

Analysis of Genetic Diversity of Red Deer (*Cervus elaphus* L.) in Bulgaria: Implications for Population Conservation and Sustainable Management

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Abstract: The red deer (*Cervus elaphus* L.) is the most important big game species in Bulgaria with marked habitat fragmentation. The species is known to be strongly anthropogenic influenced by recent human activities in the country such as selective hunting for trophies. To estimate the haplotype diversity of red deer from Northeast Bulgaria, a complete sequences of cytochrome *b* (1140 bp) was analyzed. 36 haplotypes were revealed in the investigated populations; thus much at least is clear the existence at least two different genetic lines of red deer in Bulgaria. The carried out comprehensive description of genetic structure of Bulgarian red deer's populations and comparative analysis of their phylogenetic relationships among some other subspecies of *Cervus elaphus* in Europe does not exhibit remarkable loss of genetic diversity of the deer in Bulgaria. These results are discussed in respect to population conservation, sustainable management and prevention of loss of genetic diversity of red deer in Bulgaria as well as to assess the effects of human activities indicating human impact on population structure most likely resulting from selective hunting for trophies.

Key words: *Cervus elaphus*, phylogeny, cytochrome *b*, Bulgaria, red deer, genetic diversity.

Introduction

Optimal economic activity regarding game mammals should be consistent not only with temporary economic benefit, but should also provide unlimited existence of the natural populations of resource species. Exploiting animal natural resources without view of population genetic structure of the species and historically developed regulation mechanisms allowing the populations to reproduce steady in an unlimited number of generations, leads to commonly observe adverse effects associated with abrupt reduction of their genetic diversity. Thus, studying the genetic diversity of economically valuable mammals' species in order to prevent such adverse effects becomes one of the most important tasks of modern biology for conservation of biological resources and their rational use. The rational economic activity should pro-

vide continued preservation of biological resources and conservation of genetic, and therefore, biological stability of species. Besides its purely scientific importance, the assessment of the genetic diversity uniqueness of the natural populations, each of which had been formed during a long process of selection of better adapted phenotype, has definite practical value for the management of hunting. It would help to intensify the hunting through scientifically sound influence on the populations' structure, selection, conservation and improvement of their genetic resources.

In Bulgaria, environmental and geomorphological conditions generally provide a favourable situation for the development of natural populations of red deer (*Cervus elaphus* L., 1758). Strong decline in its numbers was observed in 1915-1918, when

very few animals have remained in separated local populations. The re-acclimatization of animals, bred in farms, has contributed to the restoration of red deer's number. Its stocks have begun to increase intensively in 1950's, when there were only 1430 animals in 1952, and reached 28 370 animals in 1992. In the recent years, the species population numbers varies between 18 000 and 21 000 animals because of many complex reasons – damages from predators, severe winters, poaching, etc. Due to the appropriate ecological conditions and proper management populations with very good trophy qualities have evolved - males have mighty, beautiful and symmetric crowns, dark richly pearly beams with long evenly spaced antlers and white tines.

All the trophies of Bulgarian red deer with world records were obtained in Northeastern Bulgaria. There are 4 world records of antlers, established from Bulgarian populations of red deer. The first trophy with world record was rated 253 points (CIC) in the International Exhibition in České Budějovice in 1976; the second one received 256.78 CIC points in 1980 during the International Exhibition in Nitra; the third one was shown at the International Exhibition in Brno in 1985 and was rated 263.28 CIC points; and the fourth trophy, presented in Nitra in 1990 received 273.60 points. Bulgaria has 15 trophies with a grade over 250 points (CIC) and ten of top twenty world trophies are Bulgarian. During 2011-2012 hunting season, some of the best red deer trophies from the country again were obtained within the range of Regional Directorate of Forestry in Shumen, Northeastern Bulgaria and won gold medals. The first specimen has a grade of 231.82 point by CIC and weight of 11.320 kg. The second and the third one had 228.03 by CIC and 10.940 kg weight and 227.22 points by CIC and 10.445 kg weight respectively. A marked interest has arisen in shooting through the hunting tourism elite males from this population, which has pronounced good trophy status.

At the same time, the lack of scientific results about the genetic diversity of the red deer population from this region, which is influenced by the selective shooting of males with good trophies, determined the necessity of the present analysis of genetic diversity of the red deer (*Cervus elaphus* L.) in Northeastern Bulgaria with a view to conservation and sustainable management of this population, elimination of risk of loss of genetic diversity and deterioration of red deer biological diversity.

Materials and Methods

The genetic diversity of the Bulgarian red deer has been assessed on a sample of 35 individuals from the territory of Bulgaria. All the studied animals were adult males (between 7 and 12 years old) with very good trophy antlers. The trophy indices of the antlers have been assessed according to CIC measurement features (SCORE SHEET FOR RED DEER, 2004). The age of the animals has been established on the grounds of the development of the tooth system (BROWN & CHAPMAN, 1991). The samples for biochemical-genetic analysis have been collected during the regular shooting of red deer through the hunting tourism.

31 of the studied specimens have been obtained from lowland red deer mega-population from Shumen district in the north-eastern region of the country. The mega-population inhabiting the territory of Shumen district has been assessed as one with high trophy value and was exploited intensively. The physico-geographic characteristic of this region is rather diverse. Its topography changes from lowlands, hills and plateaus in the northern part to mountainous relief in the southern part, where it reaches the watershed of the Balkan Mountains. The altitude increases from north to south, the lowest point being situated at 50 m and the highest – at 1046 m above the sea level. Climatically the land belongs to the temperate continental subzone, where the winter is dry and the summer is hot. The average temperature throughout the year is 12.1 °C (the very minimum ever recorded is minus 32, 4 °C in January and the very maximum is +43.2°C in June). The average annual rainfall is about 580 mm. The varied natural and climatic conditions favour the growth and dissemination of over 250 kinds of trees and bushes, which from plantations with a great variety of composition, age, structure and condition. The biggest share in the timberland has the moss-capped oak (21.4%), followed by the oak (14.1%), the hornbeam (13.7%), the eastern beech (9.7%) and the lime-tree (5.3%). The Scotch fir and the black pine, which form 9.6% of the timberland, have been artificially introduced through forestation together with the common locust (5.1%) and the red oak (2.5%) (ARFD, 2012).

Samples from 22 red deer have been taken for analysis from sub-population (POP1-H1) inhabiting a total area of 130 000 ha in the western part of this lowland region of Shumen district. Forests cover about 28 000 ha of this habitat. The agricul-

tural fields with maize and other crops cover a high percentage of the land as well. The terrain is rolling. The elevation is from 125 m to 486 m.

The eastern part of the lowland region of Shumen district is characterized by pronounced hilly landscape and greater presence of forest vegetation; 9 specimens from sub-population (POP1-H2) inhabiting this area have been studied.

Another 6 specimens have been obtained from mountain and semi mountain populations in the country: 5 specimens from the Balkan Mountains, on the northern slopes of Central Balkan range (POP2) which are covered with centuries-old beech forests and succulent mountainous meadows, cut by valleys and gullies and the relief is typically mountainous to alpine. The altitude varies from 350 to 1850 m. The last 1 specimen have been taken from population from Middle Rhodope Mountains (POP3); it inhabits a region situated is from 380 m to 2000 m typical mountainous terrain, rugged and uneven in some parts. Deciduous and coniferous forests cover 95% of the whole territory (Fig. 1).

Thus, the main population groups of the Bulgarian red deer, differing by their phenotype characteristics and by the ecological conditions of their habitats (MARKOV 1998) have been included in the analysis.

To assess the phylogenetic relationships of the Bulgarian red deer to the some presumable subspecies of *Cervus elaphus* in the South-eastern part of its range, 35 sequences of specimens from zoo-geographically and taxonomically typical populations from its area (10 sequences were extracted from GenBank) have also been included in the analysis (Table 1).

In all, 73 complete cytochrome *b* sequences (1140 bp) were included in the analysis. DNA was extracted using a Diatom®DNA Prep reagent kit (Russia). PCR was conducted using standard primers for complete sequence of cytochrome *b* (KOCHER *et al.* 1989).

DNA sequences were identified using an ABI PRISM® BigDye™ reagent kit Terminator v. 3.1 on an ABI PRISM 3100Avant automatic DNA sequencer (USA).

All the sequences were aligned manually using the BioEdit program (HALL 1999). Nucleotide diversity and distance between haplogroups were revealed using MEGA 5.0 (LEMMON, MILINKOVITCH 2002). Median network of haplotypes was built in the Network 4.111 program (BANDELT *et al.* 1999).

Mean distance within established haplogroups and mean distance between them were calculated using MEGA 5.0 program (KUMAR *et al.* 2008).

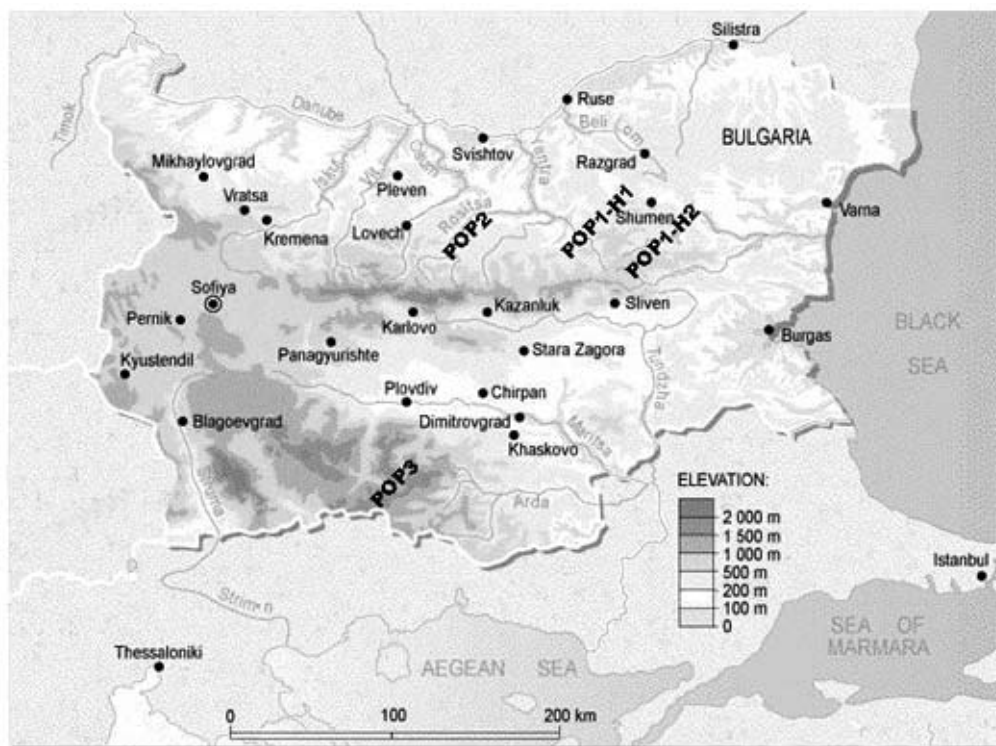


Fig. 1. Geographic location of collection sites for red deer (*Cervus elaphus* L.) in Bulgaria. Population's codes and numbers of investigated individuals are shown in Table 1.

Table 1. Sampling sites of the studied red deer and population's haplotype structure.

Sample number	Region	Population	Haplotype
Red deer from Bulgaria			
1	lowland north-eastern BG	POP1-H1	A2
2	lowland north-eastern BG	POP1-H1	unique
3	lowland north-eastern BG	POP1-H1	B2
4	lowland north-eastern BG	POP1-H1	A2
5	lowland north-eastern BG	POP1-H1	B2
6	lowland north-eastern BG	POP1-H1	A unique
7	lowland north-eastern BG	POP1-H1	A unique
8	lowland north-eastern BG	POP1-H1	B1
9	lowland north-eastern BG	POP1-H1	B1
10	lowland north-eastern BG	POP1-H1	B1
11	lowland north-eastern BG	POP1-H1	B1
12	lowland north-eastern BG	POP1-H1	A unique
13	lowland north-eastern BG	POP1-H1	B2
14	lowland north-eastern BG	POP1-H1	A unique
15	lowland north-eastern BG	POP1-H1	A unique
16	lowland north-eastern BG	POP1-H1	B1
17	lowland north-eastern BG	POP1-H1	B1
18	lowland north-eastern BG	POP1-H1	A1
19	lowland north-eastern BG	POP1-H1	A1
20	lowland north-eastern BG	POP1-H1	B2
21	lowland north-eastern BG	POP1-H1	A1
22	lowland north-eastern BG	POP1-H1	B1
23	lowland north-eastern BG	POP1-H2	unique
24	lowland north-eastern BG	POP1-H2	B3
25	lowland north-eastern BG	POP1-H2	B1
26	lowland north-eastern BG	POP1-H2	B 3
27	lowland north-eastern BG	POP1-H2	B1
28	lowland north-eastern BG	POP1-H2	A unique
29	lowland north-eastern BG	POP1-H2	B1
30	lowland north-eastern BG	POP1-H2	B unique
31	lowland north-eastern BG	POP1-H2	B unique
32	semi mountainous central BG	POP2	unique
33	semi mountainous central BG	POP2	B unique
34	semi mountainous central BG	POP2	B unique
35	semi mountainous central BG	POP2	A unique
36	semi mountainous central BG	POP2	B unique
37	mountainous south-western BG	POP3	A unique
Outgroup samples			
38	Belarus		C3
39	Belarus		C3
40	Russia, Northern Caucasus, North Ossetia		unique
41	Russia, Northern Caucasus, Adygeya Republic		unique
42	Russia, North Caucasus, Karachay–Cherkess Republic		unique
43	Russia, Belgorod region		Belg1
44	Russia, Belgorod region		Belg1
45	Ukraine, Crimea peninsula		C1
46	Ukraine, Carpatian mountains		B1
47	Ukraine, Crimea peninsula		C1
48	Ukraine, Crimea peninsula		C1

Table 1. Continued.

Sample number	Region	Population	Haplotype
49	Ukraine, Crimea peninsula		C1
50	Russia, Kaliningrad region		C2
51	Russia, Kaliningrad region		C2
52	Russia, Kaliningrad region		C2
53	Russia, Kaliningrad region		C2
54	Ukraine, the coast of Azov sea		B1
55	Ukraine, Crimea peninsula		C1
56	Belarus		unique
57	Belarus		C1
58	Belarus		C2
59	Belarus		unique
60	Ukraine, Birjuch island		unique
61	Ukraine, Birjuch island		unique
62	Northern Caucasus, Dagestan Republic		unique
Sequences from GenBank			
(63) AY044857	Austria		B1
(64) AY118197	Turkey		B2
(65) AY044858	Germany		C2
(66) AY044860	Poland		C2
(67) AY044859	Spain		C2
(68) AY070226	Norway		C2
(69) AB021099	Scotland		C2
(70) AY244489	Sardinia island		A unique
(71) AY070222	Tunisia		A unique
(73) AY118198	Tunisia		A unique

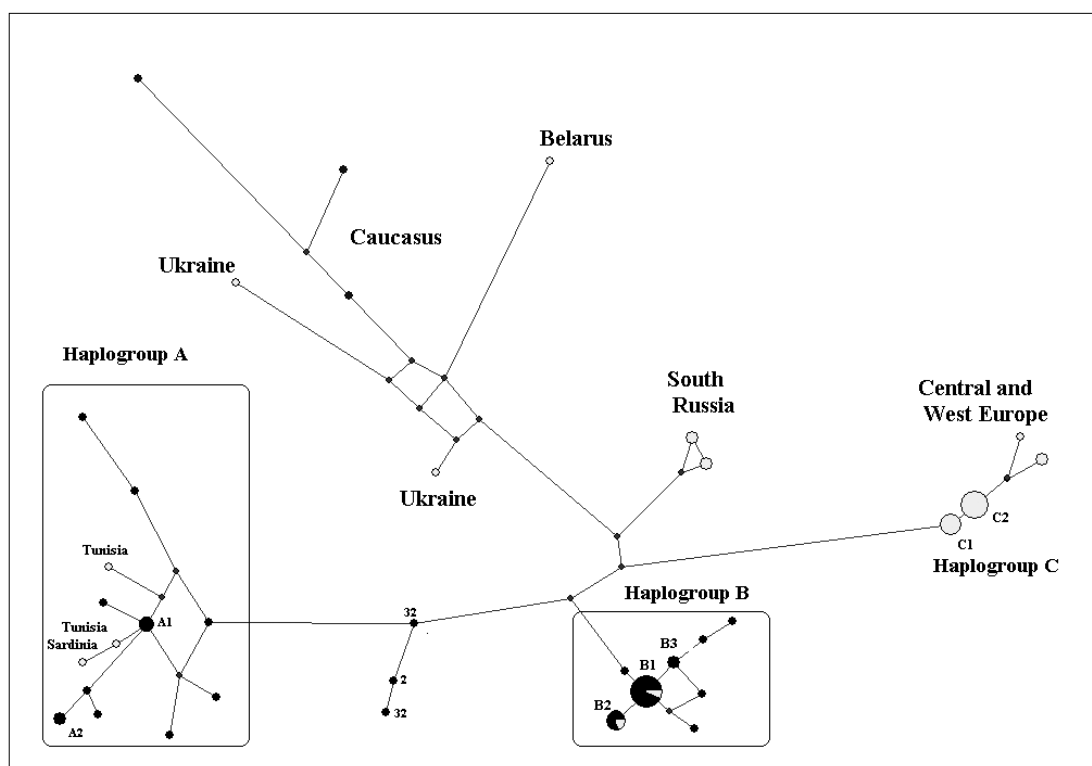


Fig. 2. Haplotype networks for red deer groups A–C. Haplotypes and associated specimens are shown in Table 1 and geographically sites of the Bulgarian populations are depicted in Fig. 1.

Results

The investigated data set encompasses 73 cytochrome *b* sequences. Totally 57 (5%) parsimony-informative sites have been identified. Nucleotide diversity of the complete set was 1.4% (according to p-distance model). Estimated number of haplotypes in the analyzed cohort was 36. The network of haplotypes was derived taking in account 179 mutations.

The reconstructed dendrogram illustrating phylogenetic relationships of Bulgarian red deer and the sequences of some other specimens of red deer from zoo-geographically and taxonomically typical populations from its area is shown in Fig. 2.

Two main groups of haplotypes were detected in Bulgarian red deer. The first group of haplotypes (haplogroup A) includes 13 haplotypes (10 characterizing Bulgarian red deer and 3 from GenBank, characterizing Tunisian and Sardinian red deer). The haplotype (A1) from this group is attributed to 3 samples, and another haplotype from this group (A2) is attributed to 2 samples all of which have Bulgarian origin and belong to individuals from POP1-H1. The other 8 unique haplotypes from this group were distributed as follows: 5 haplotypes belong to individuals from POP1-H1 and well as one each of the individuals from POP1-H2, POP2 and POP3. The haplotype belonging to the specimen from POP3 occupies an extremely distant position. This may be an evidence of existence of a different group of haplotypes in Bulgaria. Haplogroup A contains also two samples from Sardinia Island (*C. e. corsicanus*) and one from Tunisia (*C. e. barbarus*).

The second group (haplogroup B) includes 8 haplotypes of Bulgarian red deer. It combines specimens from three of the four studied red deer habitats in Bulgaria – POP1-H1, POP1-H2 and POP2. Haplotype B1 encompasses 7 samples from POP1-H1 and 2 samples from POP1-H2; haplotype B2 encompasses 3 samples from POP1-H1 and 2 samples from POP1-H2; haplotype B3 encompasses 1 sample from POP1-H1 and 3 samples from POP1-H2; the other 5 unique haplotypes of Bulgarian red deer encompass 2 samples from POP1-H2 and 3 samples from POP2.

Beyond the deer from Bulgaria, haplogroup B includes 4 samples found in Turkey, Ukraine (Carpathian Mountains and Azov sea coast), and Austria. These samples have the identical haplotypes with a majority of Bulgarian samples belonging to this haplogroup.

Three samples from Bulgaria, one of POP1-H1, one of POP1-H2 and one of POP2, each with unique haplotype, form a small haplogroup, occupying an intermediate position between haplogroups A and B. Most likely, the presence of this unique haplotype in populations of Bulgarian red deer can be seen as evidence of presence of another group of haplotypes of red deer in Bulgaria.

The evaluation of genetic diversity of the studied red deer groups from Bulgaria expressed through the number of haplotypes in each one of them has shown that:

1) POP1-H1 sub-population consists of individuals characterized by 10 haplotypes or haplotype diversity there is 0.454 per studied specimen; POP1-H2 sub-population consists of individuals characterized by 6 haplotypes or its haplotype diversity is 0.667 per specimen studied; on the whole, the megapopulation from the lowland region in Shumen district consists of specimens characterized by 15 haplotypes, only one of them being common for both sub-populations or its haplotype diversity is 0.484 per studied specimens, which is very close to the diversity of POP1-H1 sub-population.

2) POP2 population from semi-mountainous central region consists of specimens characterized by 5 unique distinguished haplotypes or haplotype diversity in this population is 1 per studied specimen; none of these 5 haplotypes has been found in the other studied Bulgarian specimens.

3) in the population POP3 inhabiting mountainous Southwestern region is found a specific only for it haplotype.

4) The high haplotype diversity of red deer from Bulgaria (21 unique distinguished haplotypes in 31 samples) determine and relatively high value – 0.677 per studied specimen for all the country.

These results revealed the relatively high at the moment value of the genetic diversity of the red deer sub-populations in Southeastern Bulgaria intensively used in hunting, as well as its specific manifestation in Bulgarian red deer, differing by their phenotype characteristics and ecological conditions of their habitats. They are consistent with previous results of biochemical-genetic investigations of genetic variation in local populations of the country which have revealed very high levels of polymorphism and heterozygosity as compared to other European populations (HARTL *et al.* 1993 a; HARTL *et al.* 1995) and high population genetic diversity of species in the country (HARTL *et al.* 1993 b; CHASSOVNIKAROVA *et al.* 1993; MARKOV 1998).

The third main group in reconstructed network of haplotypes in the red deer from Europe (haplogroup C) includes 4 haplotypes. No samples from Bulgaria belong to this group. Two main haplotypes include 6 and 10 individuals respectively, another two are unique. First haplotype (C1) from this group belong to the red deer from Crimean peninsula (Ukraine) and Belarus. Samples belonging to the second one (C2) come from different parts of Europe (Russia – west part, Belarus, Poland, Spain, Scotland, and Norway). Another two haplotypes belong to deer from Belarus.

All other samples, which were included in the network of haplotypes of the studied red deer from Eurasia, formed an additional monophyletic cluster (upper part of the network) including a number of long branches. The first branch consists of samples from Southern Russia (Belgorod region as well as two Caucasian localities, Adygei and Dagestan).

Red deer inhabiting other parts of its European range forms a separate haplogroup, which is distantly positioned towards the two above mentioned Bulgarian genetic lines (Fig. 2).

Thus, we revealed at least two separate genetic lines in the red deer population inhabiting Bulgaria. One of these lines is most similar to the red deer inhabiting central and western Europe (*C. e. carpathicus* and *C.e. hipelaphus*), whereas the second line is closely related to red deer inhabiting the Mediterranean Islands (*C. e. corsicanus*) and northern Africa (*C. e. barbarus*).

The evaluation of diversity in the main 3 haplogroups of the studied red deer populations (Fig. 3) have revealed that: the haplotype diversity assessed through the number of haplotypes per studied specimen was the highest in the haplogroup A and the lowest in the haplogroup C (Fig. 3a); a nucleotide diversity and mean distance within groups were the highest in the haplogroup C and lowest in the haplogroup B (Fig. 3b,c). Between the two main haplogroups (A and B) containing all the haplotypes found in the Bulgarian red deer, the haplogroup A had relatively higher number of haplotypes per studied specimen, nucleotide diversity and mean distance within groups.

The distances established between the obtained 3 main haplogroups (Fig. 3d) have revealed that: A

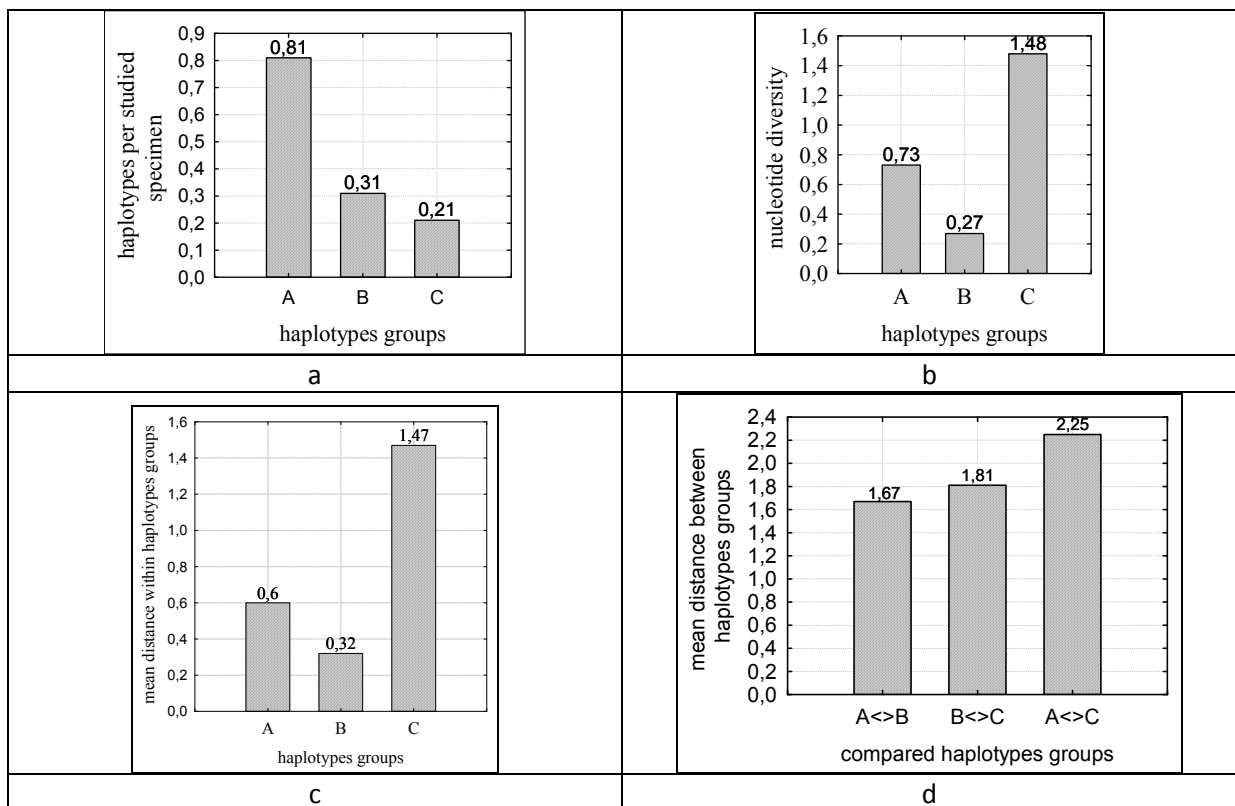


Fig. 3. The haplotypes (a) and nucleotide diversity (b) and mean distance within (c) and between groups (d) of the established 3 main haplotypes groups of the studied red deer. The haplotypes revealed in them and the geographic localization of investigated individuals are shown in Table 1.

and B haplogroups, which included Bulgarian red deer, showed the highest similarity; The haplogroup B, in which red deer from all the studied populations in the country have been included, showed relatively higher similarity with the haplogroup C (red deer from Central and Western Europe) than haplogroup A.

The integrative evaluation of the results about the diversity of Bulgarian red deer cytochrome *b* haplotypes suggested that unique distinguished haplotypes have been retained in the Bulgarian populations and despite the increased anthropogenic pressure on the lowland red deer mega-population from Shumen District expressed through intensive trophy hunting, it has retained to a great extent its natural haplotype diversity and the most diverse genetic structure in the country.

Discussion

Genetic resources of red deer are essential for the development of hunting business. The overexploitation of the populations, among the other main factors causing directly the loss of biodiversity, such as habitat change, pollution, invasive alien species and climate change, could entail massive loss of biodiversity. If the genetic structure of the populations currently exposed to intensify anthropogenic exploitation will be appreciably impaired in the nearest future, their further chances to survive and adapt will be lost forever.

The analysis of the obtained results discussed in respect to population conservation, sustainable man-

agement and prevention of loss of genetic diversity of red deer in Bulgaria, together with the assessment of the human impact on population structure as a result of selective hunting for trophies, has shown that the largest group of red deer in Bulgaria – the lowland mega-population from Northeastern Bulgaria – is not threatened by direct loss of genetic diversity for the present. The data about the genetic variation of Bulgarian red deer obtained through detailed analysis in the mega-population from Northeastern Bulgaria and description of the degree of similarity between the Bulgarian haplotypes and the haplotypes of the species populations from Southeastern Europe represent an initial scientific basis for genetic assessment of the biological resources of this species and should be used for regulation of hunting in the nearest future.

The change in haplotype structure of economically exploited red deer populations in Bulgaria should be monitored annually in order to obtain a proper assessment of the degree of the species biodiversity, which represents one of the main indicators of its viability. Carrying out hunting activities without such assessment leads to a high risk of degradation of exploited red deer populations, if their genetic diversity decreases under certain threshold or critical values. In the nearest future, it will be extremely difficult to prevent the further losses of biodiversity in the resource species conditioned by human activities, if we have no scientifically sound information about its condition today.

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