GENETIC VARIABILITY AND POPULATION STRUCTURE OF GREY WOLF (*Canis lupus*) FROM BOSNIA&HERZEGOVINA

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Summary: Previous analysis of mtDNA control region (CR) sequence variability for grey wolf population from Bosnia&Herzegovina has revealed its high genetic variability and absence of population structure. Due to limited informativeness of CR mtDNA, additional analyses of population genetic variability and structure were done, applying high polymorphic genetic markers. Microsatellites were applied in order to explore level of genetic variability and population genetic structure. Eighteen microsatellites have been amplified in a total sample of 20 grey wolves, from different localities across Bosnia&Herzegovina. Observed values of the basic genetic parameters indicated high level of genetic variability ($H_o=0.66$; $H_e=0.76$), similar to other populations belonging to the grey wolf Dinaric-Balkan population. STRUCTURE analysis showed differentiation into two genetic clusters. These results are important for future management plans and estimation of the conservation units.

Key words: Bosnia&Herzegovina, *Canis lupus*, genetic differentiation, grey wolf, microsatellites

Introduction

The grey wolf (*Canis lupus*) is a very mobile species with wide area of distribution. Reduction of its distribution area, mainly due to habitat changes and overhunting, led to decline of the population, which was observed in Europe at the end of 19th and beginning of 20th century (Boitani, 2000; Chapron et al., 2014). Today, due to the efforts of local communities, wolf population is in the process of recovery in many European countries, with evident increase in population size.

According to previous researches, grey wolf population from Bosnia&Herzegovina counts approximately 400 individuals (Boitani, 2000; Milenković et al., 2007) with a distribution which includes karstic fields and mountains (Sofradžija and Muzaferović, 2007) at the central parts of the Dinaric region and at borders with Croatia and Montenegro. This population is a part of continuous large Dinaric-Balkan population (Štrbenac et al., 2005, 2008), together with populations from Slovenia, Croatia, Serbia, Montenegro, Albania and FYR Macedonia, with documented gene flow among them (Gomerčić et al., 2010).

Grey wolf population from Bosnia&Herzegovina has been analysed primarily as a part of the Dinaric-Balkan population (Fabbri et al., 2013, Gomerčić et al., 2010, Djan et al., 2014, Lucchini et al., 2004; Moura et al., 2013), thus the lack of information of its genetic variability and population structure is present. Šnjegota et al. (2014) analysed control region (CR) mtDNA sequences of grey wolf from Bosnia&Herzegovina, but in order to perform in depth population structure analyses, an application of more informative polymorphic markers was necessary.

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Highly polymorphic microsatellite markers have been applied in this study of genetic analysis of grey wolves from Bosnia&Herzegovina. The specific aims were to determine the level of genetic variability; to explore population genetic structure and to estimate level of gene flow.

Material and Methods

In total, 20 grey wolf muscle tissue samples from different localities across Bosnia&Herzegovina (Fig. 1) were collected during regular hunts. After sampling, tissues were preserved in 95% pure ethanol and stored at -20°C. Total DNA was isolated using a phenol–chloroform DNA extraction method according to Sambrook and Russel (2001).

Figure 1: Map showing sampling localities of grey wolves from Bosnia&Herzegovina.

Multiplex Polymerase Chain Reaction (PCR) was performed for a panel of 19 markers (18 autosomal microsatellite markers and Amelogenin sex determination locus), using The Canine Genotypes™ Panel 1.1 kit (*Finnzymes Diagnostics*), following the manufacturer recommendations. Genotyping was done by capillary electrophoresis on the ABI3730xl Genetic Analyzer.

Genotypes were scored using Gene Marker (*Softgenetics*) software. ARLEQUIN 3.5.1.2 (Excoffier and Lisher, 2010) was used to calculate basic genetic variability parameters (average number of alleles per locus - Na, observed heterozygosity - Ho and expected heterozygosity - He). The same software was used for observation of deviations from Hardy-Weinberg and linkage equilibrium test. In order to define the population structure, we performed Bayesian cluster analyses implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009). STRUCTURE was run with three independent chains and for number of groups (K) between 1 and 5, with following parameters: burning period 100,000 and Markov Chain Monte Carlo (MCMC) repetitions 1,000,000. We used the admixture model with correlated allele frequencies and no prior population information. The results were assessed using STRUCTURE HARVESTER (Earl et al., 2012). The most likely number of groups was assessed based on the likelihood and the ΔK method (Evanno et al., 2005), using CLUMPAK (Kopelman et al., 2015) and STRUCTURE HARVESTER (Earl et al., 2012).

Results and Discussion

Amplification of all 19 loci was successful for the total number of 20 samples of grey wolves from Bosnia&Herzegovina. The total number of alleles was 126 and all microsatellite loci were polymorphic. The number of observed alleles per locus varied from 5 (locus AHTk211) to 11 (locus FH2054). We have found high level of genetic diversity for grey wolf population, according to the basic genetic diversity parameters ($H_o=0.66$; $H_e=0.76$), (Tab. 1), which was expected due to the fact that grey wolves from the Balkans show high genetic variability (Gomerčić et al., 2010; Fabbri et al., 2013; Djan et al., 2014). In Croatian grey wolf population, Fabbri et al. (2012) observed H_o (0.66) and H_e (0.72) with $N_a=152$, indicating similar level of genetic variability.

Table 1: Basic parameters of genetic variability in grey wolf population from Bosnia&Herzegovina.

Locus	Na	Ho	He
AHTk211	5	0.350	0.553
CXX279	7	0.600	0.774
REN169018	7	0.800	0.767
INU055	6	0.750	0.769
REN54P11	6	0.700	0.726
INRA21	5	0.550	0.768
AHT137	10	0.750	0.869
REN169DO1	7	0.800	0.856
AHTh260	8	0.550	0.774
AHTk253	5	0.700	0.715
INU005	6	0.600	0.805
INU030	6	0.750	0.722
FH2848	8	0.750	0.801
AHT121	10	0.800	0.833
FH2054	11	0.750	0.789
REN162CO4	6	0.500	0.666
AHTh171	6	0.500	0.768
REN247M23	7	0.700	0.798

Na- average number of alleles per locus; *Ho*-observed heterozygosity; *He*-expected heterozygosity

There is an overall systematic departure of the majority of loci ($He > Ho$), although non-significant (Fig 2.), indicating systematic homozygosity excess (Tab1). Statistically significant deviation from the HWE was observed for only one locus (AHTh171). The small number of analyzed individuals may affect statistical power of HWE test. But, even then, homozygosity excess might represent the signal of the Wahlund effect and might point to presence of population genetic structure.

Figure 2. Plot of Ho vs. He ($X=Ho$, $Y=He$) showing systematic departure of all loci from HWE

The STRUCTURE analysis with implemented Bayesian model-based clustering method revealed division of Bosnia&Herzegovina grey wolf population into two clusters (Fig. 3). ΔK was estimated to be at first peak $K=2$, and CLUMPAK analyses revealed structuring for assumed K (Fig.3). No clear geographic pattern or possible barrier could be revealed according to the obtained results, but those clusters might be explained as a genetic signal of separate gene pools, reflecting biology of species and its high mobility (Fig 4.). The obtained population structure was not revealed in previous analysis of the same population using CR mtDNA, thus this result highlight the fact that microsatellites are more appropriate markers for detection of population structure, and represent important tool which should be used for future management plans and estimation of the conservation units. In order to fully explain obtained results on population genetic structure, higher number of individuals must be included in further studies and from broader geographic region.

Figure 3: STRUCTURE results for *Canis lupus* individuals from Bosnia&Herzegovina

Figure 4. Map representing clusters defined in STRUCTURE analysis. Line colorus correspond to colors in Fig 3

Fabbri et al. (2014) stated division in grey wolf population from Croatia in three clusters: Dalmatia, Gorski Kotar and Lika regions, and nine grey wolf individuals from Bosnia&Herzegovina were included in Dalmatia cluster. Pilot et al. (2006) observed genetic divergence within the Dinaric-Balkan grey wolf population and Djan et al. (2014) observed internal subdivision of grey wolves from the central Balkans into two subpopulations on axis corresponding to the Drina River.

Both detected genetic clusters possessed similar average number of alleles (5.9 and 6.5). Basic genetic diversity parameters were calculated for both detected genetic groups. In first cluster (marked in red on Figs. 3&4) following heterozygosities values were detected $H_o=0.67$ and $H_e=0.74$; and similar in the second cluster ($H_o=0.65$ and $H_e=0.78$), confirming high genetic variability. Nevertheless, larger number of samples should be included in further research in order to give comprehensive genetic characterization of the grey wolf population in Bosnia&Herzegovina.

Conclusion

Microsatellite analyses of grey wolves from Bosnia&Herzegovina confirmed their high genetic variability which was inferred from the CR mtDNA analyses. Such results were expected due to their central position at the Balkans, which is well known as a biodiversity hotspot for many species. In order to reveal grey wolf population structure from the Bosnia&Herzegovina in detail, larger number of samples should be included in further research.

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