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MITOCHONDRIAL DNA VARIATION IN ROE DEER POPULATION FROM LITHUANIA

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Summary: In order to investigate the roe deer population from Lithuania, data on the 457 bp mtDNA control region sequences were analysed. In the samples of 20 roe deer from Lithuania we found 6 different haplotypes, based on 38 variable sites, and observed haplotypes belonged to two haplogroups. Genetic diversity was estimated by haplotype diversity H_d=0.800, nucleotide diversity P_i=0.03031, average number of nucleotide differences k=13.853, and sequence conservation C=0.917. Haplotypes Hap_1 and Hap_2 were the most common in Lithuanian roe deer population. Comparative analysis of the data was performed using homologous mtDNA control region sequences downloaded in GenBank database. Analyses of control region mtDNA sequences indicated widespread introgression of Siberian roe deer (*C. pygargus*) mtDNA in the European roe deer genome, and introgressed individuals constituted 20% of the deer studied. Phylogenetic findings demonstrated distinction between two clades. Hap_2, Hap_3 and Hap_4 haplotypes were specific in roe deer from Lithuania, Hap_1 and Hap_5 haplotypes were identified in roe deer populations from Russia, and also Hap_5 and Hap_6 were identified in roe deer from Poland.

Key words: mtDNA, roe deer, genetic variability, Lithuania.

Introduction

The roe deer is widespread in Palearctic and continental Asia and includes two polytypic species the European roe deer (*Capreolus capreolus* Linnaeus 1758*)* and Siberian roe deer (*Capreolus pygargus* Pallas 1771) (Grubb, 1993; Danilkin, 1996; Vernesi et al., 2002). These two species have different morphometric traits, body sizes and karyotypes (Sokolov and Gromov, 1990; Danilkin, 1996). Roe deer species contact zone appears to lie in far Eastern Europe, in a narrow range between the rivers Volga and Don (Danilkin, 1996). Whether the two species may generate viable fertile offspring and where the suture zone actually lies are question that deserve attention (Lister, Grubb & Summer, 1998; Lorenzini et al. 2014). The evolutionary history of the European roe deer during last 2-3 millions years is not known (Vernesi et al., 2002). These two species have probably lived in allopatry for most of their evolutionary history. In prehistoric times, because of alternating contractions and expansions of their overlapping areas they must have come into contact more than once (Hewison and Danilkin, 2001; Lorenzini et al., 2014).

The European roe deer is the most numerous ungulate species of the continent with a population size of approximately 10 million animals occurring in Europe (Linnell et al., 1998; Apollonio et al., 2010), the current Lithuanian roe deer population is abundant, consisting of approximately 115 000 individuals (Statistics Lithuania, 2014). For at least 400 years roe deer has been a popular game species and has undergone frequent local extinctions, translocations and reintroductions (Lorenzini and Lovari, 2006). Siberian roe deer were introduced to the distribution range of the European roe deer for hunting purposes since the 19th century (Danilkin, 1996). According to Hewison and Danilkin (2001) introgression and persistence of the Siberian form hardly occurs in the wild, there are strong reproductive barriers developed during and after speciation. Usually small females of European roe deer die in kidding, when they mate with

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larger Siberian roe deer males. F1 hybrids bred in captivity are totally or partially infertile (Sokolov and Gromov, 1990), it is likely that this leads to differences in the karyotypes (Danilkin, 1996).

Siberian genotypes are lost in the areas of introduction and these releases do not seem to have left signs in the resident European roe deer species (Hewison and Danilkin, 2001; Lorenzini et al., 2014).

Data on the genetic variability of roe deer in Lithuania is still sparse, studies on the morphometric data and genetic diversity of roe deer have only been launched recently (Pėtelis and Brazaitis, 2003; Lorenzini and Lovari, 2006; Narauskaitė et al. 2011; Pūraitė et al., 2013). The aim of this study was to assess the genetic status of roe deer in Lithuania using D-loop sequences analysis.

Material and Methods

Samples of 20 roe deer were collected from 11 different Lithuania regions (Fig. 1). Specimens were collected from native populations, any reintroductions have never been documented in Lithuania. Tissue samples were obtained from legally hunted animals in period from 2008 to 2013. Genomic DNA was extracted from small pieces of muscle tissue using a "Genomic DNA Purification Kit" (Thermo Scientific). Before performing the PCR, all samples were diluted up 50 ng/µl.

Amplification of the mitochondrial control region (457 bp) was performed using primers pairs: L-Pro (5'-CGTCAGTCTCACCATCAACCCCCAAAGC-3') and H-493 (5'-TGAGATGGCCCTGAAGAAA GAACC-3') (Douzery and Randi, 1997; Vernesi et al., 2002; Royo et al., 2007). The PCR protocol consisted of 20 µl reaction mix containing approximately 50 ng DNA, 0.2 µM each primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 U of Taq polymerase (Thermo Scientific). The PCR consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, with final elongation step of 10 min at 72 °C after the last cycle.

Products were separated on 1.5% agarose gel and visualized by etidium bromide. PCR amplification products of D-loop were purified by "GeneJET Gel Extraction Kit" (Thermo Scientific) and sequenced with "Big Dye® Terminator v3.1 Cycle Sequencing Kit" (Applied Biosystems) following the manufacturer's recommendations, which run on 3130xl Genetic Analyzer (Applied Biosystems). Sequences were aligned using CLUSTAL W algorithm (Thompson et al., 1994) implemented in BioEdit 7.2.5 (Hall, 1997) and MEGA 6 (Tamura et al., 2013) software, and haplotypes were identified by DnaSP v.5 (Librado and Rozas, 2009) software.

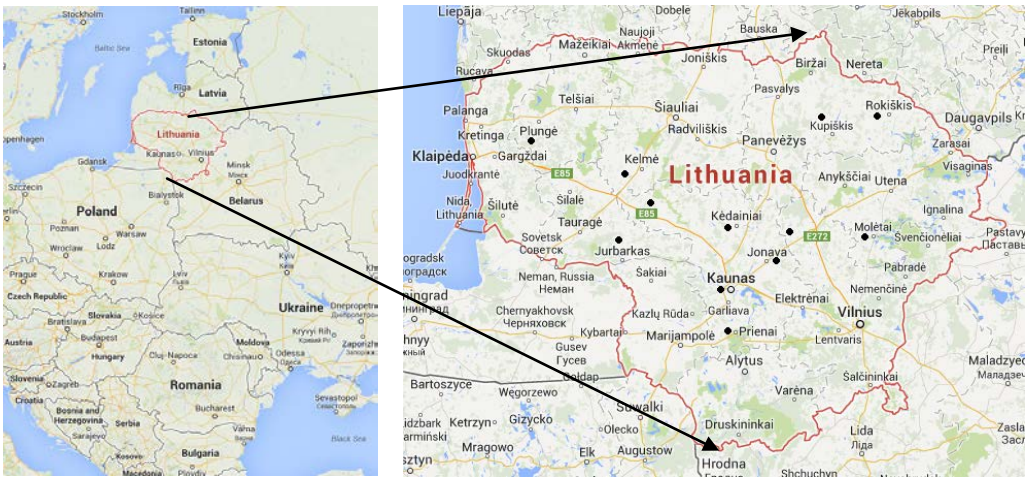

Figure 1. Collection sites of roe deer samples in Lithuania (Kupiskis, n=1; Kelme, n=2; Rietavas, n=2; Ukmerge, n=2; Jonava, n=2; Kaunas, n=2; Rokiskis, n=2; Kedainiai, n=1; Moletai, n=2; Prienai, n=2; Jurbarkas, n=1; Raseiniai, n=1).

Results and discussion

The 457 bp mtDNA control region sequence and 38 variable nucleotide sites (Table 1) were determined in 20 roe deer individuals. Six haplotypes and two haplogroups were identified in population

of roe deer in Lithuania (GenBank accession numbers KM215767-KM215786). Haplotype diversity value was H_d=0,800, variance of haplotype diversity was 0,00445, nucleotide diversity value P_i=0,03031, Theta (per site) from Eta was 0,02344, the average number of nucleotide differences was k=13,853, and the sequence conservation value was C=0,917. Haplotypes Hap_1 and Hap_2 were the most common in Lithuania roe deer population. Comparative analysis of the data was performed using homologous mtDNA control region sequences retrieved from the GenBank database (Table 2). Analyses of control region mtDNA sequences indicated widespread introgression of Siberian roe deer (*C. pygargus*) mtDNA in the European roe deer genome, introgressed individuals constituted 20% of the deer studied. Hap_2, Hap_3 and Hap_4 haplotypes were specific in roe deer from Lithuania. Haplotypes Hap_1 and Hap_5 were identified roe deer populations from Russia (Zvychainaya et al., 2011) and Lithuania. The phylogenetic tree indicated that the mtDNA haplotypes of the roe deer are split into two well divergent clades (European roe deer and Siberian roe deer) (Fig. 2).

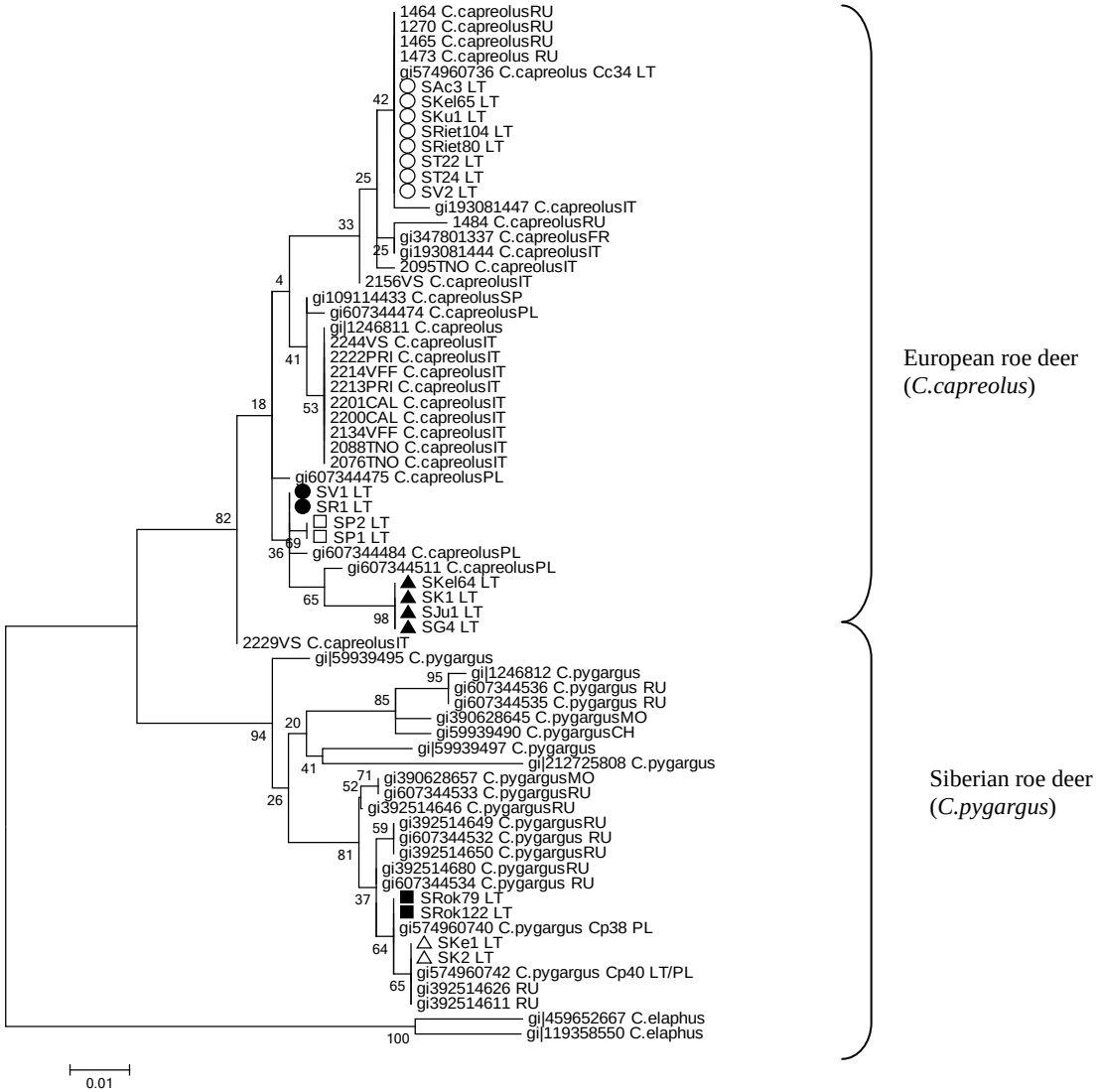

Figure 2. Neighbour-joining tree based on pairwise nucleotide divergence of roe deer mtDNA haplotypes. Numbers at the nodes show support from 1000 bootstrap replicates, *Cervus elaphus* used as an outgroup.

Six haplotypes identified in roe deer population of Lithuania: ○ – Hap_1, ▲ – Hap_2, ● – Hap_3, □ – Hap_4, Δ - Hap_5, ■ – Hap_6.

Table 1. Mitochondrial haplotypes found in this study.

	Variable sites																																												
									1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**4**	**4**	**4**	**4**	**4**	**4**
Haplotypes, individual code	**1**	**2**	**4**	**7**	**8**	**8**	**9**	**9**	**0**	**0**	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	**6**	**7**	**7**	**0**	**2**	**2**	**3**	**3**	**3**	**4**	**4**	**6**	**6**	**6**	**7**	**9**	**2**	**3**	**4**	**7**	**7**	**0**	**0**	**0**	**0**	**2**	**3**
	7	**8**	**2**	**4**	**5**	**7**	**4**	**5**	**2**	**6**	**3**	**6**	**1**	**6**	**9**	**0**	**1**	**6**	**0**	**0**	**2**	**4**	**5**	**8**	**2**	**3**	**4**	**1**	**5**	**3**	**7**	**8**	**7**	**5**	**0**	**1**	**2**	**2**	**7**	**5**	**6**	**7**	**9**	**3**	**2**
Hap_1 (**LT***: SKu1, SKel65, SRiet80, SRiet104, SAc3, SV2, ST22, ST24. **RU**+:1270, 1465, 1464, 1473; **LT**+: gi574960736)	C	T	C	C	C	C	T	A	C	C	G	G	G	T	G	A	T	C	A	T	G	T	A	C	G	A	A	A	G	T	A	A	A	T	C	A	C	T	G	A	A	A	C	T	T
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Hap_2 (**LT***: SKel64, SG4, SK1, SJu1)	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	G	.	.	C	T	.	T	.	A	.	.	.	.	C	C
	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	G	.	.	C	T	.	T	.	A	.	.	.	.	C	C
	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	G	.	.	C	T	.	T	.	A	.	.	.	.	C	C
	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	G	.	.	C	T	.	T	.	A	.	.	.	.	C	C
Hap_3 (**LT***: SR1, SV1)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	T	.	.	.	.	.	.	.	.	.	C
	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	T	.	.	.	.	.	.	.	.	.	C
Hap_4 (**LT***: SP1, SP2)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	G	C	T	.	.	.	.	.	.	.	.	.	C
	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	G	C	T	.	.	.	.	.	.	.	.	.	C
Hap_5 (**LT***: SKe1, SK2. **LT/PL**+: gi574960742; **RU**+: gi392514626, gi392514611)	T	A	T	T	T	T	C	C	T	T	A	A	.	C	A	G	C	T	G	C	A	C	G	A	A	.	.	.	T	.	.	.	.	.	T	.	.	.	A	G	G	G	.	.	C
	T	A	T	T	T	T	C	C	T	T	A	A	.	C	A	G	C	T	G	C	A	C	G	A	A	.	.	.	T	.	.	.	.	.	T	.	.	.	A	G	G	G	.	.	C
Hap_6 (**LT***: SRok79, SRok122. **PL**+: gi574960740)	T	A	T	T	T	T	C	C	T	T	A	A	.	C	A	G	C	T	G	C	A	C	G	A	A	.	.	.	T	.	.	.	.	.	T	.	.	.	A	G	G	.	.	.	C
	T	A	T	T	T	T	C	C	T	T	A	A	.	C	A	G	C	T	G	C	A	C	G	A	A	.	.	.	T	.	.	.	.	.	T	.	.	.	A	G	G	.	.	.	C

Vertical numbers refer to the aligned sites in the 457 bp data set (only variable sites are shown). Dots indicate identity with Hap_1.

* - Our study results, + - sequences from GenBank.

Table 2. Accession numbers and references of sequences derived from GenBank. [+]Unpublished data, [*]our study results.

Title	Accession number	References
gi390628645_MO	JQ958971	Bayarlkhagva et al.[+]
gi390628657_MO	JQ958978	Bayarlkhagva et al.[+]
gi1246811	Z70318	Douzery and Randi, 1997
gi1246812	Z70317	Douzery and Randi, 1997
gi119358550	AM419026	Fajardo et al.[+]
gi109114433_SP	AM279273	Fajardo et al., 2007
gi193081447_IT	EU600315	Gentile et al.[+]
gi193081444_IT	EU600312	Gentile et al.[+]
gi347801337_FR	JN632610	Hassanin et al., 2012
gi459652667	KC294007	Karaiskou et al.[+]
gi212725808	FJ416669	Liu and Zhang[+]
gi574960736_LT	KF724435	Lorenzini et al., 2014
gi574960740_PL	KF724439	Lorenzini et al., 2014
gi574960742_PL	KF724441	Lorenzini et al., 2014
gi607344474_PL	KJ558225	Matosiuk et al., 2014
gi607344475_PL	KJ558226	Matosiuk et al., 2014
gi607344484_PL	KJ558235	Matosiuk et al., 2014
gi607344511_PL	KJ558262	Matosiuk et al., 2014
gi607344536_RU	KJ558287	Matosiuk et al., 2014
gi607344535_RU	KJ558286	Matosiuk et al., 2014
gi607344533_RU	KJ558284	Matosiuk et al., 2014
gi607344532_RU	KJ558283	Matosiuk et al., 2014
gi607344534_RU	KJ558285	Matosiuk et al., 2014
SR1 – SRok79	KM699017864 - KM699017883	Pūraitė et al.*
2095TNO_IT	HM121295	Vernesi et al.[+]
2156VS_IT	HM121339	Vernesi et al.[+]
2244VS_IT	HM121313	Vernesi et al.[+]
2222PRI_IT	HM121259	Vernesi et al.[+]
2214VFF_IT	HM121299	Vernesi et al.[+]
2213PRI_IT	HM121256	Vernesi et al.[+]
2201CAL_IT	HM121231	Vernesi et al.[+]
2200CAL_IT	HM121232	Vernesi et al.[+]
2134VFF_IT	HM121297	Vernesi et al.[+]
2088TNO_IT	HM121271	Vernesi et al.[+]
2076TNO_IT	HM121268	Vernesi et al.[+]
2229VS_IT	HM121314	Vernesi et al.[+]
gi59939495	AY785545	Xiao et al., 2007
gi59939490_CH	AY785540	Xiao et al., 2007
gi59939497	AY785547	Xiao et al., 2007
1270_RU	JQ906107	Zvychainaya et al., 2011
1464_RU	JQ906126	Zvychainaya et al., 2011
1465_RU	JQ906127	Zvychainaya et al., 2011
1473_RU	JQ906129	Zvychainaya et al., 2011
1484_RU	JQ906131	Zvychainaya et al., 2011
gi392514646_RU	JQ906148	Zvychainaya et al., 2011
gi392514649_RU	JQ906151	Zvychainaya et al., 2011
gi392514650_RU	JQ906152	Zvychainaya et al., 2011
gi392514680_RU	JQ906182	Zvychainaya et al., 2011
gi392514626_RU	JQ906128	Zvychainaya et al., 2011
gi392514611_RU	JQ906113	Zvychainaya et al., 2011

The first four haplotypes (Hap_1, Hap_2, Hap_3 and Hap_4) depend on European roe deer lineage, last two haplotypes (Hap_5 and Hap_6) on Siberian roe deer lineage. Haplotype 5 was established in central part of Lithuania (Kaunas and Kedainiai), haplotype 6 was established in northern part of Lithuania (Rokiskis).

Conclusion

Results of samples of roe deer individuals from different regions of Lithuania analysis revealed high level of molecular genetic variation in mtDNA control region. The majority of sequences (16) represented 4 haplotypes and passed into a European roe deer lineage. Similar phylogenetic patterns based on mtDNA were identified in animals from Russia, Italy, France, Spain and Poland. The two haplotypes (Hap_5 and Hap_6) belonged to the Siberian roe deer lineage and these sequences were similar to sequences of mtDNA from Mongolia, China, Russia roe deer populations. Results indicated widespread introgression of Siberian roe deer (*C. pygargus*) mtDNA in the European roe deer genome, introgressed individuals constituted 20% of the roe deer studied, similar results (16.6%) were obtained in studies of roe deer in Poland (Matosiuk et al., 2014).

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