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Short Communication

Genetic diversity and structuring of the grey wolf population from the Central Balkans based on mitochondrial DNA variation

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ABSTRACT

The Dinaric-Balkan grey wolf population used to be at a border between the large remaining Eastern European populations and the largely eradicated Western European populations. During the last few decades we have witnessed the Western European wolf population recovery. Substantial genetic variation has previously been reported in the Balkan wolf population, but rigorous genetic characterization has not been done for its central parts. The aims of this research were to determine genetic diversity based on mtDNA sequence variability, to infer possible population structuring, to find genetic signals of population expansions or bottlenecks and to evaluate phylogenetic position of the grey wolf population from the Central Balkans. Six haplotypes were detected, of which three have only been found in the Balkan region. These haplotypes belong to both haplogroups previously determined in Europe. Based on our mtDNA sequence analyses, the Dinaric-Balkan wolf population is vertically differentiated into "western" (Croatia/Bosnia and Herzegovina) and "eastern" (Serbia/Macedonia) subpopulations. None of the results support assumption of population expansion. Instead, significantly positive values for Tajima's *D* and Fu's Fs may suggest recent population bottleneck. Obtained data may be helpful in observation to which extent gene pool from the Balkans contribute to newly founded populations in Western Europe.
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The grey wolf (*Canis lupus*) was abundant and widely distributed over Eurasia, North America and North Africa until the end of the 19th and beginning of 20th century (Boitani, 2000). Widespread destruction of the wolf's habitats, direct eradication, and the decrease of natural prey led to their disappearance from Central and Western Europe (Delibes, 1990; Randi et al., 2000; Randi, 2011). Only two isolated populations survived, one in Italy (Boitani, 1992) and one in Iberia (Delibes, 1990), while larger populations remained in the Balkans and Eastern Europe (Boitani, 2000; Lucchini et al., 2004; Gomerčić et al., 2010). The Balkan grey wolf population represented the border between the Eastern European population and the largely extinct Western European population.

Legal protection, together with high dispersal and breeding potential of the wolves, led to European wolf recovery in the last few decades (Boitani, 1992; Salvatori and Linnell, 2005; Hausknecht et al., 2010). Wolves have expanded rapidly along the Apennine ridge, recolonizing the West Italian and French Alps (Valière et al., 2003; Lucchini et al., 2004). Genetic monitoring of those populations suggested that wolves in Italy are partially isolated from other populations in Europe. Lucchini et al. (2004) stated that wolves with distinct genotypes from the east expanded from Slovenia towards the Italian border in the eastern Alps (Lucchini et al., 2004). Several previous genetic studies suggest that wolf population(s) in the Balkan region have retained a significant portion of historical variation on the pan-European scale (Randi et al., 2000; Lucchini et al., 2004; Gomerčić et al., 2010). No wide-ranging genetic characterization of grey wolf populations from the Central Balkan area has been done so far. Only some individuals from this region have been included in previous population genetic studies (Vilà et al., 1999; Randi et al., 2000; Pilot et al., 2010). Milenković (1997) suggested, based on morphometric analyses, that biogeographic features of the Central Balkan region, specifically Morava-Vardar valley, influence the Dinaric-Balkan grey wolf population structure.

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According to the recent estimates, there are now ~700–800 wolves in Serbia, over 1000 in Macedonia, around 400 in Bosnia and Herzegovina (Boitani, 2000; Milenković et al., 2007) and around 250 in Croatia (Fabbri et al., 2013). Wolves underwent severe declines in Croatia during the 20th century, but since then the population has grown to its current estimated size during the last two decades (Kusak and Huber 2010; Fabbri et al. 2013). Population decline has also been reported in Bosnia and Herzegovina (Boitani, 2000). On the contrary, in Serbia and Macedonia the wolf populations are growing (Boitani, 2000; Milenković et al., 2007).

Wolves in Europe do not have large-scale phylogeographical structure (Vilà et al., 1999; Randi et al., 2000; Pilot et al., 2010). The genealogic network of Eastern European wolf populations does not seem to exhibit an explicit geographical pattern in mtDNA haplotypes distribution (Hausknecht et al., 2010); neither does in the Dinaric-Balkan population (Gomerčić et al., 2010).

The main aims of this research were to: (1) analyze mtDNA sequence variability of grey wolf population from the Central Balkans, (2) to infer possible population structuring of the Dinaric Balkan grey wolf population caused by biogeographical features of the Central Balkans (e.g. Morava-Vardar valley), (3) to study the demographic history and to find genetic signals of population expansions or bottlenecks and (4) to evaluate the phylogenetic position of Central Balkan wolves.

The Dinaric-Balkan grey wolves' dataset consisted of 192 mtDNA control region sequences. Muscle tissue samples of 87 grey wolves from Serbia (53), Macedonia (18), and Bosnia and Herzegovina (16) were collected during legal hunts, during winter seasons (1997–2010) and analyzed in this research (Fig. 1). Remaining 114 sequences were retrieved from GenBank (Gomerčić et al., 2010; Vilà et al., 1999; Randi et al., 2000) and a respective number of individuals per haplotype were inferred from the original reference. Haplotype lu9 reported by Vilà et al. (1999) and found for one individual from Croatia has not been uploaded to the GenBank and therefore was not included in our dataset.

Total DNA was extracted from ethanol preserved muscle tissue samples using standard phenol chloroform isoamylalcohol extraction with proteinase K digestion (Sambrook and Russel, 2001). Partial fragment of mitochondrial control region was amplified with CR1 and CR2R primers published by Palomares et al. (2002) with a target sequence length of 280 bp. Approximately 100 ng of genomic DNA was amplified in a total volume of 25 μl containing 0.2 mM dNTP, 0.5 μM of each primer, 3 mM $MgCl_2$, 1U Taq polymerase and 1× reaction buffer. PCR amplification conditions were set as follows: initial step of denaturation at 95 °C for 5 min, followed by 35 cycles of amplification – each cycle being 94 °C for 40 s, 55 °C for 50 s and 72 °C for 1 min – and a final extension step at 72 °C for 10 min. The PCR products were purified using QIAquick PCR Purification Kit (QIAGENE). Sequencing was conducted on an ABI3730xl genetic analyzer (Applied Biosystems).

The sequences were aligned using the Clustal W algorithm (Thompson et al., 1994) implemented in BioEdit 7.0.9.0. (Hall, 1999), and final adjustments were done by eye. The length of analyzed sequences after alignment was 261 bp. DNA polymorphism (h-haplotype diversity, π-nucleotide diversity, k-mean number of pairwise differences), parameters of overall genetic variability, haplotype frequencies and distances between haplotypes were calculated using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010). Nucleotide diversity for total dataset was calculated under the Kimura 2P (Kimura, 1980) model of nucleotide substitution with Gamma correction for among-site variation in substitution rates ($\gamma = 0.05$) as suggested by a model test in MEGA version 5 (Tamura et al., 2011).

During the initial analyses samples were organized into seven sampling groups based on their geographic proximity and biogeographic features of the sampling areas: Western Serbia, Eastern Serbia, Southern Serbia, Eastern Macedonia, Western Macedonia, Bosnia and Herzegovina and Croatia. Basic genetic indices for each sampling group, analysis of molecular variance (AMOVA) among and within analyzed groups and calculation of pairwise Φst values among seven sampling groups were calculated using ARLEQUIN 3.5.1.2. The pairwise Φst values were used for the construction of UPGMA tree in MEGA version 5.

Our initial analyses clearly supported the presence of two distinctive genetic groups or subpopulations within the Dinaric-Balkan wolf population (Table 1). Therefore above-mentioned and further analyses were conducted for these subpopulations separately as well as for the whole dataset. Basic genetic indices for each subpopulation were calculated and analysis of molecular variance among and within subpopulations was done in ARLEQUIN 3.5.1.2. To gain insight into the possible historical changes in demography of grey wolves from the Central Balkans, mismatch distribution analyses were carried out, under the null hypothesis that the observed data fit the sudden expansion model. The mismatch analysis was performed in ARLEQUIN 3.5.1.2. The significance of the fit of observed mismatch distribution to the expected was estimated by means of the sum of the squared deviations (SSD). Furthermore, two neutrality tests, often used to investigate demographic changes, were performed in DnaSP v5 (Librado and Rozas, 2009). The mismatch distribution analysis and Fs test were run with a transition–transversion weight ratio of 1:1.

To perform an analysis of phylogenetic relationships among wolf mtDNA haplotypes, we collected all available haplotypes from the GenBank and combined them with our Dinaric-Balkan dataset. This dataset comprised of 84 haplotypes and the final alignment of this dataset was 223 bp. Few previously published haplotypes collapsed together in this fragment alignment (for details see Table S1). To avoid further complications with haplotype designation, we adopted the designation presented by Pilot et al. (2010) in our study. For the analysis on phylogenetic relationships of our combined dataset a median-joining (MJ) network (Bandelt et al., 1999) was constructed with the software Network 4.6.0.0 (available at http://www.fluxus-engineering.com/sharenet.htm). Network approaches are more suitable in determining the relationships among haplotypes in intraspecific studies as they allow for the presence of ancestral haplotypes in a sample (Posada and Crandall, 2001; Hausknecht et al., 2010; Zachos et al., 2010).

The analysis conducted on 192 mtDNA control region sequences from the Dinaric-Balkan grey wolf population, with a total length of 261 nucleotides revealed six different haplotypes (Table 1). There were in total 12 polymorphic sites, of which all were parsimoniously informative transitions. Haplotype diversity value was 0.775 ± 0.014, nucleotide diversity (π) was 0.020 ± 0.011 and the average number of nucleotide differences (k) was 5.444 ± 2.632. We observed a relatively high amount of mtDNA variation in the Dinaric-Balkan grey wolf population. Genetic diversity levels of other Balkan wolves at mtDNA control region were also high (Randi et al., 2000; Pilot et al., 2010; Gomerčić et al., 2010; Moura et al. 2013; Fabbri et al., 2013) as compared with other European wolf populations (Randi et al., 2000; Valière et al., 2003; Ellegren et al., 1996; Hausknecht et al., 2010; Sastre et al., 2011). The high genetic variability found in the Balkans might have originated from a past continuous large grey wolf population, that has retained despite human and environmental influences, as it was indicated for Bulgarian grey wolves (Randi et al., 2000), Croatian wolves (Gomerčić et al., 2010; Fabbri et al., 2013), and all Balkan populations (Pilot et al., 2010). Furthermore, the employment of microsatellite markers in population genetic studies of grey wolves also detected the highest diversity in samples from the Balkan wolves (Lucchini et al., 2004; Moura et al. 2013).

No support for the presumed subdivision of the population along the Morava-Vardar valley was obtained based on mtDNA

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