

# Indication for Genetic Diversity of European Roe Deer *Capreolus capreolus* (L.) in Southeastern Europe Revealed By mt DNA Markers

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**Abstract:** A molecular-genetic analysis of a control region (936 np) and cytochrome b gene (1140 np) of the mtDNA roe deer from Pannonian mixed forests ecoregion in Central south-eastern Europe (Hungary) and roe deer of Rila-Rhodope biogeographical region in Balkan Peninsula (Bulgaria) has been carried out. It was found that 97.02% of the samples from Pannonian mixed forests ecoregion had a mitotype typical for European roe deer (*Capreolus capreolus*). In the roe deer population inhabiting this region, 2.98% of animals possessed a mitotype typical for Siberian roe deer (*Capreolus pygargus*). The roe deer inhabiting in Rila-Rhodope biogeographical region in Balkan Peninsula also had typical for the European roe deer mtDNA sequences. Because of the assessment of biochemical-genetic uniqueness of the investigated roe deer, it was postulated that its populations in Pannonian mixed forests ecoregion and Rila-Rhodope biogeographical region represent distinct gene pools. This conclusion formed the basis of incurred recommendation to avoid admixing populations in both studied European bio-geographical regions by introductions of roe deer from abroad.

**Key words:** European roe deer, Siberian roe deer, genus *Capreolus*, control region, cytochrome b gene, mtDNA, Hungary, Bulgaria

## Introduction

In the geographical range of the genus *Capreolus* Gray, 1821, the roe deer populations exhibit a wide population biological (SOKOLOV 1992) and ecological diversity (DANILKIN 1996). Two closely related species, *Capreolus capreolus* (L., 1758) (European roe deer) and *Capreolus pygargus* (Pallas, 1771) (Siberian roe deer) are present (for details, see WILSON & REEDER 2005).

Recently, the European roe deer, as an important game species, has undergone hunting-economic exploitation across the European continent, from the Mediterranean to Scandinavia (OLANO-MARIN *et al.* 2014); together with the anthropogenic habitat fragmentation (WANG & SCHREIBER 2001), this factor

has modified the species population genetic diversity and predetermined the necessity to study the genetic structure of the main roe deer populations in the continent (COULON *et al.* 2006).

In the present, the European roe deer (*C. capreolus*) is one of the genetically best studied species but the studies of its genetic structure, based on the assessment of variation, cover mainly the western and eastern part of the species range (LORENZINI *et al.* 2014, MATOSIUK *et al.* 2014). At the same time, the new approach to characterise the genetic structure of the roe deer population by application of a set of molecular markers (sequences of the mitochondrial DNA) has been demonstrated to be a powerful tool

in studies of cervids (ZACHOS *et al.* 2007).

The aims of the present study were to determine the genetic diversity of roe deer (*C. capreolus*) in the Pannonian mixed forests ecoregion in Central south-eastern Europe (Hungary) and Rila-Rhodope biogeographical region of the southeastern Balkan Peninsula (Bulgaria) based on the variability of the control region and cytochrome b gene in mtDNA. With this study, we are attempting to (i) clarify the main mtDNA haplogroups in examined populations, (ii) infer the potential presence of individuals with Siberian mitotype (previously recorded in other European roe deer populations, and (iii) evaluate the phylogenetic position of studied roe deer population from the two examined biogeographic regions in Europe.

## Material and Methods

Our study sites in Pannonian mixed forests ecoregion are located in Hungary. This ecoregion consists of the depression surrounded by the Carpathian Mountains, Alps and Dinaric Mountains. It is situated in the Temperate Broadleaf and Mixed Forests biome and many natural habitats have been lost due to the agriculture (FUND & HOGAN 2012). Roe deer from Pannonian mixed forests ecoregion were sampled from two regions of Hungary: three locations from the Transdanubian Hills Region near the town Kaposvar (POP1, n=55 and POP2, n= 16) and the town Zselic (POP3, n=16), and one location from Great Alföld Region near the town Szeged (POP4, n=50). The mosaic of barriers separating these two sampling regions, the most significant among which are agricultural lands, fragmented by a motorway, major roads, railways, villages and rivers, prevent wildlife migration between them.

To assess the levels of genetic differentiation of roe deer populations from the Pannonian mixed forests ecoregion in comparison to populations inhabiting the south parts of its European range, one roe deer population was sampled from Rila-Rhodope biogeographical region (POP5, n=7), Southern Bulgaria. The region has a pronounced temperate-continental mountainous climate. The Rila-Rhodope Massif, which is the core of the Rila-Rhodope biogeographical region, consists of three distinct mountains in close proximity to each other: Rila Mountain, Pirin Mountain and Rhodope Mountains. Roe deer from Rila-Rhodope biogeographical region were sampled from Rhodope Mountains.

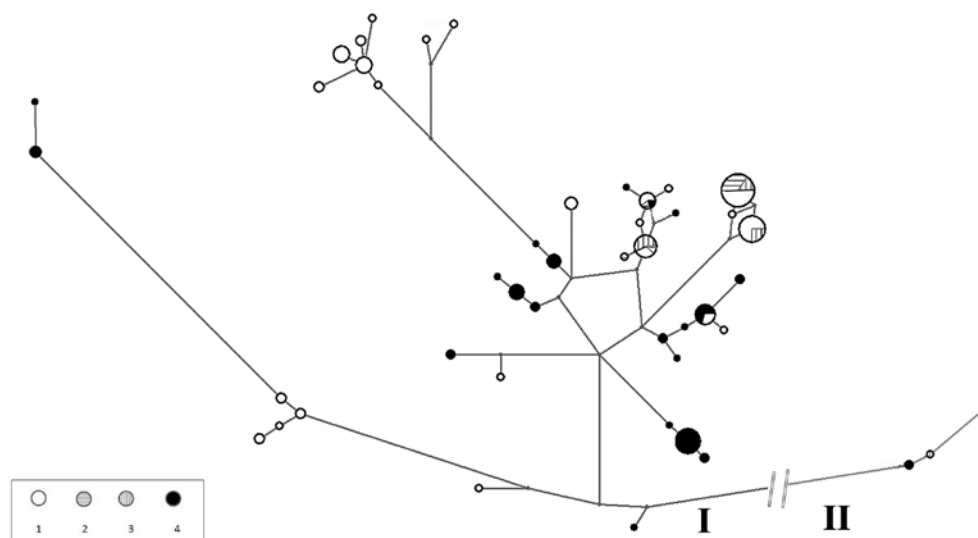
The roe deer were hunted legally, in the course of regular hunting programs organized by the local hunting association.

As molecular genetic markers, we used a control region (936 np) and cytochrome b gene (1140 np) of mtDNA. For characterisation of the genetic distinction of the roe deer populations from the Pannonian mixed forests ecoregion and Rila-Rhodope biogeographical region in the phylogeographic framework of genus *Capreolus*, we included in the analyses the following nucleotide sequences: (1) Siberian roe deer from native and non-native populations, n=88) and (2) European roe deer from South-eastern Europe (n=45) (ZVYCHAYNAYA *et al.* 2011a,b, 2013, KHOLODOVA *et al.* 2009) as well as sequences published in GenBank (LORENZINI *et al.* 2014). As an outgroup in the phylogenetic analysis, we used a combined sequence of the corresponding mtDNA fragments of *C. pygargus* (ZVYCHAYNAYA *et al.* 2011a) from Krasnoyarsk Region, Russia.

DNA isolation, amplification and sequencing as well as data analysis of the genetic distinction of the studied populations were carried out following the algorithms described in our previous studies (ZVYCHAYNAYA *et al.* 2011a) of variability of the control region and cytochrome b gene of mtDNA of Siberian roe deer (*C. pygargus*).

## Results

Complete nucleotide sequences of cytochrome b gene and mtDNA control region have been obtained for 134 samples of roe deer from Hungary. Length of the combined alignment was 2076 np; 143 variable positions were found, 13 of them were single replacements and 130 were replacements informative for the parsimony method. Of all Hungarian samples, 130 (97.02%) had a mitotype typical for the European roe deer and 4 (2.98%) had a mitotype typical for Siberian roe deer. A total of 43 haplotypes have been described: 40 European and 3 Siberian. Siberian haplotypes have been found in roe deer of POP4 inhabiting the Great Alföld region in the vicinity of the town of Szeged (2 samples, one haplotype) and in roe deer from POP3 inhabiting Transdanubian Hills near the town of Zselic (2 samples, 2 haplotypes) (Fig. 1). European haplotypes formed two haplogroups. The most numerous haplogroup (a) has a pronounced “core” of 27 haplotypes, which were found in all of the studied regions, and a detached branch of eight haplotypes, found only in POP1 from the Transdanubian Hills region near the town of Kaposvar. The relatively rare haplogroup (b) was presented by 6 haplotypes and was found only in POP3 (4 haplotypes) inhabiting in Transdanubian Hills region near the town of Zselic and in POP4 (2 haplotypes) inhabiting in Great Alföld region,



**Fig. 1.** Median network of mtDNA haplotypes of Hungarian roe deer, created on the grounds of phylogenetic analysis of 134 combined nucleotide sequences of cytochrome b gene and control region of mtDNA (2076 np) in Network program. I – *C. capreolus* haplogroup, II – *C. pygargus* haplogroup; number of symbols for displaying individuals belonging to different populations: 1- POP1, 2- POP2, 3- POP3, 4- POP4; a – haplogroup a, b – haplogroup b. Length of branches is proportional to the number of mutations, and the size of knots – to the number of specimens

near the town of Szeged; the individuals from these two regions were divided by 14 mutations. Two haplotypes took basal position on the median haplotype network and cannot be definitely classified to any haplogroup. The obtained results have been confirmed by the phylogenetic analysis through the methods of Neighbor-Joining, Maximum Likelihood and Maximum Parsimony.

The nucleotide diversity of the entire sample of Hungarian roe deer computed from combined sequences of both mtDNA fragments, excluding the “Siberian” haplotypes, was  $\pi=0,005$  (S.E.=0.001).

The greatest nucleotide diversity among the studied roe deer populations from Hungary demonstrated POP1 from Transdanubian Hills region and POP4 from the Great Alföld; the lowest nucleotide diversity was found in the roe deer from POP3.

The values of the nucleotide diversity of all the studied roe deer populations from Hungary were calculated with the same error of the mean arithmetic value. Weighted distance between the investigated roe deer populations from Hungary was close to zero.

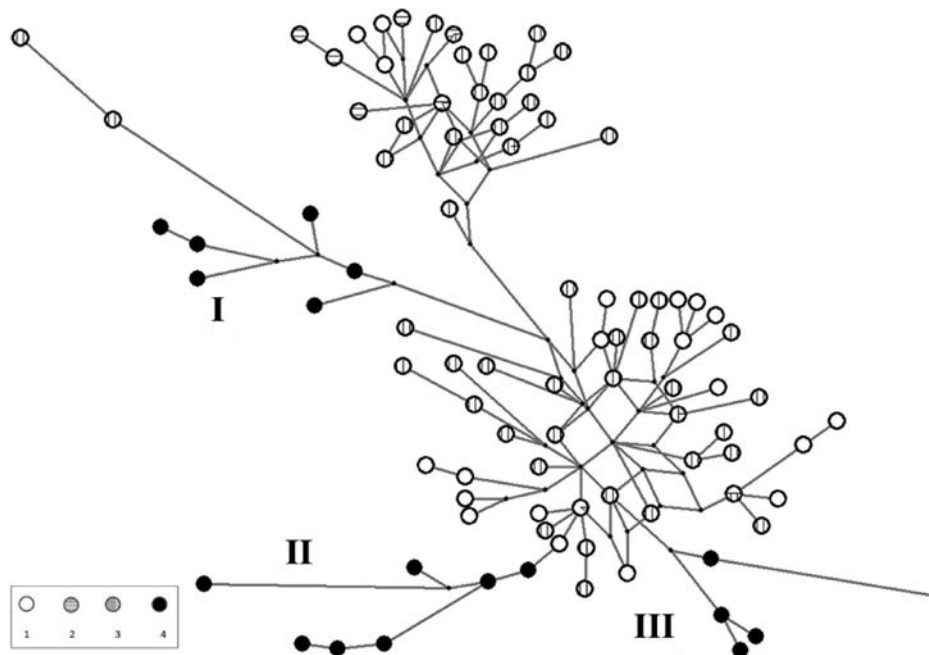
The roe deer from POP1 and POP2 originating from Transdanubian Hills showed quite similar haplotype composition; calculated weighted distance was 0.000 but actually, the small sample size of POP2 did not allow revealing the actual variation of this population and does not allow conclusions about their common origin.

Comparative analysis of the mtDNA sequences found roe deer in Pannonian mixed forests ecoregion in Central south-eastern Europe (Hungary) and Rila-

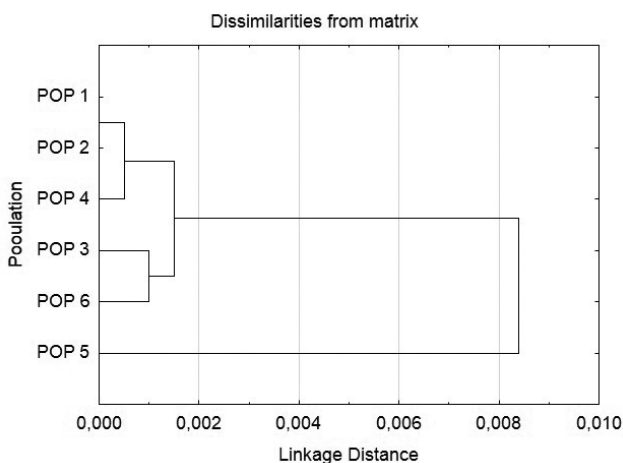
Rhodope biogeographical region in Balkan Peninsula (Bulgaria) with some known nucleotide sequences of mtDNA control region of the European roe deer (ZVYCHAYNAYA *et al.* 2011a, b, 2013, KHOLODOVA *et al.* 2009, LORENZINI *et al.* 2014) clarifies the place of the roe deer of both studied ecoregions in the phylogenetic scheme (Fig. 2) of European deer.

All investigated Bulgarian specimens had mtDNA typical for *C. capreolus*. In addition, all the identified in Bulgarian roe deer sequences belong to a relatively rare, but widespread in Europe haplogroup b (found in Russia, Lithuania, Ukraine, Hungary, Greece and Northern Italy).

All Hungarian haplotypes fall into two widely represented in Western, Central and Eastern Europe haplogroups: similar sequences occur in the species range from North-East Italy and France to Lithuania, Ukraine and the Moscow Region of Russia. Hungarian roe deer are mainly represented by its own (unique) haplotypes, which are very close to the other sequences and complement the overall picture of distribution of roe deer mitochondrial lines in Europe. Only three haplogroups are standing apart: from Central and Southern Italy (LORENZINI *et al.* 2014), from Central and Southern Spain as well as from Crimean Peninsula and Krasnodar Region of Russia (ZVYCHAYNAYA *et al.* 2013). South Italian sequences occupy a basal position in the median haplotype network and, in general, are similar to other European mtDNA sequences of the roe deer. Iberian haplotypes form a single, well-differentiated branch: they are separated from all other sequences



**Fig. 2.** Median network of mtDNA haplotypes of European roe deer created on the grounds of analysis of mtDNA fragment of the control region (880 np) without mononucleotide repeats of the right domain; number of symbols for displaying individuals belonging to different populations: 1- specimens from Hungary, 2-specimens from Bulgaria, 3-specimens from Central and Eastern Europe: from North-eastern Italy and France to Lithuania, Ukraine and Russia, 4 (I) – specimens from Central and South Spain, 4 (II) – specimens from Crimea, Krasnodar region, 4 (III) – specimens from Central and South Italy. Length of branches is proportional to the number of mutations; size of the nodes is standard regardless of number of specimens



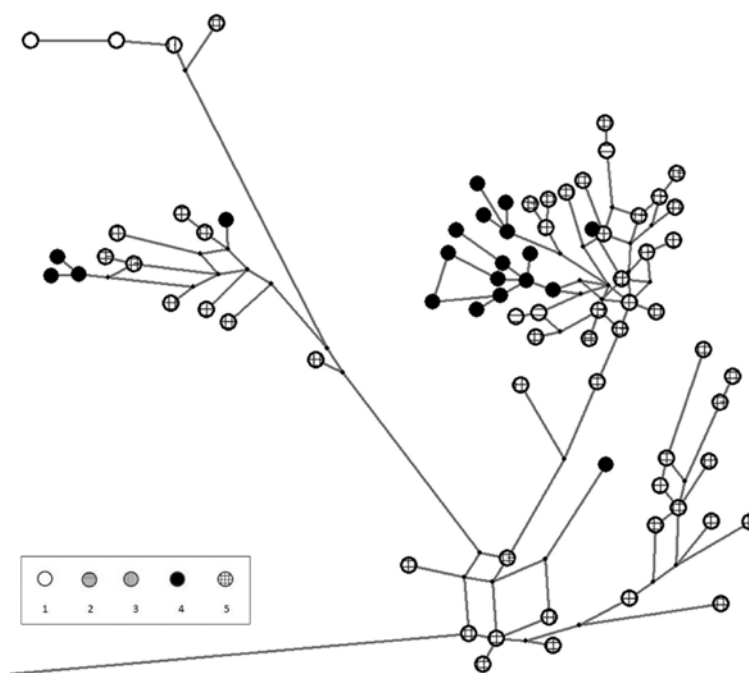
**Fig. 3.** Weighted distance (Net distance) between the roe deer populations from Pannonian mixed forests ecoregion in Central south-eastern Europe (Hungary) (POP1, POP2, POP3, POP4), Rilo-Rhodope biogeographical region in Balkan peninsula (Bulgaria) (POP5) and Carpathian geographic region of Ukraine (POP6).

by 6 mutations. Related haplotypes (apparently derived forms of Southern Spanish haplotypes) were found in two individuals from the Moscow Region (ZVYCHAYNAYA *et al.* 2011b). Crimean and Krasnodar sequences form a separate, well-defined branch but

they differ from other haplotypes (including from the nearest Hungarian) only by one mutation.

The comparative analyses of weighted distance (Net distance) between the roe deer populations from Pannonian mixed forests and Rila-Rhodope biogeographical region, in which a population from Carpathian geographic region in Ukraine is included for comparison, show: (i) among the group of populations from Pannonian mixed forests ecoregion, POP1, POP2 and POP4 are most similar to each other and differed from POP3, which in turn is more similar to the roe deer population from Carpathian geographic region of Ukraine, and (ii) there is a large distance between Bulgarian and Hungarian haplotypes. This large distance could be explained exactly by the low frequency of occurrence of that haplogroup. Despite a very close similarity of some Bulgarian and Hungarian sequences, the average weighted distance between them (Net distance) was 10 times higher than between Hungarian populations only.

Comparative analysis of the Siberian mtDNA sequences found in Hungary and the available sequences of the control region of *C. pygargus* from China, Russia (Far East, Central and Eastern Siberia, the Urals, Ural foothills, Moscow Region, Krasnodar Region), Poland, Lithuania (ZVYCHAYNAYA 2011a,



**Fig. 4.** Median network of mtDNA haplotypes of Siberian roe deer created on the grounds of analysis of mtDNA fragment of the control region (880 np) without mononucleotide repeats of the right domain; number of symbols for displaying individuals belonging to different populations: 1- specimens from Hungary, 2- specimens from Poland, Lithuania, 3- specimens from Krasnodar region, 4- specimens from Moscow region (Russia), 5- specimens of Siberian roe deer from Orenburg region and Kazakhstan to Far East (Russia) and China. Length of branches is proportional to the number of mutations; size of the nodes is standard regardless of number of specimens

b, KHOLODOVA *et al.* 2009, LORENZINI *et al.* 2014) showed the uniqueness of the Hungarian haplotypes (Fig. 7). In Poland, Lithuania, Moscow Region of Russia and in the western part of the range of *C. pygargus*, other mitochondrial lines are distributed. We have found similar sequences only in two animals from Krasnodar Region and one from Altai Region.

## Discussion

Generally, the nucleotide variation of maternal lineages of Hungarian roe deer was high but slightly lower than in other European populations. For comparison, the nucleotide variation of these mtDNA fragments of roe deer from Ukraine was  $\pi = 0.007$  (S.E. = 0.001) (ZVYCHAINAYA *et al.* 2013), from Poland – 0.008 (S.E. = 0.004) (MATOSIUK *et al.* 2014). The Hungarian populations POP3 from Transdanubian Hills region and POP4 from Great Alföld Region could be recognized as the most prosperous with regard to well-balanced genetic structure.

Variation pattern of the studied mtDNA fragments of Hungarian roe deer fits into the overall picture of distribution of mitochondrial lines of this species in Europe. Only widely and rather chaotically distributed haplogroups of European roe deer were found here, without any unique sequences

such as South Spanish, South Italian, Crimean and Krasnodar sequences.

Probably, in the recent past territory of Hungary functioned as a “transit” crossroad of the flows of roe deer mitochondrial genes in the east-west and north-south. It should be noted that Hungary as all the other European countries, with few exceptions, has its own set of haplotypes, which is probably due to the sedentary way of life of European roe deer. It is most likely that the large number of described haplotypes and “continuity” of variation of the mitochondrial lines of Hungarian roe deer indicate good condition of its populations during periods of maximum abundance depression and fragmentation of the range of *C. capreolus* in XIX-XX centuries.

In Hungary, we found haplotypes of *C. pygargus* described previously only in animals from Krasnodar Region (KHOLODOVA *et al.* 2009) but mitochondrial lines observed in Poland, Lithuania and Moscow Region were not detected.

The population genetic structure inferred from mitochondrial (CR and cyt b) markers of the European roe deer (*C. capreolus*) in Hungary moved further to the west the border of introgression of Siberian roe deer (*C. pygargus*) mtDNA in the European roe deer genome in Eurasia, which according to the recent studies lies at more than 2000 km from the current distri-

bution range of *C. pygargus* and is widespread among the roe deer from Poland (MATOSIUK *et al.* 2014).

Perhaps in historical time, there were several ways of natural distribution (introgression) of *C. pygargus* mtDNA in Europe, and the Hungarian mitochondrial line represents the “Southern European way” of penetration of *C. pygargus* mtDNA to the west. On the other hand, “direct transfer” of foreign mtDNA accompanying artificial resettlement of Siberian roe deer in Central Europe cannot be excluded. This hypothesis was supported by the fact that this particular mitochondrial line was not found in the western part of the *C. pygargus* area, but was observed by us only in the Altai region and in the Krasnodar Region of Russia, where in 1958 and 1962 animals from Central Siberia were released.

For detailed explanation of the observed unexpected invasion of the mtDNA genome from Siberian roe deer into the population genetic structure of European roe deer in Central Europe, see MATOSIUK *et al.* (2014).

All the studied roe deer from the Rila-Rhodope biogeographical region in Balkan Peninsula had typical for European roe deer sequences, and they were presented by only one haplogroup. No traces of introgression of Siberian roe deer mtDNA in the European roe deer genome were found in this population from the southern part of Balkan Peninsula. The absence of interspecific mtDNA introgression in the recent European roe deer from South Bulgaria is in accordance with the available paleontological data about the presence of this species in the territory of present-day Bulgaria during the late Pleistocene. The paleontological records of roe deer from the territory of Bulgaria also showed that the species remained in this territory during the last glacial period. The abundance of European roe deer in Bulgaria has significantly increased during the Holocene, from

the Neolithic period till the Middle Ages, and shows constant and abundant presence in the territory of Bulgaria (SPASSOV & POPOV 2007, VASILEV 1985).

The obtained data about the genetic diversity of the roe deer population from Bulgaria, especially the recorded polymorphism of the control region and cytochrome b gene in mtDNA, suggest that the genetic variation of this population has been reduced. This suggestion is in contrast with the data about the allozyme diversity within and among the populations of *C. capreolus* from South-eastern and Central Europe, which showed higher genetic diversity of Bulgarian roe deer, when compared with populations from Hungary or different populations from Western (France) or Central (Austria or Switzerland) Europe (HARTL *et al.* 1990, 1993).

Analysis of an additional sample of this group of roe deer would contribute to a more unambiguous assessment of genetic variation of South-Balkan population. However, on the basis of the data obtained in this study, revealing the geographic structure of the polymorphism of the control region and cytochrome b gene in mtDNA of the roe deer from both studied ecoregions, we can propose several conclusions. Our results support the results of the assessment of the allozyme diversity among populations of *C. capreolus* of South-eastern and Central Europe (HARTL *et al.* 1993), which showed their genetic uniqueness, a conclusion that may have conservation and management implications. Furthermore, the populations of the two studied ecoregions represent distinct gene pools, which should not admix with other geographical populations by introductions of roe deer from abroad.

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