sample was stored according to an optimized protocol (Fedorca et al. 2018). Seven microsatellite loci, described for capercaillie (TUD7, TUT4, TUD8, TUD3, TUT1, TUT2 and TUD6) (Segelbacher et al. 2000) and two described for black grouse were used (BG15 and BG18) (Piertney and Hoglund 2001), was divided into two multiplexes (multiplex 1: BG15, TUT2, TUT1, TUT4) and multiplex 2: TUD3, TUD6, BG18, TUD7, TUD8). PCR reactions were performed in 15µl mixture, containing 7.5µl of Qiagen Multiplex PCR Kit, 2µl of DNA and fluorescently labelled markers (concentration depending on each marker). Fragment analysis was performed in a mixture of 40 µl consisting of SLS, Size Standard and PCR product, using the FRAG-3 method on the GenomeLab™ GeXP Genetic Analysis System. Alleles were scored using GenomeLab™ Software (Beckman Coulter, Inc).

Statistical analysis

Quality assurance

A first step in the analyses of the database consisted in testing for null alleles. This analysis was conducted using MICROCHECKER software (Van Oosterhout et al. 2004), no evidence for allele dropout or null alleles were found for the analysed set of markers.

Genetic structure

For testing the population genetic structure and possible spatial genetic discontinuity were implemented in software STRUCTURE v 2.3.4 (Pritchard et al. 2000, Evanno et al. 2005) and GENELAND v 4.03 (R package) (Guillot 2005, Guillot et al. 2012) applying the Bayesian algorithm Markov Chain Monte Carlo (MCMC). The stochastic model for STRUCTURE v 2.3.4. consisted in setting the number of iterations to 5, for each number of expected subpopulations (K), K=1 to 10, and 500.000 MCMC repetitions, after 1 million simulations. In order to estimate the posterior probability of appearance of a certain number of subpopulations, we used the Ln(K)

value given by the STRUCTURE software, and ΔK was calculated (Evanno et al. 2005). In GENELAND (Guillot 2005, Guillot et al. 2012) software were implemented 100.000 iterations with a narrow of 100 (1000 iterations have been saved), for K=1 to 10 subpopulations. The repetitions have been conducted including also the geographic position of all the analysed samples. Additionally, for determining population structuring we used TESS software (Chen et al. 2007), choosing values from K=2 to 10, for each subpopulation five repetitions, were conducted using 1.200 simulations with 200 repetitions.

Genetic diversity

In order to see if the genotypes are distributed randomly in the study area or if they show a genetic structure determined by a high level of relatedness, we used spatial autocorrelation analyses and the relatedness coefficient r (Smouse et al. 2008), and the software GenAlex v 6.5 (Peakall & Smouse 2012). We used two different tests in order to determine the differences between the relatedness coefficient of a null model (9999 random permutation of all the genotypes in space) and all the observed genotypes between given distances. In order to determine the unique genotypes of the class distances, we calculated the median coordinates of the multiple geographic positions (Rösner et al. 2014).

Both F_{st} and FIS indices (Hartl & Grant, 1997, Rousset 1997) were calculated using FSTAT v 2.9.3.2 software (Goudet 1995), in order to determine the level of differentiation. In order to test if the Romanian capercaillie population have passed recently through a genetic bottleneck, we used the software BOTTLENECK v 1.2.02 (Piry et al. 1999), with the Wilcoxon test and IAM and S.M.M. model of determining mutations, using 9999 iterations (Cornuet & Luikart 1996).

Nei genetic distance is closely correlated/ influenced by the private alleles (are alleles that are found only in a single population among a broader collection of populations) found in the chosen subpopulations. This distance has a maximum value when each of the subpopulations has a different allele. In order to determine Nei genetic distance, GeneAlEx software has been used.

To estimate the relation between the genotype and the distance among the samples we have used the GenAlEx software (Peakall & Smouse 2012). This software calculates the multilocus autocorrelation coefficient r between the genotypes of the individuals which are between certain distance classes, having values between 1 and -1, and a confidence interval of 95% (Neville et al. 2006).

In order to test the spatial autocorrelation between samples, we have used distance classes of 60 km in each interval, in which, 60 km being less than the maxim dispersal rate of an individual (Segelbacher 2002).

Results

Genetic structure

The sex of the individuals was determined in our laboratory according to the faeces size (Jacob et al. 2010) and the majority were males, further differentiated sex analyses were not implemented.

For determining the number of capercaillie subpopulations, the whole results from STRUCTURE were analyzed using STRUCTURE HARVESTER. However, our hypotheses for K = 2 (Figure 3, a), 3 (Figure 3, c), 4 (Figure 3, d), or more have not been supported by STRUCTURE HARVESTER (Figure 2, a) and TESS results (Figure 2, b and Figure 3, b). Further, GENELAND analysis indicated the presence of 4 subpopulations (Figure 2, c), but this have not been supported by the spatial analysis (Figure 4).

For a better understanding of the results, we have used GIS software to plot the genetic clusters obtained from STRUCTURE on a map using the location of the samples, and for TESS and GENELAND software (Figure 4). The results

from TESS are similar with the results from STRUCTURE, showing that the capercaillie population is not subdivided, and the GENELAND results which show a possibility of the capercaillie to be divided into 4 subpopulations.

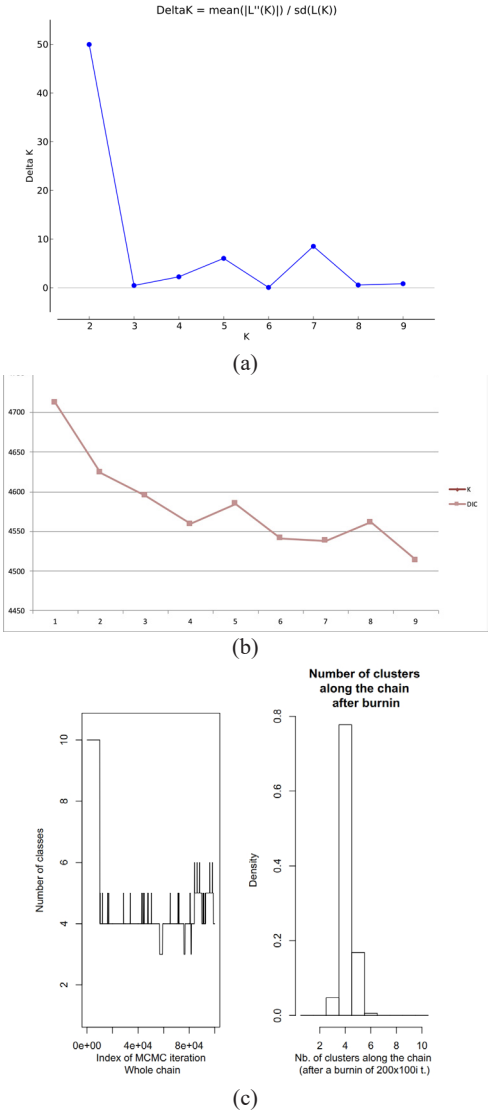


Figure 2 (a) ΔK values resulted from STRUCTURE software; (b) K values resulted from TESS with the vertical axis representing the DIC values, and the horizontal axis representing the number of clusters (K max); (c) Index of MCMC iteration whole chain from GENELAND software and number of clusters.

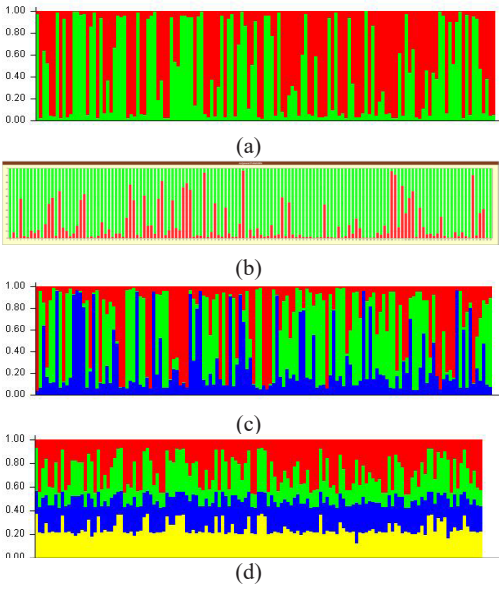


Figure 3 Genetic structure of capercaillie revealed by nine microsatellite markers; Bayesian histogram for K=2, without LOCPRIOR with the genotypes sorted per area from STRUCTURE (a), histogram resulted from TESS software K=2 (b), Bayesian histogram for K=3, without LOCPRIOR with the genotypes sorted per area from STRUCTURE (c) and Bayesian histogram for K=4, without LOCPRIOR with the genotypes sorted per area from STRUCTURE (d).

Genetic diversity

Regarding the number of alleles/locus (Table 1), the highest value was observed at TUD6 marker (Na=9) while the mean value for this parameter at all the analysed marker was Na=4.89.

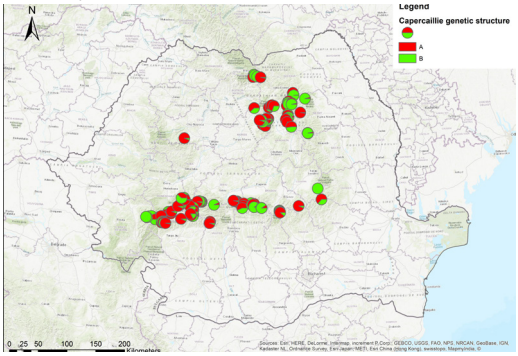
The overall observed heterozygosity was Ho=0.859, the highest value of the observed heterozygosity was registered by TUT2 marker (Ho=0.985), while the smallest value was registered by TUT4 marker (Ho=0.484). The mean expected heterozygosity was He=0.58, with the highest value registered by the marker TUD 6 (He=0.778).

Allelic richness was calculated for the entire population (AR=2.8), the highest value was registered

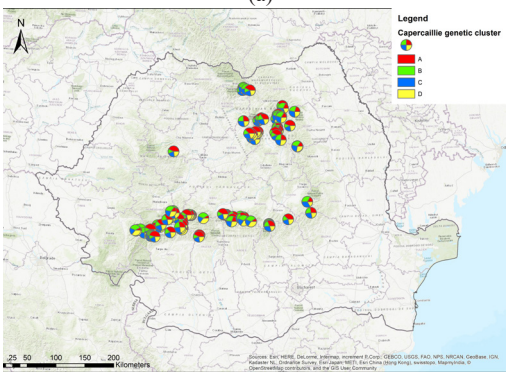
by the TUD6 marker (4.44) while the smallest for the TUD7 and TUT2 markers (2.0) (Table 2).

The results from the genetic distances Nei indicated that the highest distance is between the individuals located in Harghita Mountains and the ones located in Curvature Carpathians (0.121), followed by the ones located Neamț and Curvature Carpathians (0.102). The smallest values have been registered between Făgăraș and Retezat Mountains (0.007). When calculating the Nei genetic distances between Southern Carpathians and Eastern Romanian Carpathians, the results indicated a very close genetic structure (Nei = 0.015). Furthermore, after visualizing these results on dendrograms, the results showed that the Curvature Carpathians are differentiated in a single class with a small distance from the other mountain ranges (Figure 5).

In order to test if the individuals located in different mountain ranges are grouped in different genetic structures, the results showed that the Romanian capercaillie population is not isolated by distance, the p-value = 0.509. However, in order to see the differences between the mountain ranges we used F_{st} values (Table 3).



(a)



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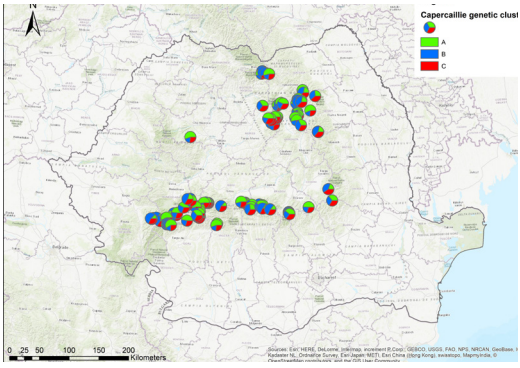

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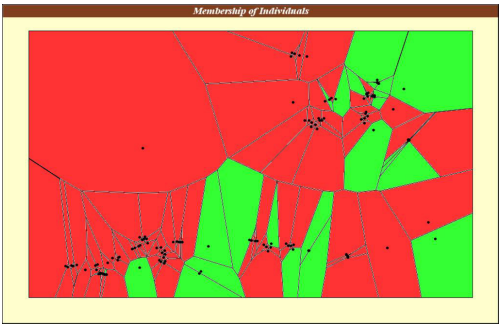

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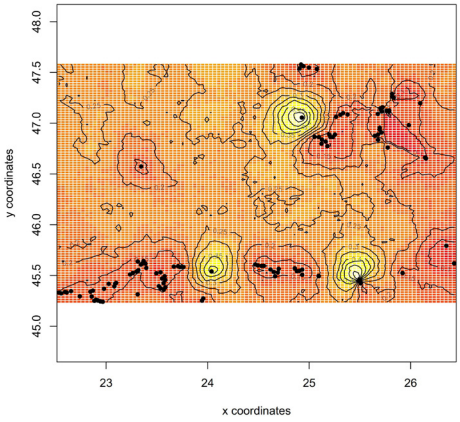

(e)

Figure 4 Distribution of genetic cluster using STRUCTURE (a) - K=2; b) - K=3; c) - K=4), TESS(d) and GENLAND (e)

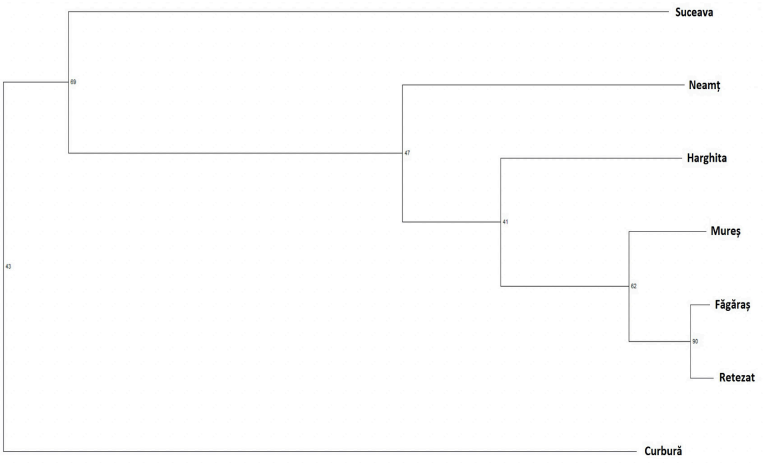

Figure 5
UPGMA (unweighted pair group method with arithmetic mean) dendrogram for the capercaillie subpopulations.

Table 1 Genetic parameters of capercaillie for the entire Romanian population calculated with GenAlEx software

Locus	AR	Na	Ne	Ho	He	F
TUD7	2.00	2	2.000	0.970	0.500	-0.941
TUT4	3.19	5	2.137	0.484	0.532	0.091
TUD8	2.03	3	2.027	0.955	0.507	-0.884
BG15	2.19	5	2.080	0.913	0.519	-0.759
TUD3	3.25	5	3.153	0.908	0.683	-0.329
TUT1	3.08	7	2.655	0.946	0.623	-0.517
TUT2	2.00	2	2.000	0.985	0.500	-0.970
BG18	2.99	6	2.713	0.926	0.631	-0.466
TUD6	4.44	9	4.499	0.643	0.778	0.173
Average	2.80	4.889	2.585	0.859	0.586	-0.511

AR - allelic richness, Na - number of alleles per locus, Ne - effective number of alleles, Ho - observed heterozygosity, He - expected heterozygosity, F - fixation index

Table 2 Allelic richness (AR) of capercaillie per population/subpopulation and per locus resulted from FSTAT software

Locus	Romania	Suceava	Neamț	Harghita	Mureș	Curvature	Făgăraș	Retezat
TUD7	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
TUT4	3.19	3.00	1.54	3.16	3.09	4.94	3.38	3.23
TUD8	2.03	2.00	2.00	2.00	2.00	2.00	2.00	2.21
BG15	2.19	2.00	2.00	2.45	2.56	2.00	2.00	2.32
TUD3	3.25	3.00	3.58	2.93	2.93	3.67	3.38	3.26
TUT1	3.08	3.00	2.82	2.85	2.95	3.82	2.91	3.21
TUT2	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
BG18	2.99	3.00	4.05	2.71	2.50	2.93	2.85	2.89
TUD6	4.44	4.00	5.07	4.92	4.09	4.00	4.73	4.28
Total	2.80	2.67	2.78	2.78	2.68	3.04	2.80	2.82

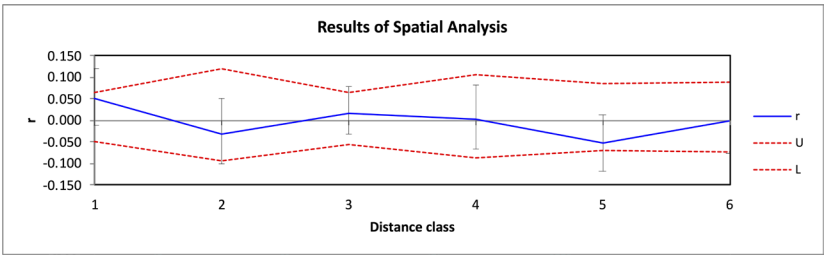

Figure 6 Correlogram (GenAlEx) showing pairwise genetic relatedness of capercaillie, using six equal distance classes, r - correlation coefficient, r is the coefficient of similarity among the individual genotypes within a distance class (solid line). Bootstrap error bars are also shown for each distance class. The red dotted lines (U and L) represent the upper and lower confidence intervals for the null hypothesis (no autocorrelation among the genotypes within the distance classes (r = 0).

The results for the two tests (SPM and Mode-Shift) indicated that capercaillie population had not passed recently through a bottleneck effect (Table 4), moreover, the results obtained from the pairwise genetic relatedness were not significant (Figure 6).

Table 3 F_{st} values among subpopulations of capercaillie calculated with GENEPOP software

Subpopulation	Suceava	Neamț	Harghita	Mureș	Curvature	Făgăraș
Neamț	0.0479					
Harghita	0.0210	0.0181				
Mureș	0.0056	0.0138	-0.0098			
Curvature	0.0126	**0.0554**	0.0388	0.0265		
Făgăraș	0.0039	0.0280	0.0077	-0.0092	0.0020	
Retezat	0.0030	0.0238	0.0132	-0.0053	0.0040	-0.0047

Table 4 Bottleneck effect testing for the entire population of capercaillie

Probability	SPM	Mode-Shift
Standardized differences test	0.21771	0.068
Wilcoxon Test	0.32617	
Sign Test	0.36339	

Discussion

Using three different genetic approaches, we were able to determine the number of subpopulations of capercaillie in Romania.

Furthermore, we found evidence that the Curvature Carpathians act as a connectivity bridge between the capercaillie population across the country, and by maintaining the capercaillie habitat in these regions, we can ensure the gene flow among the subpopulations.

Because of the STRUCTURE software which not reliably recover the correct population structure when sampling is uneven (Puechmaille 2016, Janes et al. 2017), we also compared the results obtained by STRUCTURE with the ones resulted from TESS and GENELAN. STRUCTURE and TESS results indicated that the genetic clusters are not divided between mountain ranges, suggesting that the Romanian capercaillie population is not genetically isolated and the gene flow is still ongoing. However, the GENELAND results should be interpreted with caution; samples collected from capercaillie leks, where the majority of the males are related (Regnaut et al. 2006), could bias the software's results by suggesting the presence of family structuring among them (Segelbacher - personal comments) and also because this computer software tends to overestimate genetic structure (Frantz et al. 2009).

The autocorrelation interval has not been passed; a fact can be attributed to the maxim distance that the capercaillie could travel (70

km according to Segelbacher 2002), which is larger than the interval considered within this study. Overall, the results indicated that capercaillie population from Romania is panmictic (Kimura & Weiss 1964).

Regarding the number of alleles/locus (Na=4.89), the value obtained by us is higher than Na=2.85 resulted from a capercaillie study in Spain (Rodríguez-Muñoz et al. 2007), and also higher with 7.7% (Na=4.54) than all the European capercaillie populations (excepting Spain) (Segelbacher et al. 2003).

The observed heterozygosity (Ho=0.859) registered higher values than the ones obtained for other capercaillie populations: like Ho=0.66 in Germany and Italy (Segelbacher et al., 2003); Ho=0.72/0.71 in Northern and Southern Alps (Rodríguez-Muñoz et al. 2007); and much bigger than the ones in Spain (Ho=0.44) (Alda et al. 2011). However, the high values of the observed heterozygosity and F negative values suggests the selection in favour of heterozygotes. These results are different from other studies which show that the breeding system of this species can lead to an increase of inbreeding potential because only a small proportion of the males transmit their genes (Segelbacher et al. 2007). We assume that our results can be attributed to the dominant males culling during the breeding season, which facilitated the participation in matting of more individuals, ensuring thus a permanent gene flow. This was confirmed by species behaviour in Pyrenees, where each year has registered a reorganisation of the territory of males (Catusse 1993).

Regarding the expected heterozygosity, our values are similar with the Bavarian Alps calculated by Segelbacher et al. (2003) (He=0.59-0.66) and higher than the one obtained by Rodríguez-Muñoz et al. (2007), for some areas from Cantabrian Mountains (He=0.36), where the populations are small and isolated and exhibit inbreeding effect (Alda et al. 2011).

Some authors assimilate the heterozygote excess with population decline (Cornuet & Luikart 1996, Segelbacher 2002) while considering that there is a need for a certain number of individuals in order to preserve the genetic diversity (Soulé 1987). In fragmented habitats it is still uncertain how individuals could move between the patches (Cushman et al. 2006, Segelbacher et al. 2008); however, that is not applicable in the Romanian capercaillie population, which is a continuous one, and the habitat is unfragmented.

Compared with other studies conducted in Europe, the value of allelic richness was smaller than the ones obtained for Black Forest (AR=4.35) (Segelbacher et al. 2008) and Poland (AR=4.075) (Rutkowski et al. 2005), and approximately equal with Jura Mountains (AR=2.78) (Regnaut et al. 2006). However, lower values were registered by capercaillie in the Cantabrian Mountains (Rodríguez-Muñoz et al. 2007).

Taking into consideration the F_{st} values, we can assume that the genetic differences between the mountain ranges are very small (Rutkowski et al. 2005), the one exception is Curvature Carpathians and Neamt area of Eastern Romanian Carpathians, the F_{st} value is 0.0554, indicating a small genetic differentiation (Hartl & Grant 1997, Segelbacher 2002), which also indicated that the higher pairwise Fst-values is not in relation with the distance between subpopulations. The Fst value calculated between the biggest mountain ranges in Romania (Eastern Romanian Carpathians and Southern Carpathians) suggest a very small differentiation among the individuals from the two areas (F_{st} = 0.0064).

Even though our study assesses the capercaillie population from all the mountain ranges in Romania, the number of samples is not very high, also mitochondrial analyses could be applied. We intend to analyse the capercaillie population using a higher set of markers on a bigger set of samples and also utilising complex analyses.

Conclusion

Our study highlighted that the capercaillie population from Romania is genetically not subdivided, and gene flow among individuals from the different regions appears sufficient. Another very important aspect regarding the genetic status of the population is, that it has not recently passed through a genetic bottleneck, furthermore, random mating within the population occurs.

Taking into consideration that capercaillie inhabits most of the mountain ranges in Romania, and its characteristic to cover a variety of habitats, from old forest, to pastures and young forests, require protection of such areas inhabited by the species, which will conserve and protect other habitats and species of high ecological value but which received less attention from both general public and decision makers, simultaneously.

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