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MITOCHONDRIAL DNA CONTROL REGION VARIABILITY IN WILD BOARS FROM WEST BALKANS

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The wild boar (*Sus scrofa*) is one of most abundant game species in hunting areas of Balkan region. The large fraction of pre-glacial genetic diversity in wild boar populations from the Balkans was addressed due to high proportion of unique mtDNA haplotypes found in Greece, indicating Balkan as main refugial area for wild boars. The aim of the present study is to characterize mitochondrial DNA control region variability in wild boars from different areas in the West Balkan region, in order to evaluate level of genetic variability, to detect unique haplotypes and to infer possible structuring. The total number of 163 individuals from different sampling localities were included in the study. A fragment of the mtDNA control region was amplified and sequenced by standard procedures. Population genetic analyses were performed using several computer packages: BioEdit, ARLEQUIN 3.5.1.2., Network 4.6.0.0 and MEGA5. Eleven different haplotypes were identified and haplotype diversity was 0.676, nucleotide diversity 0.0026, and the average number of nucleotide differences (k) 1.169. The mismatch distribution and neutrality tests indicated the expansion of the all populations. It is shown that high level of genetic diversity is present in the wild boars from the West Balkan region and we have managed to detect regional unique haplotypes in high frequency. Genetic diversity differences have been found in regional wild boar groups, clustering them in two main clusters, but further speculations on the reasons for the observed clustering are prevented due to restricted informativness of the single locus marker. Obtained knowledge of genetic variation in the wild boar may be relevant for improving knowledge of

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the phylogeny and phylogeography of the wild boars, but as well as for hunting societies and responsible authorities for the effective control of wild boar populations.

Key words: wild boar, mtDNA control region, West Balkan

INTRODUCTION

The wild boar (*Sus scrofa*) is one of most abundant game species in hunting areas of Balkan region. Beside its important biological influence on ecosystems of the region, it has important economical role (UROSEVIC *et al.*, 2011). Wild boar is native at Europe, Asia and northern Africa. Present distribution of wild boar populations has been influenced by Earth's climate history, but also by more recent factors, such as agricultural practice changes and increased human impact on forest systems and landscape. During Last Glacial Maximum, between 26.500 and 19.000–20.000 years ago as many other temperate species, wild boars were repressed to southern refugia and recolonized Europe in Postglacialization period (HEWITT, 1999, 2000). The Balkan Peninsula acted as a main refugial area and habitat of the source population for recolonization of European continent (HEWITT, 1999). The previous phylogenetic and phylogeographic researches showed that large fraction of preglaciation genetic diversity remained in wild boars in Italy and southern part of Balkan Peninsula (SCANDURA *et al.*, 2008; ALEXANDRI *et al.*, 2012).

Moreover, present diversity and distribution across Europe has been further influenced by strong demographic decline and regional extinctions of wild boar populations in several European countries during nineteenth and early twentieth centuries, mainly due to human impacts such as hunting pressure, agricultural changes, habitat transformation, etc. (APPOLONIO *et al.*, 2010; MASSEI and GENOV, 2000; LINNEL and ZACHOS, 2011). After the registered decline, during the last several decades, the recovery of wild boar populations across their range and remarkable increase in population density were detected (FONSECA, 2004; FERREIRA *et al.*, 2009; APPOLONIO *et al.*, 2010; SCANDURA *et al.*, 2011). The increase of wild boar populations has been registered in hunting areas in the Balkans as well (JERINA, 2006; TONCIC *et al.*, 2006; UROSEVIC *et al.*, 2011). This increase caused severe impact on agroecosystems and forestry and led to addressing the wild boar as a pest species for which the management strategy is highly needed (ĐORĐEVIĆ *et al.*, 2010).

Due to the all named factors, accompanied by the developments of molecular genetic tools for wildlife management and conservation, wild boar populations in Europe and worldwide have become in a scope of interest in different studies (FERREIRA *et al.*, 2006, 2009; SCANDURA *et al.*, 2008, 2011; ALEXANDRI *et al.*, 2012; ALVES *et al.*, 2003, 2010), dealing with the investigations of genetic diversity and phylogeny of this game species. The large fraction of pre-glacial genetic diversity in wild boar populations from the Balkans was addressed due to high proportion of unique mtDNA haplotypes found in Greece, indicating Balkan as main refugial area for wild boars (ALEXANDRI *et al.*, 2012). The genetic diversity of wild boar populations from the other parts of the Balkans was inferred from microsatellite analyses. In wild boar populations from Bulgaria high level of genetic diversity and different genetic clusters were found (NIKOLOV *et al.*, 2009), and same was proved for several wild boar populations from Serbia, Croatia and Bosnia (VELIČKOVIĆ *et al.*, 2010, 2012).

The aim of the present study is to characterize mitochondrial DNA control region variability in wild boars from different areas in the West Balkan region (Slovenia, Croatia,

Bosnia and Herzegovina, Serbia, Montenegro and Macedonia), with main goals to evaluate level of genetic variability, to detect unique haplotypes and to infer possible structuring of large continuous wild boar population.

MATERIALS AND METHODS

Material

The sampling area in this study was located in the West Balkan region (Slovenia, Croatia, Bosnia and Herzegovina, Serbia, Montenegro and Macedonia) (Figure 1). Muscle tissue samples of 163 wild boars from different sampling localities were analyzed (Figure 1). Tissue samples were collected during regular hunts and stored at -20°C prior to the analyses.

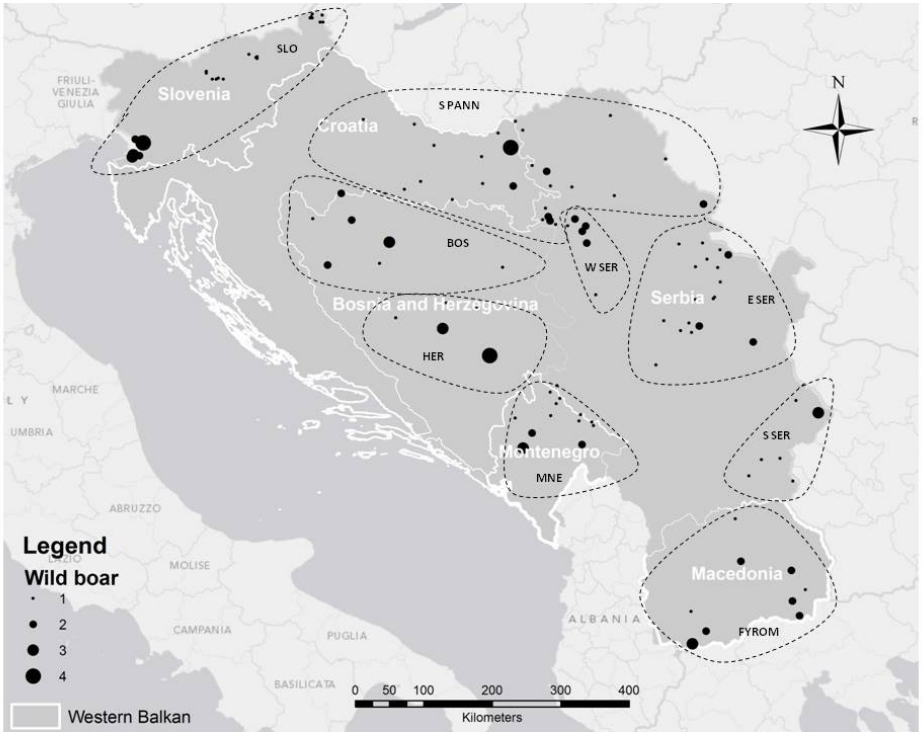

Figure 1. Location map of the wild boar sampling areas. For acronyms of localities groups in block letters see section Statistical analysis in Material and Methods. Circle sizes indicate number of samples per locality.

DNA amplification and sequencing

Total DNA from each muscle sample was extracted by phenol chlorophorm extraction (SAMBROOK and RUSSEL, 2001). A fragment of the mtDNA control region, between sites 15378 to 15900 (according to reference sequence AJ002189), was amplified with primers: 5′GGAGACTAACTCCGCCATCA3′ and 5′ TGGGCGATTTTAGGTGAGATGGT 3′, forward and reverse, respectively. PCR reactions were performed in total volume of 20µl containing 50ng of DNA, 1x Reaction Buffer (Fermentas), 2.5mM $MgCl_2$, 100µM of each dNTP, 0.10µM of each primer and 1U Taq DNA Polymerase (Fermentas). Touchdown PCR was performed with conditions as follows: 94°C for 4 minutes, followed by 20 cycles 94°C for 30 s, 65-55°C for 30 s, and 72°C for 30 s, followed by 30 cycles 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 minutes. The PCR products were purified using ExoSAP (Fermentas) following the manufacturers recommendations. Sequencing was conducted on an ABI3730xl genetic analyzer.

Statistical analysis

Obtained sequences were aligned using the Clustal W algorithm implemented in BioEdit (HALL, 1999), and final adjustments were done by eye.

Basic parameters of genetic diversity: haplotype (h) and nucleotide (π) diversity, mean number of pairwise differences (k) and theta estimator S (θs) were estimated using ARLEQUIN 3.5.1.2. (EXCOFFIER and LISCHER, 2010). All samples were pooled in nine groups based on geographic criterion (Figure 1). The factors for pooling the samples from close localities into the same geographically determined group were absence of obvious artificial and natural barriers, wide rivers and high mountains among localities and biogeographical characteristics of the area. Following these criteria individual sequences were divided in nine groups and named according to the geographic region: South Pannonian (S PANN); West Serbia (W SER); East Serbia (E SER); South Serbia (S SER); Former Yugoslav Republic of Macedonia (FYRM); Montenegro (MNE); Herzegovina (HER); Bosnia (BOS) and Slovenia (SLO) (abberviation for each group is given in parantheses).

Population genetic structure among and within analyzed groups was estimated by an analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.5.1.2. (EXCOFFIER and LISCHER, 2010). In order to explore the demographic history of wild boar population from the West Balkan we followed two approaches. We analyzed the mismatch distribution and the sum of the squared deviations (SSD) was computed to test goodness-of-fit of the observed mismatch distribution to that expected under sudden population expansion model as implemented in ARLEQUIN 3.5.1.2. (EXCOFFIER and LISCHER, 2010). Next, we run Fu's Fs and Tajima's D neutrality tests of the total number of segregating sites, both were performed in ARLEQUIN 3.5.1.2. program (EXCOFFIER and LISCHER, 2010) and were applied for each geographical area.
The geneological relationships among determined mtDNA control region haplotypes of wild boars from the West Balkan region were inferred by median-joining (MJ) network (BANDELT *et al.*, 1999) calculated in the software Network 4.6.0.0 (available at http://www.fluxus-engineering.com/sharenet.htm), using equal weights for all the mutations and setting parameter to zero in order to restrict the choice of feasible links in final network. The neighbour-joining tree clustering nine wild boar groups was constructed in MEGA5 (TAMURA *et al.*, 2011).

RESULTS

In total, 11 different haplotypes were identified by 9 variable sites (Table 1). Haplotype diversity (h) for the presented Balkan wild boars was 0.676, nucleotide diversity (π) 0.0026, and the average number of nucleotide differences (k) was 1.169. Indel positions and missing data were excluded from all further analyses.

Table 1. Control region mtDNA haplotypes in wild boars from the Balkans with associated variable positions. N – number of individuals sharing the same haplotype. % - percentage of total number of haplotypes. Group refers to geographical region from which the individuals of cetrain haplotypes were sampled. List of regions with associated designation number is given in the Material Section and footnote of the Table 1.

haplotypes	Variable positions									N	%	Group
	124	182	241	280	283	307	324	388	406			
H1	T	C	C	C	C	C	C	A	T	1	0.006	1
H2	A	T	T	.	.	.	.	.	.	85	0.521	1-5; 7-9
H3	.	.	T	.	.	.	T	.	.	10	0.061	7-9
H4	A	T	T	.	.	.	T	.	.	34	0.208	1; 4-8
H5	.	.	T	.	.	.	.	.	.	14	0.087	1,2,8
H6	.	T	T	.	.	.	.	.	.	11	0.068	1,5,9
H7	.	T	T	.	.	.	.	.	C	1	0.006	3
H8	.	T	T	.	.	T	.	.	.	1	0.006	8
H9	A	T	T	T	.	.	T	.	.	4	0.025	9
H10	.	.	T	.	.	.	.	G	.	1	0.006	4
H11	A	T	T	.	T	.	.	.	.	1	0.006	3

1 – South Pannonian (S PANN); 2 – West Serbia (W SER); 3 – East Serbia (E SER); 4 – South Serbia (S SER); 5 – former Yugoslav Republic of Macedonia (FYRM); 6 – Montenegro (MNE); 7 – Herzegovina (HER); 8 – Bosnia (BOS); 9 – Slovenia (SLO)

Basic molecular diversity indices were calculated for each group (Table 2). The mismatch distribution didn't show a statistically significant deviation from expectations, indicating the expansion of the all populations. Fu's Fs test also didn't show statistically significant positive or negative Fs values, indicating that there is no recent expansion or recent population bottleneck.

The AMOVA analysis showed significant genetic differentiation within groups (75.90%), while precentage of variation among groups was 24.10% (Table 3).

The neighbor-joining tree was constructed based on pairwise fixation indices (Fst) among groups (Figure 2).

Table 2. Basic molecular diversity indices per each wild boar group, mismatch distibution and neutrality tests results

Population	SPANN	W SER	E SER	S SER	FYRM	MONT	HER	BOS	SLO
Number of individuals	38	6	20	7	16	17	12	10	37
Molecular diversity indices									
Number of haplotypes	5	2	4	3	3	1	3	5	4
Ti/Tv ratio	3	1	3	3	1	0	2	3	3
Haplotype diversity (h)	0.6543	0.333	0.284	0.524	0.425	0.00	0.591	0.844	0.652
Nucleotide diversity (π)	0.0028	0.0016	0.00097	0.0028	0.0011	0.00	0.002	0.0046	0.0035
Pairwise differences (k)	1.297	0.759	0.441	1.289	0.487	0.00	0.912	2.12	1.577
θs	0.952	0.876	1.127	1.633	0.603	0.00	0.993	1.414	0.958
Mismatch analysis									
Sum of squared deviations SSD	0.049	0.071	0.00042	0.0388	0.011	/	0.0066	0.0295	0.013
p	0.21	0.21	0.66	0.50	0.16	/	0.54	0.17	0.42
Neutrality tests									
Fs	0.966	1.609	-2.074	0.263	-0.571	/	0.289	-0.577	1.439
p	0.736	0.725	0.01*	0.505	0.264	/	0.479	0.312	0.80
D	0.541	-1.132	-1.867	-1.434	-0.649	0.00	-0.579	1.109	0.998
p	0.738	0.13	0.017*	0.06	0.242	1.00	0.28	0.854	0.839

Table 3. Analysis of molecular variance among and within wild boar groups

Source of variation	Total variance (%)	Fixation index (Fst)
Among groups	24.10	0.241*
Within groups	75.90	

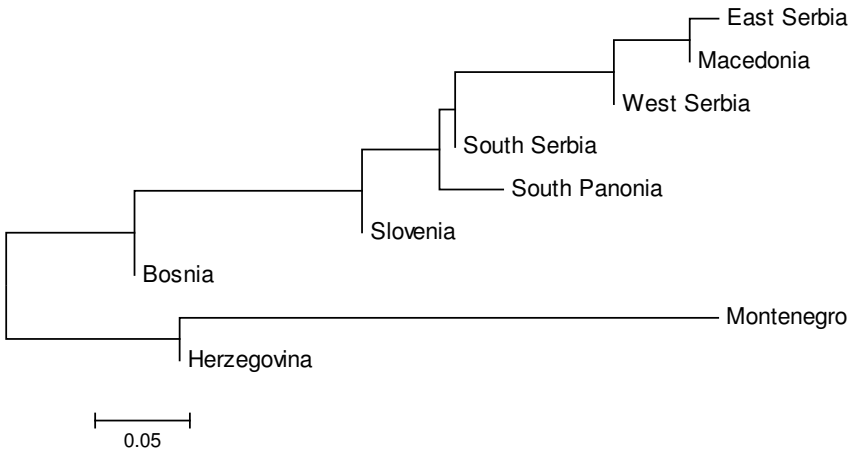

Figure 2. Neighbour-joining tree clustering nine wild boar groups. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

In order to overview haplotype distribution and relationships in wild boars from the Balkan, median-joining network has been constructed (Figure 3).

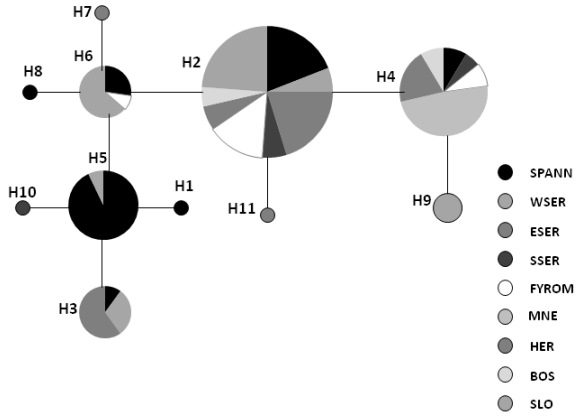

Figure 3. Median-joining network of 11 determined mtDNA control region haplotypes. Circle sizes correspond to the frequencies of the haplotypes. Pie charts in each circle indicate frequency of haplotype in wild boar groups. The number of mutational steps is one between each pair of haplotypes.

DISCUSSION

Mitochondrial DNA regions (COI, cytb and rRNAs) were oftenly used in the population genetics and phylogenetics studies of different game species, hares (KASAPIDIS *et al*., 2005; SERT *et al.*, 2009; STAMATIS *et al.*, 2009), red dear (ZACHOS and HARTL, 2011), roe dear (HAMMER *et al.*, 2008) and wild boars (FERREIRA *et al.*, 2006, 2009; SCANDURA *et al.*, 2008, 2011; ALEXANDRI *et al.*, 2012; ALVES *et al.*, 2003, 2012). Control region of mtDNA was proved to be adequate molecular marker in estimations of phylogeny, phylogeography and processess of introgression and hybridization (MELO-FERREIRA *et al.*, 2011a, 2011b), but also as an informative marker for the determination of genetic diversity and population structure (KASAPIDIS *et al*., 2005; BEN SLIMEN *et al.*, 2008; SERT *et al.*, 2009). The selected part of the control region in this study proved to be informative enough for the determination of genetic diversity in wild boar population and revealed high level of genetic variability in wild boars from the West Balkan region, eventhough the genetic diversity indices and number of unique haplotypes were lower comparing to wild boar population from Greece (ALEXANDRI *et al.*, 2012). It seems that at a regional scale, a general trend exist in Europe, and on the Balkans as well, indicating decrease of genetic variability in south-north direction. NIKOLOV *et al.* (2009) identified two main subpopulations in Bulgaria, one north and one south of the Thracian valley, indicating the later as population with higher genetic diversity. The Thrace has been indicated as the region with the highest levels of genetic diversity in other species, e.g. brown hares (KASAPIDIS *et al*.,2005; STAMATIS *et al.*, 2009), suggesting that a south-north gradient of genetic variation exists on the Balkans. This pattern of genetic variation might be consequence of past expansion from the source region of recolonization in postglacialization period. Our data support the existence of high genetic diversity in the West Balkan region as well, and together with five regional unique haplotypes might indicate that the region of high genetic diversity is extended to larger geographic area on the Balkans. The latest conclusion is speculative and in order to define delineation of subpopulations in the region, more molecular markers should be employed including nuclear genetic markers.

Basic molecular diversity indices in each wild boar group from the West Balkan region and significantly lower precentage of variation among groups compared to within groups suggest shallow differentiation and existing gene flow among groups. This was also supported by pairwise Fst values (not shown) and Neighbor –joining dendogram constructed, which shows clustering of two groups, Montenegro and Herzegovina into one clade, while all other groups collapsed into second clade. Moreover, surprisingly high level of homogeneity was observed in the Montenegro group. A possible bias introduced by the single marker used may have affected this result and thus prevent further speculations. Since it is known that even in the presence of continuous habitats, wild boar populations are seldom homogenous and mostly split into genetically differentiated subpopulations (SCANDURA *et al.,* 2011), it is indicated by mtDNA control region sequence variability in this study that genetic structuring might exist in the region, but as mentioned before, further nuclear gene markers must be explored. The mismatch distribution analyses and neutrality tests performed for each group, indicated past population expansion and no recent event of expansion or bottleneck. It seems that selected mtDNA control region has a signature of post-glacial demographic expansion, which has been proved by other phylogenetic studies of wild boars (FERREIRA *et al.*, 2006, 2009; SCANDURA *et al.*, 2008, 2011; ALEXANDRI *et al.*, 2012; ALVES *et al.*, 2003, 2012). This is also supported by network analysis, in

which the star-like distribution with a single central and very frequent haplotype may reflect ancient expansion.

The present study gives first overview of the genetic variability of the mitochondrial DNA control region of wild boars from different areas in the West Balkan region. It is shown that high level of genetic diversity is present in the wild boars from the West Balkan region and we have managed to detect regional unique haplotypes in high frequency. The obtained data can be relevant for improving knowledge of the phylogeny and phylogeography of the wild boar. Genetic diversity differences have been found in regional wild boar groups, clustering them in two main clusters, but further speculations on the reasons for the observed clustering are prevented due to restricted informativness of the single locus marker. Obtained knowledge of genetic variation in the wild boar may be relevant to hunting societies and responsible authorities for the effective control of wild boar populations and avoiding mistakes in population management.

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VARIJABILNOST KONTROLNOG REGIONA MITOHONDRIJALNE DNK DIVLJIH SVINJA ZAPADNOG BALKANA

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Izvod

Divlja svinja (*Sus scrofa*) je jedna od najbrojnijih vrsta divljači u lovnim područjima Balkana. Na osnovu velikog broja jedinstvenih haplotipova u populacijama divljih svinja Grčke, zaključeno je da se veliki deo pre-glacijalnog genetičkog diverziteta ovih populacija zadržao na Balkanskom poluostrvu, koje je označeno kao glavni refugijum za divlje svinje. Cilj ovog rada je karakterizacija varijabilnosti kontrolnog regiona mitohondrijalne DNK kod divljih svinja sa različitih lokaliteta Zapadnog Balkana, u svrhu utvrđivanja genetičke varijabilnosti, detekcije jedinstvenih haplotipova i definisanja moguće struktuiranosti populacije. Ukupno su analizirane 163 jedinke sa različitih lokaliteta. Deo kontrolnog regiona mtDNK je umnožen i sekvenciran standardnim metodama. Populaciono-genetička analiza urađena je pomoću programskih paketa za analizu sekvenci: BioEdit, ARLEQUIN 3.5.1.2., Network 4.6.0.0 and MEGA5. Nađeno je jedanaest haplotipova i diverzitet haplotipova je iznosio 0.676, diverzitet nukleotida 0.0026, dok je prosečan broj nukleotidnih razlika bio 1.169. Test unimodalne distribucije i testovi neutralnosti pokazali su ekspanziju svih populacija. Pokazan je visok nivo genetičkog diverziteta u populacijama divljih svinja Zapadnog Balkana i detektovani su haplotipovi jedinstveni za regione u visokoj frekvenciji. Razlike u genetičkom diverzitetu pokazane su u regionalnim grupama, i klaster analiza je pokazala prisustvo dve odvojene grane, ali konačan zaključak o stepenu struktuiranosti nije mogao biti donet, zbog ograničene informativnosti jednog genskog markera. Dobijeni rezultati o genetičkoj varijabilnosti divljih svinja relevantni su za rekonstrukciju filogenije i filogeografije ove vrste divljači, ali i za organizacije odgovorne za efektivnu kontrolu populacija divljih svinja.

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