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GENETIC DIFFERENTIATION OF GREY WOLF POPULATION (CANIS LUPUS L.) FROM BALKAN AND CARPATHIANS

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Summary: Genetic differentiation of grey wolf (*Canis lupus*) populations from the Balkans (Serbia and Bulgaria) and the Western Carpathians was studied by means of using tissue and bone samples from legally hunted wolves. In total, 339 samples of tissues and bones and small number of feaces, blood and urine samples were used. Genotyping was based on 15 microsatellites of nuclear DNA. Genetic differentiation was studied using Bayesian approach (STRUCTURE software). Obtained results showed the differenetiation between the Balkan and the Carpathian populations as well as between the Bulgarian population and both populations from Serbia and Slovakia. There are indications of migration towards west. Although these results are based on the nuclear markers, it would be advised to reanalyze the samples using mtDNA markers.

Key words: Carpathians, Balkan, genetic differentiation, *Canis lupus*

Introduction

Despite grey wolf (*Canis lupus*, L.) fascinates humans, its competition with men led to overhunting and population decline which even brought the species to the edge of extinction in some parts of Europe. This negative trend resulted with several isolated wolf populations in Iberian, Apennine and Balkan peninsulas as well in eastern parts of Europe. Nevertheless recent increase of environmental awareness among Europeans supported wildlife managers' and scientists' efforts to recover abundance of wolves. Wolves were able to spread back in suitable areas of northern and central Slovakia, while their number in Bulgaria and Serbia is stabilized again.

Several authors (Rueness et al. 2003; Dalén et al. 2005) find that carnivores with large distributions, such wolf is, have cryptic genetic structures that cannot be described by geographic or historic factors. Mobility of wolves is not restricted by the types of landscapes, natural and man-made barriers, but more likely some ecological factors which could restrict migrations and, in some cases, lead to isolation. Thus in the recent years phylogeography of wolf in Europe became common research topic. According to Pilot et al. (2010) European haplotypes of wolf belong to two large haplogroups which differ between south-western Europe (Iberian and Apennine peninsula) and Eastern Europe (Balkan, eastern and south-eastern Europe). Same authors found phylogeographic differences between northern lowland populations and mountain populations from southern Europe. Shortcoming of this work is insufficiency of experimental material from the whole Carpathian range, except southern Poland, and Balkan.

Aim of this study was to compare the genetic diversity of two wolf populations – Balkan one, represented by the sample of wolves from Serbia and Carpathian one, represented by the sample of wolves from Slovakia, the westernmost group within the Carpathians. These two populations will be compared with the sample from Bulgaria which might be a possible source for possible gene flow towards western Balkan and/or to central Europe.

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Material and Methods

A total of 339 bones, skin, tissue, faeces, blood and urine samples of grey wolf (*Canis lupus*, L.) were collected from Slovakia (194), Serbia (120) and Bulgaria (25). Tissue and faeces samples were stored in 96 % ethanol in fridge with ±4 °C temperature. Snow samples with blood and urine were stored in freezer with –20 °C temperature. Bones and skins samples were stored in a paper envelope or micro tubes at the room temperate.

DNA from tissue was isolated with Chelex100 Resin (Biorad) method according Walsh et al. (1991) and also with method according to Doyle and Doyle (1987), which was modified for animal tissues by Oliveira et al. (2007). Bones sample were grinded at first and DNA was isolated by NucleoSpin® Tissue kit (Machery-Nagel). DNA from faeces was isolated by Qiagen Stool MiniKit (Qiagen, Germany) according to producer`s manual. Blood samples found in the snow, were dissolved and centrifuged in 2 ml tubes at 14,000 rpm for 10 minutes and urine samples find in the snow were dissolved in 30 ml tubes and centrifuged at 3,000 rpm. DNA isolation from both blood and urine samples then continued with transferring upper layer to another 1.5 ml tube and DNA isolation continued with Chelex 100 Resin method according Walsh et al. (1991). To detect possible contamination, one or two negative controls in each isolating batch were used.

Samples were amplified by fifteen microsatellites loci published by UCB 250. UCB 253(Ostrander et al.1993), CPH2,CPH 4, CPH 5, CPH7,CPH 8, CPH 12 (Fredholm and Wintero 1995), CPH6, CPH9 (Dolf et al. 2000) and FH2010.FH2088, FH2096, FH2137, FH2145 (Francisco et al. 1996). Sex was determined by MS34 locus (Sundqvist et al. 2001). Primers were divided into two panels (Table 1). PCR reactions were performed in 10 µl mixtures containing 1 µl DNA, 4 µl Qiagen Multiplex Kit (Qiagen, Germany) (Souto et al. 2004), 0.8 µl Q´solution and appropriate concentration of each fluorescently labelled 10 µM primers (Table 1). Minimum one negative control in each analysed plate was used to detect possible contamination. The optimized PCR conditions were the same for all two sets with an initial step of denaturation at 95 °C for 15 min, followed by 35 cycles for tissue samples and 40 cycles for faeces samples of 94 °C for 45 s, Ta 58 °C for 30 s and 72 °C for 1 min. Final extension was at 72 °C for 10 minutes. Amplified PCR products were analysed by ABI 3130 Genetic Analyzer (Applied Biosystem) and an internal size standard GeneScan^{TM} – 500LIZ (Applied Biosystems) was used. The GENEMAPPER software (Applied Biosystems) was used to analyse electrophoregrams and obtain genotypes.

Possible occurrences of null alleles, which may cause genotyping errors due to non-amplified alleles, were detected by MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004). Deviations from Hardy-Weinberg equilibrium (HWE) as well as linkage equilibrium (LE) were estimated for the microsatellite allele distribution of the grey wolf population, using ARLEQUIN version 3 (Excoffier et al. 2006).

The possible bottleneck effect was analysed using microsatellites evolution model: the Two-Phased Model – TPM (Di Rienzo et al. 1994) implemented in BOTTLENECK 1.2 software (Cournet and Luikart 1996). According to authors we used Wilcoxon test to detect complete differences between expected and observed heterozygosity.

Genetic differentiation between populations based on allelic identity F_{ST} (Weir and Cockerham 1984) was computed using the program FSTAT (Goudet 2001) and GENETIX 4.04 (Belkhir et al. 2001) and R package PopGenReport (Gruber and Adamack 2013). Based on pairwise F_{ST} values, we found out the number of migrants between populations per generation *Nm* (Wright 1969). For this computation software GENETIX 4.04 was used (Belkhir et al. 2001).

Population structure was analysed by Bayesian cluster method, implemented in STRUCTURE 2.2 (Pritchard et al.2007). At first we exposed the value of *K* (number of clusters), with simulation of 10 runs for each value of *K*, where we used 200,000 burn-in and 1 mil Markov Chain Monte Carlo (MCMC) replications. We choose admixture mode, because we expect gene flow between populations and we choose independent allele frequencies to exclude overvalue of cluster number (Falush et al.2003).The number of groups was determined using ΔK method according to Evanno et al. (2005). The most probable value of *K* was defined using software R 2.15.2 by implemented function STRUCTURE-SUM (Ehrich et al. 2007), where *K* with highest probability in each runs was chosen. Similarity between runs was calculated by Rosenberg et al. (2002) moderately modified by Ehrich et al. (2007).

Principal coordinate analysis was constructed using R package PopGenReport (Gruber and Adamack 2013). This plot, using the first two axes, visualizes genetic diversity among sampled individuals. Missing data are

replaced by the mean of the allele frequencies. Colours indicate subpopulation if specified in the genetic data set (Gruber and Adamack 2013).

Results

For fragmentation analysis 15 microsatellite loci of nuclear DNA were used. In entire material we found 187 alleles, while the number of alleles per locus varied between 8 and 19. The number of private alleles is rather high for individual populations – Slovakia 30, Serbia – 8 and Bulgaria – 6.

Table 1. Overall population genetic characteristics in studied loci.

Loci	H_o	H_e	F_{is}	F_{st}
CPH12	0.5290	0.6690	0.2067	0.0710
CPH5	0.5320	0.6152	0.1118	0.0670
CPH7	0.5410	0.7348	0.2615	0.0900
CPH8	0.5860	0.8373	0.2858	0.1330
CPH9	0.6400	0.8020	0.1666	0.0890
FH2010	0.5910	0.7931	0.2689	0.1120
FH2145	0.4280	0.8191	0.5022	0.2630
UCB250	0.6280	0.8072	0.1479	0.0940
UCB253	0.6860	0.8405	0.2286	0.0960
CPH2	0.6670	0.8261	0.1912	0.0320
CPH4	0.5490	0.7903	0.3900	0.1620
CPH6	0.6650	0.8456	0.2459	0.0560
FH2088	0.6280	0.8482	0.2490	0.1010
FH2096	0.4720	0.7193	0.3512	0.0740
FH2137	0.7810	0.9055	0.1299	0.0290
Mean	**0.5949**	**0.7902**	**0.2492**	**0.0979**

In Tables 1 and 2 there is an overview of the population genetic characteristics in overall population as well as split over individual subpopulations. As it is shown in both tables there is a difference between observed and expected heterozygosities which caused also significant values of fixation indices meaning the deficiency of heterozygotes. General deficiency of heterozygotes could be explained by populations structure and also on the fragmented populations.

Comparing the genetic differentiation of three populations studied, we found significant differences between the Bulgarian population and both Slovak and Serbian ones, while the differences between Serbian and Slovak populations were not so expressed. There still exist gene flow along the Balkan penninsula from Bulgaria westwards, but mostly towards the Serbian population (Figure 1). Concerning the individual loci, most of them are suitable to estimate differentiation between populations as measured by F_{ST} characteristics (Figure 2).

Table 2. Observed and expected heterozygosities and fixation indices of three wolf populations – Slovakia (Carpathians), Serbia and Bulgaria split over studied loci.

Observed heterozygosity – H_o				**Expected heterozygosity – H_e**				**Fixation index – F_{IS}**			
Loci	**Population**			**Loci**	**Population**			**Loci**	**Population**		
	SVK	**RS**	**BG**		**SVK**	**RS**	**BG**		**SVK**	**RS**	**BG**
CPH12	0.594	0.435	0.474	CPH12	0.592	0.574	0.785	CPH12	0.000	0.249	0.419
CPH5	0.393	0.789	0.550	CPH5	0.487	0.609	0.771	CPH5	0.197	–0.291	0.310
CPH7	0.519	0.409	0.250	CPH7	0.764	0.437	0.645	CPH7	0.323	0.069	0.628
CPH8	0.570	0.495	0.556	CPH8	0.758	0.655	0.835	CPH8	0.251	0.249	0.360
CPH9	0.635	0.583	0.412	CPH9	0.745	0.618	0.677	CPH9	0.151	0.065	0.417
FH2010	0.515	0.621	0.667	FH2010	0.634	0.803	0.756	FH2010	0.192	0.233	0.146
FH2145	0.495	0.143	0.353	FH2145	0.773	0.315	0.640	FH2145	0.364	0.550	0.473
UCB250	0.586	0.929	0.333	UCB250	0.745	0.620	0.807	UCB250	0.217	–0.495	0.603
UCB253	0.738	0.443	0.667	UCB253	0.836	0.627	0.838	UCB253	0.120	0.297	0.228
CPH2	0.676	0.526	0.619	CPH2	0.775	0.825	0.837	CPH2	0.130	0.367	0.283
CPH4	0.336	0.413	0.500	CPH4	0.614	0.594	0.738	CPH4	0.456	0.310	0.348
CPH6	0.559	0.667	0.591	CPH6	0.782	0.793	0.817	CPH6	0.288	0.168	0.298
FH2088	0.641	0.640	0.800	FH2088	0.825	0.719	0.775	FH2088	0.227	0.114	0.020
FH2096	0.463	0.388	0.235	FH2096	0.616	0.675	0.656	FH2096	0.252	0.430	0.659
FH2137	0.775	0.825	0.733	FH2137	0.870	0.889	0.893	FH2137	0.113	0.081	0.212
MS34	0.141	0.108	0.385	MS34	0.498	0.441	0.707	MS34	0.720	0.759	0.487
Mean	0.539	0.526	0.508	Mean	0.707	0.637	0.761	Mean	0.250	0.197	0.368

Results of the STRUCTURE analysis show at k=2 splitting the data in two different clusters – the Balkan and the Carpathian ones although within the Carpathian groups there are several individuals which would belong to the Balkan group and vice versa. At k=3 the Bulgarian group of individuals differ from the Serbian one. There are however, visible numerous individuals which might be migrants from Eastern Balkan, both to Serbian and further to Carpathian population.

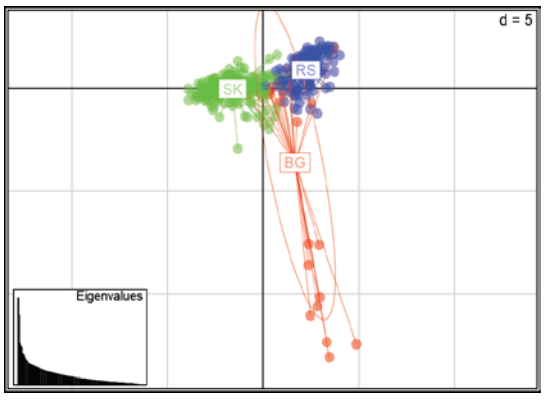


Figure 1. Principal coordinate analysis of genetic differentiation of Balkan and Carpathian wolf population.

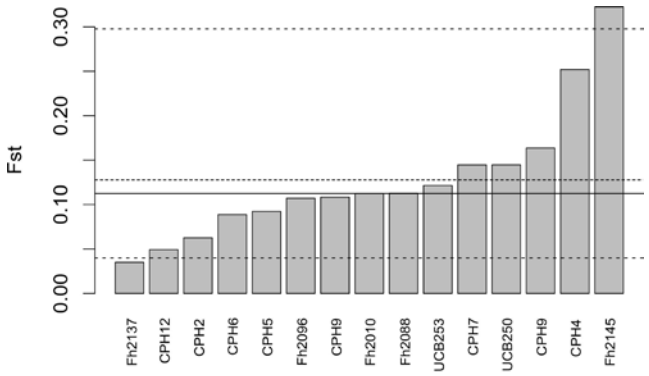
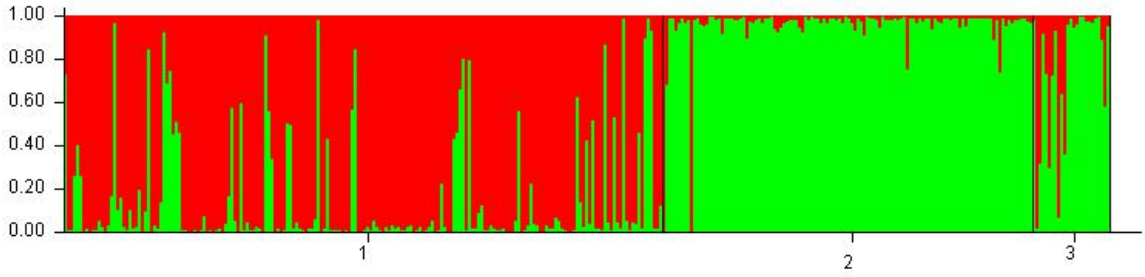


Figure 2. Distribution of F_{ST} values for individual loci.

(a)



(b)

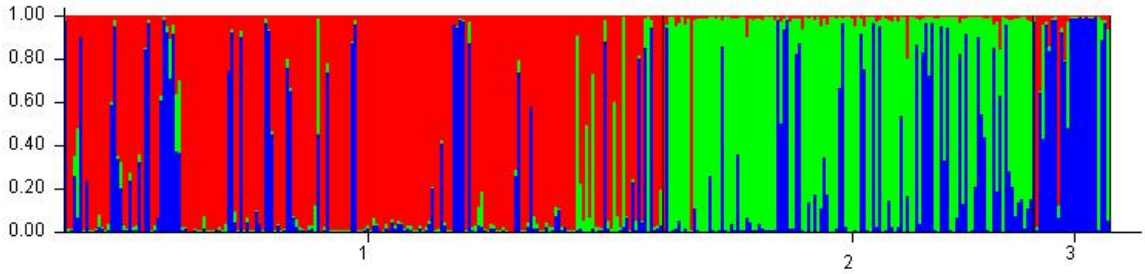


Figure 3. Results of STRUCTURE analysis allocating individual samples to clusters (a) case of two clusters k=2, and (b) case of three clusters k=3. Populations: 1 – Carpathians, 2 – Serbia and 3 – Bulgaria.

Discussion

Two previous studies concerning Balkan grey wolf populations were based on the mtDNA variation. Gomerčić et al. (2010) and Djan et al. (2014) used 280 bp of mitochondrial control region Djan et al. (2014) found two haplogroups (western and eastern) and they revealed very high genetic variation with significant differences between both haplogroups. In contrast in the previous paper Gomerčić et al. (2010) found in Croatian population in total four haplotypes (including a new one not previously described) and thus the Croatian wolf population is considered to be with the highest mtDNA variation.

Czarnomska et al. (2013) found in total 6 haplotypes over wolf natural range Poland while four of them were more frequent and based on their frequency four subpopulations could be recognized. There is a north-south trend of the occurrence of the individual haplotypes and the Carpathian subpopulation is clearly differentiated from the lowland ones. These results were also concordant with the results of the fragmentation analysis of 11 nuclear microsatellites which shown clear differentiation of Carpathian and Roztocze subpopulations from the remaining lowland occurrences.

Wolves in Serbia are a part of a bigger Dinaric population, with a continuous distribution covering the entire Balkan Penninsula from Slovenia to Greece. However, the effective gene flow has been documented between several neighboring countries e.g. Slovenia and Croatia and/or Croatia and Bosnia and Herzegovina, but in fact there are no barriers for gene flow along the Balkan penninsula. The Dinaric population is estimated to be 5,000 individuals (Linnell et al. 2007). A local decrease in the size of fragmented Balkan population during the last two decades of 20th century could lead to changes in genetic diversity. Due to protection status the population seems to recover by migration from adjacent territories and/or recolonization of suitable habitats.

Similar situation seems to be along the Carpathians with a continuous population from Central Slovakia, southern Poland through Ukrainian Carpathians to Romania. In Ukraine the Carpathian population is connected to wolf occurrence in Pre-Carpathians and adjacent plains.

Carpathians host the wolf population about 3,000–4,000 individuals, most of them in Romania and Ukraine (Linnell et al. 2007).

Stronen et al. (2013) in their pioneering study using SNPs and covering the entire Southern and Eastern Euorpean range presented well the differentiation of North Central population from the Croatia and Carpathian ones as well as from the Ukrainian Steppe and Bulgaria and Greece ones. The only drawback of this study was lack of samples from Carpathians and Central Balkan to allow better proof on their position within entire European scale.

It was observed for several taxa, including Italian wolves (Lucchini et al. 2004), that nuclear diversity assessed by microsatellites was considerably higher than mtDNA diversity. This was also the reason why we decided to deal with the genetic differentiation based on bi-parentally inherited nuclear microsatellites. In accordance to Czarnomska et al. (2013), we found also pretty good differentiation between all three groups of samples with indication of the migration pattern between the Eastern and Central Balkan and Carpathians.

The results of this study showed on clear clustering Balkan and Carpathian populations with indication of migration process between the Bulgarian and Serbian populations. Although this results are based on the nuclear markers, it would be advised to reanalyze the samples using also mtDNA markers.

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