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ANIMAL GENETICS

Genetic Variability and Population Structure of Grey Wolf (*Canis lupus*) in Serbia[1]

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Abstract—Results of previous morphometric and genetic analyses of grey wolf (*Canis lupus* L.) population from Serbia indicated different patterns of population subdivision. In order to explore population structure, level of genetic variability, genetic drift, inbreeding and signals of bottleneck for grey wolves from Serbia, we applied highly polymorphic genetic markers (microsatellites). Obtained data are valuable in determination of conservation units and creation of appropriate management plans. We have amplified 18 highly polymorphic microsatellites, in a total sample of 75 grey wolves, from different localities across Serbia and multilocus genotypes were analyzed using appropriate software. Observed values of the basic genetic parameters ($H_O = 0.69$; $H_E = 0.75$) indicated moderate level of genetic variability, similar to genetic variability in other populations belonging to the Dinaric-Balkan population of grey wolf. In STRUCTURE analysis, although ΔK was estimated to be at first peak $K = 2$, and second peak $K = 4$, CLUMPAK analyses showed that there's no structuring for any of assumed K, and therefore the population of grey wolf from Serbia may be considered as one continuous population and treated as one conservation unit in future management plans. Signals of bottleneck haven't been observed (Wilcoxon test two phase mutation model $p = 0.247$; and stepwise mutation model $p = 0.815$).

Keywords: Serbia, grey wolf, microsatellites, genetic differentiation
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INTRODUCTION

The grey wolf (*Canis lupus*) is a highly mobile species, occupying large ranges and dispersing for long distances [1]. It was very abundant and widely distributed in Europe until the end of the 19th and beginning of the 20th century, when the decline of its populations was observed [2], with the main reasons such as destruction of its habitats, human prosecution and decreases in natural prey [3–5]. Decline was primarily pronounced in Western Europe, while in Eastern Europe continuous population remained. Delibes [3] stated that during the population decline period in Europe, two isolated populations survived in Western Europe, one in Italy and one in Iberia, while larger populations remained in the Balkans and Eastern Europe [2, 6, 7]. Individuals from Eastern Europe are the primary source of recolonization of Western European populations, and specifically grey wolf population from the Balkans may play key role in recolonization of the Alps [8]. After centuries of population decline, wolves are now increasing in number and range in European countries [5, 9]. Their strict legal protection, together with the recovery of natural prey populations, are probably the main causes for recent recovery and range expansion of their populations [5, 10, 11]. Wolves extended their occurrence, increased in numbers and re-colonized areas, where they had become extinct [12–17]. Wolf has recovered to around 12000 individuals in Europe what is partially a result of their ability to colonize a diverse range of modified habitats, reflecting its high adaptability level [18].

The Balkans is well known as a biodiversity hotspot and glacial refugium for many species [19–23]. Genetic diversity of grey wolf population from the western Balkans was characterized using microsatellites and mtDNA variability data [7, 24]. Djan et al. [25] performed wide-range genetic characterization of grey wolf populations from the Central Balkan area using mtDNA control region (CR) marker, and observed the genetic signal of population structuring on north-south axis along Drina River. Previous morphometric analysis by Milenković [26] suggested that wolf population from the Balkans is divided into two subpopulations (western and eastern) along Morava-Vardar valley due to different biogeographical features in the area. This latter population subdivision was not

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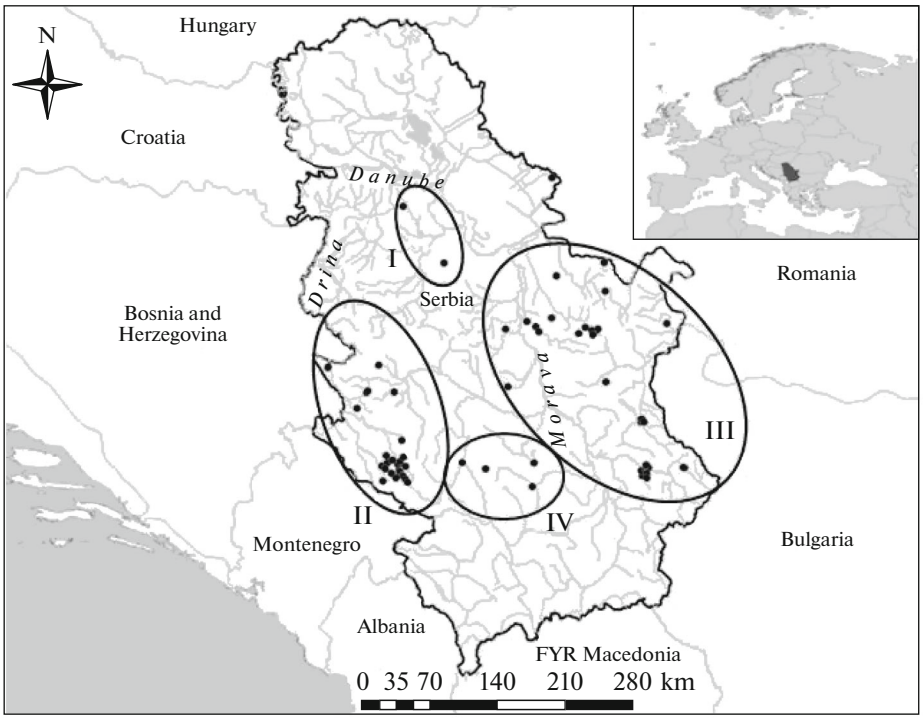

Fig. 1. Map showing sampling localities of grey wolves from Serbia. Geographic regions are marked as follows: I—Northern Serbia; II—Western Serbia; III—Eastern Serbia; IV—Southern Serbia.

supported by Djan et al. [25], but due to the limited information from single locus marker (CR mtDNA) used in this study, in depth structure analysis was not possible to be performed.

As an expanding species, wolf affects populations of wild and domestic ungulates [27], increases human-wolf conflicts and hybridizes with domestic dogs [28–31]. Long-term conservation of wolf populations demands solving biological and human-dimension problems [32, 33], thus importance, appropriate conservation projects and management strategies must be established [10, 34]. According to above mentioned, determining of wolf population structure and detailed insight into its genetic diversity are of great importance [35]. The knowledge of wolves' genetic diversity and structure is one of the most important aspects in their management and conservation. Genetic data could help us to: define population structure and management units, measure gene flow between subpopulations and identify potential risks associated with demographic changes and inbreeding [36]. Genetic characterization of grey wolf population from Serbia is crucial for development of proper management plans due to central position of this population on the Balkans where from it can play important role in recolonization processes. Definition of population structure could provide valuable data for the determination of conservation management units.

In order to measure genetic variability and determine population structure of grey wolf in Serbia, highly polymorphic microsatellite markers were applied in this study. Microsatellites are addressed as suitable molecular markers for resolving population genetic structure in many studies [37–39]. The specific aims were (a) to determine the level of genetic variability; (b) to explore population genetic structure; (c) to estimate level of gene flow, inbreeding and search for potential genetic signals of recent bottleneck.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

The total number of 75 grey wolf muscle tissue samples from different localities across Serbia (Fig. 1) was collected, during regular hunts. After sampling, tissues were preserved in 95% ethanol and stored at −20°C. Total DNA was isolated using a phenol-chloroform method by Sambrook and Russel [40].

Microsatellite Genotyping

Multiplex Polymerase Chain Reaction (PCR) for a panel of 19 markers (18 autosomal microsatellite markers (17 dinucleotides and 1 tetranucleotide) and Amelogenin sex determination locus) was performed 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 (Applied Biosystem). Before fragment analysis all samples were mixed with Hi-Di formamide and size standard GS500 Lys. The results of fragment analysis were analyzed using Gene Marker (Softgenetics) software. Fragment analysis was also done for control DNA sample (H12) of known genotype for the purpose of precisely genotype scoring.

Statistical Analysis

Analyses of genetic variation. Basic genetic parameters: average number of alleles per locus (N_a), observed (H_O) and expected (H_E) heterozygosities were calculated using ARLEQUIN 3.5.1.2 [41]. The same software was used for observation of deviations from Hardy–Weinberg and linkage equilibrium test. Genetix v. 4.02 [42] was used to calculate per-locus F_{IS} [43].

Bayesian clustering, admixture analysis and population assignment. In order to define population structure, in our sample of grey wolf from Serbia, we performed Bayesian cluster analyses implemented in STRUCTURE 2.3.4 [44–47] which uses multilocus genotypes to infer population structure and simultaneously assign individuals to populations. This model assumes that there are K populations, each characterized by a set of allele frequencies at each locus. STRUCTURE was run with three independent chains and for number of groups (K) between 1 and 5, with following parameters: burn in 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 [48]. The most likely number of groups was assessed based on the likelihood and the ΔK method [49], using CLUMPAK [50] and STRUCTURE HARVESTER [48]. CLUMPAK was also used for detailed inspection of convergence between independent runs for each K and graphical interpretations of the results.

Bottleneck analysis. The signature of a genetic bottleneck was detected using the program BOTTLENECK 1.2.02 [51]. To simulate the distribution of alleles and estimate expected heterozygosity three mutation models could be applied: IAM—Infinite Allele Model, SMM—Stepwise Mutation Model and TPM—Two-Phase Model (which is the most appropriate for microsatellite data) [52, 53]. Infinite Allele Model assumes that each mutation leads to existence of new allele [54]; Stepwise Mutation Model assumes that new alleles arise by gain or loss of one repeat unit (one microsatellite motive) [55], and Two-Phase Model assumes that the most mutations follow SMM allowing a certain percentage by IAM model [53]. We tested for bottleneck signatures under all three models and using the one-tailed Wilcoxon's test checked for significance of the heterozygosity excess [56].

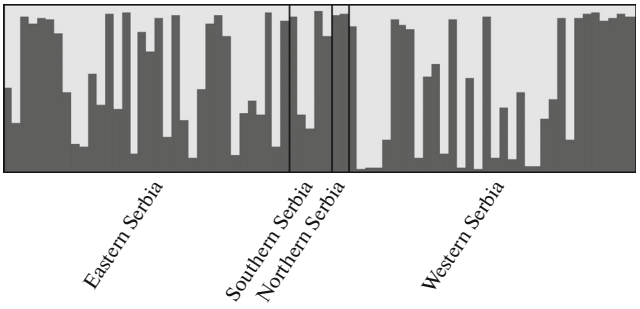

Fig. 2. Estimated population structure of grey wolf (*Canis lupus*) from Serbia. Each individual is represented by a vertical line; a thin black lines separate individuals from different sampling groups (no prior sampling group information was used in admixture analysis).

RESULTS

Amplification of all 19 loci was successful for total number of 75 samples. Among 75 multilocus genotypes, for two individuals genotype could not be scored at AHTk211 locus, which might be a consequence of the presence of null alleles.

Total number of alleles at 18 microsatellite loci was 147 and all microsatellite loci were polymorphic. Number of observed alleles per locus varied from 5 (locus AHTk211) to 14 (locus FH2848), with an average value of 7.8. Observed heterozygosity H_O values (average 0.69) were lower than expected (H_E) (average 0.75). The average F_{IS} value was 0.088, and none of the values was statistically significant. Linkage disequilibrium was observed only for two loci (REN169O18 and INU030) and 14 out of 153 combinations showed low level of linkage disequilibrium.

Grey Wolf Population Structure in Serbia

Bayesian model-based clustering method implemented in STRUCTURE didn't reveal any population structuring in grey wolves from Serbia (Fig. 2). Although ΔK was estimated to be at first peak $K = 2$, and second peak $K = 4$ [43], CLUMPAK analyses showed that there's no structuring for any of assumed K (Fig. 2), so we can conclude that, based on genetic data, no subpopulations can be defined in grey wolves from Serbia.

The significant excess of heterozygosity, which is characteristic of bottlenecked population [51], was not observed in grey wolf population from Serbia, considering two phase mutation model and using Wilcoxon test for combining the results for all 18 loci ($p = 0.24754$). Considering stepwise mutation model the same result was observed (Wilcoxon test $p = 0.81539$), while the significant excess of heterozygosity was

Basic parameters of genetic variability in grey wolf population from Serbia. N_a—average number of alleles per locus; H_E—expected heterozygosity; H_O—observed heterozygosity; F_{IS}—coefficient of inbreeding

Locus	N_a	H_O	H_E	F_{IS}
AHTk211	5	0.65556	0.68001	–0.06609
CXX279	9	0.73913	0.81367	0.03485
REN169018	7	0.71739	0.82591	0.1643
INU055	7	0.6413	0.69874	0.06114
REN54P11	6	0.67391	0.69874	0.03482
INRA21	6	0.51087	0.6713	0.22699
AHT137	10	0.63043	0.72565	0.08161
REN169DO1	9	0.73913	0.81718	0.09334
AHTH260	10	0.66667	0.79379	0.10772
AHTk253	8	0.76087	0.81005	0.03822
INU005	9	0.70652	0.84307	0.11311
INU030	6	0.52174	0.62622	0.2294
FH2848	7	0.69565	0.70011	–0.00944
AHT121	14	0.81522	0.8429	0.03091
FH2054	12	0.81522	0.85252	0.02248
REN162CO4	8	0.66304	0.78344	0.11161
AHTH171	7	0.6413	0.78053	0.14169
REN247M23	7	0.71739	0.76426	0.02608

detected using infinite allele model (Wilcoxon test $p = 0.00001$).

DISCUSSION

This study is the first one that applied panel of 18 autosomal microsatellite loci (The Canine Genotypes™ Panel 1.1 kit) for characterization of grey wolf population from Serbia. Analysis of genetic diversity for grey wolf from Serbia, based on usage of microsatellites, was performed earlier by Paule et al. [57] and they applied 15 autosomal microsatellite loci, but none of them was included in our panel. The panel of 18 loci used in our research, showed to be informative for analyses of genetic diversity and migration corridors in the Western Carpathians [58].

We have found moderate level of genetic diversity for grey wolf population in Serbia, according to the basic genetic diversity parameters ($H_O = 0.69$; $H_E = 0.75$). Those values were higher as compared to the results obtained from Paule et al. [57], ($H_O = 0.53$, $H_E = 0.64$) for grey wolf from Serbia, which may reflect the higher number of microsatellite loci employed in our study and higher informativness of selected markers. Higher values of $F_{IS} = 0.197$ observed by Paule et al. [57] could be explained by general deficiency of heterozygotes in their sample. In comparison to the genetic diversity of grey wolves from Bulgaria ($H_O = 0.526$; $H_E = 0.637$; $F_{IS} = 0.368$) and Slovakia ($H_O = 0.539$; $H_E = 0.707$; $F_{IS} = 0.250$), reported by Paule et al. [57], we also observed higher values of H_E and H_O and lower values of F_{IS}. In Croatian grey wolf population Fabbri et al. [59] observed H_O (0.66) and H_E (0.72) with $N_a = 152$, and $A = 7.4$, indicated slightly lower values in comparison to our results. Lucchini et al. [6] used 18 microsatellite loci for detection of genetic diversity in 11 regional grey wolf populations across Europe. Populations expressing similar level of genetic diversity, as found in our study, were from Croatia, Bulgaria and Greece, which supported previous presumption that the Dinaric-Balkan grey wolf population represents continuous large population with high genetic diversity and extensive gene flow across whole population area on the Balkan Peninsula.

Population structure for grey wolves from Serbia based on Bayesian clustering method was not revealed, so we didn't confirm previously suggested subdivision based on morphological characteristics by Milenković et al. [60], to western and eastern subpopulation, along Morava-Vardar valley. This internal subdivision was also not found by Djan et al. [25] based on CR mtDNA marker. We may suggest that above mentioned division could be a consequence of different ecological factors and/or possible hybridization (wolves with dark coat colors were observed in the Eastern Serbia and high number of feral dogs), but there is no molecular evidence. Better insight in population structuring could be observed by increasing of samples number.

Analyses of grey wolf genetic differentiation from the Balkans and Carpathians by Paule et al. [57] revealed existence of two different clusters, with first peak at $K = 2$, the Carpathian (Slovak) as one cluster and the Balkan (Bulgaria and Serbia) as another cluster, with evident migrations between Bulgaria and Serbia. This confirms existence of gene flow between grey wolf regional groups on the Balkans. On the other hand, Pilot et al. [61] observed genetic divergence within the Dinaric-Balkan grey wolf cluster in larger study area identifying that grey wolf individuals from Croatia make one cluster, while individuals from Bulgaria and Greece were grouped in the second cluster. This is partially in congruence with the results of Djan et al. [25] that found internal subdivision of grey wolves from the central Balkans into two subpopulations on axis corresponding to Drina River. Furthermore, Fabbri et al. [59] stated division in grey wolf population from Croatia in three clusters: Dalmatia, Gorski Kotar and Lika regions and included nine grey wolf individuals from Bosnia and Herzegovina in Dalmatia cluster.

Considering our results we can conclude that grey wolf population from Serbia has no internal subdivision and that it may be considered as one conservation management unit in future conservation plans. Populations that have passed through a demographic bot-

tleneck have the risk of extinction in correlation with reduction of genetic diversity and increase in effects of inbreeding, decreasing the potential for adaptation [62, 63]. Therefore, the detection of bottlenecked populations is important for the implementation of suitable conservation and management plans. In our research we haven't detected recent population decline in bottleneck tests as it was slightly indicated from mtDNA analyses by Djan et al. [25]. They revealed that in Serbian grey wolf population genetic signal of bottleneck was weak which may indicate past and not severe decline in population size. Undetected signal of bottleneck in our research might be consequence of the usage of microsatellites (nuclear DNA markers) that are reliable for reflecting more recent demographic changes comparing to mtDNA markers.

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