

# DNA Sequence Variation in the Mitochondrial Control Region of *Oryctolagus cuniculus* from Croatia

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## Summary

In Croatia, wild rabbits (*Oryctolagus cuniculus*) are present in several East Adriatic islands, while their origin is not known (documented). To characterize their maternal origin, we have analysed partial mitochondrial DNA control region (CR-mtDNA) sequence (317-bp) in 36 wild rabbits sampled in three Croatian islands (11 in Dolin, 10 in Dugi otok and 15 in Trstenik). After comparison with other worldwide distributed wild and domestic rabbit sequences (208), two distinguished haplotypes (H1 and H2) were found in Croatian rabbits. H1 was equal to the reference rabbit sequence (GenBank accession number NC\_001913) that is the most frequent haplotype present in wild and domestic rabbits and belongs to the lineage B. H2 haplotype, present in the one rabbit from Dugi otok, was just one mutation, transversion T→C at 15803, remote from the H1. The results presented in this study show extremely low CR-mtDNA diversity of Croatian rabbits and indicate their feral origin.

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## Key words

*Oryctolagus cuniculus*, mitochondrial DNA, haplotype diversity, Croatia

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## Introduction

The European rabbit (*Oryctolagus cuniculus*), progenitor of domestic rabbits, is native to South-West Europe and North-West Africa. It is one of the most successful invasive animals introduced by people throughout the world (Flux and Fullagar, 1983). It is generally accepted that wild rabbits originate from the Pyrenees Peninsula (Spain, Portugal), Western France and North Africa while it is considered they are introduced throughout Western Europe since Roman times (Gibb, 1990; Mitchell-Jones et al., 1999). On the other side, a number of lagomorph species, including rabbits have been archaeologically documented in Croatia (Miracle et al., 2010; Farbstein et al., 2012). Thus, according to Janković et al., (2006) wild rabbits were present in Croatia in the Upper Paleolithic or even before. Today, only two lagomorph species, European wild rabbit (*Oryctolagus cuniculus*) and European brown hare (*Lepus Europaeus*) have survived. Although systematically not recorded, it is known that in Croatia wild rabbits are present only on several North-East Adriatic islands (Brijuni, Cres, Iž, Kornati, Lavdara, Levrnaka, Lošinj, Pag, and Rab) see Long, (2003). Thus, rabbits were present on Cres and Lošinj as early as in 771 (Dulic, 1987). No information about the population size neither number of culled rabbits is presently documented. In addition, up to our knowledge, no single genetic analysis on the origin and/or diversity of wild rabbits in Croatia have been performed.

MtDNA analyses have been widely used in the genus *Oryctolagus cuniculus*. The study of restriction fragment length polymorphism (RFLP) of the entire rabbit mtDNA molecule revealed the existence of two (A and B) very distinct maternal lineages (Ennafaa et al., 1987; Biju-Duval et al., 1991). Lineage A was found in the south-west of the Iberian Peninsula while lineage B was found in the rest of Europe, including Northern Spain, and domestic modern rabbit breeds. A clear subdivision into two clusters was also found when analyses were based on the nuclear genes (van der Loo et al., 1991, 1999; Ferrand, 1995), with a geographical distribution that largely coincides with two mitochondrial clades A and B. Thus, clade A was tentatively associated with *O. cuniculus algirus* and clade B with *O. cuniculus cuniculus* and the same pattern was observed in analyses based on nuclear gene (Biju-Duval et al., 1991; van der Loo et al., 1991; Ferrand, 1995; Branco & Ferrand, 1998).

The aim of the present study was to characterize maternal genetic diversity and origin of wild rabbits in Croatia.

## Materials and methods

A total of 36 tissue samples of *Oryctolagus cuniculus* from three Croatian island were collected, Dolin Island (11), Dugi otok (10) and Trstenik Island (15). DNA extraction was performed using the EZ-10 spin column genomic DNA miniprep kit (Bio Basic, Canada) according to the manufacturer's instructions. A 509-bp fragment of the *Oryctolagus cuniculus* mitochondrial D-loop region (between nucleotides 15460-15968) was amplified by PCR using forward (5'-CACCATCAGCACCCAAAG-3') (Melo-Ferreira et al., 2007) and reverse (5'-ATTTAAGAGGAACGTGTGGG-3'), (Pierpaoli et al., 1999) primers. PCRs were performed in a 25 µL volume containing 0.2 µM of each primer and using Emerald

AMP GT PCR Master Mix (Takara Bio Inc., Japan) according to manufacturer's protocol. The amplification reactions were performed on a iCycler (Biorad, Germany) comprised of an initial denaturation at 95 °C for 5 min, 38 cycles of denaturation at 95 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1 min and final extension at 72 °C for 1 min. PCR products were purified using Wizard SV Gel and PCR Clean-Up Kit (Promega, USA) and sequenced on an ABI 3130 DNA automated sequencer (Applied Biosystems, USA) using the ABI Prism Big Dye Terminator 3.1 Sequencing Kit (Applied Biosystems, USA). Referent sequence for *Oryctolagus cuniculus* under accession number NC\_001913 (Gissi et al., 1998) and total of 300 sequences were retrieved from the GenBank for comparison (Table S1). The sequences were visualized and aligned using MEGA 7 (Kumar et al., 2016). Haplotypes were calculated using DNA Sp 5.10 (Librado and Rozas, 2009) and median-joining network (Bandelt et al., 1999) was constructed by PopART (Leigh and Bryant, 2015; <http://popart.otago.ac.nz>).

## Results and discussion

The analysis was performed on the 317-bp long sequence fragment in the CR-mtDNA using a data set of 336 wild and domestic rabbits, 36 typed in this study and 300 retrieved from the GenBank (Table S1). All singletons (92), with exception of relevant haplotypes from Croatian (H2), Spanish (H37-H40) and Portuguese (H35, H36) wild rabbits, and some domestic rabbits from Europe (H17, H21-H27, H29-H32) and Asia (H18), were removed (Table 1). In the analysis based on 244 sequences we identified 40 different haplotypes (Figure S1). Phylogenetic reconstruction of 40 CR-mtDNA haplotypes, presented with the median-joining tree in Figure 1. The lineage A is presented on the right with seven haplotypes (H6, H35, H36, H37, H38, H39 and H40) while the lineage B is presented on the left and below by 33 haplotypes (H1-H5, H7-H35).

Two lineages (A and B) were clearly separated, with lineage A composed of wild rabbits (Europe: France, Portugal and Spain) and lineage B including both domestic (Africa: Egypt; Asia: China Japan; Europe: Austria, Belgium, France Germany, Great Britain, Hungary Spain) and wild rabbits (Europe: Croatia, France, Spain). This is consistent with results presented in van der Loo et al. (1997), Long et al. (2003), Seixas et. al (2014) and Emam et. al (2016). Two most frequent haplotypes within lineage B, here H1 and H3, are observed in both, domestic and wild (Africa, Asia, Australia, and Europe) rabbits, see also Long et al. (2003). Mostly, sequences fell within only one substitution from these two central haplotypes. Haplotype H1 is the most frequent and except Croatian wild rabbits from this study (35/87), appears in wild rabbits from Australia (2/87), Spain (7/87) and in domestic rabbits from China (9/87), Egypt (25/87), Japan (2/87), Germany (4/87) and Hungary (3/87), see Table 1. Only one individual from Croatian island Dugi otok belongs to haplotype H2 which is one (T→C at 15803) transversion remote from the H1 rabbit reference sequence reported in GenBank under accession number NC\_001913 (Gissi et al., 1998). Haplotype H3 is found in wild rabbits from Australia (2/46) and Spain (15/46), as well in domestic rabbits from Egypt (12/46), Germany (2/46), Spain (13/46) and Hungary (2/46). Four haplotypes, H1, H3, H15 and

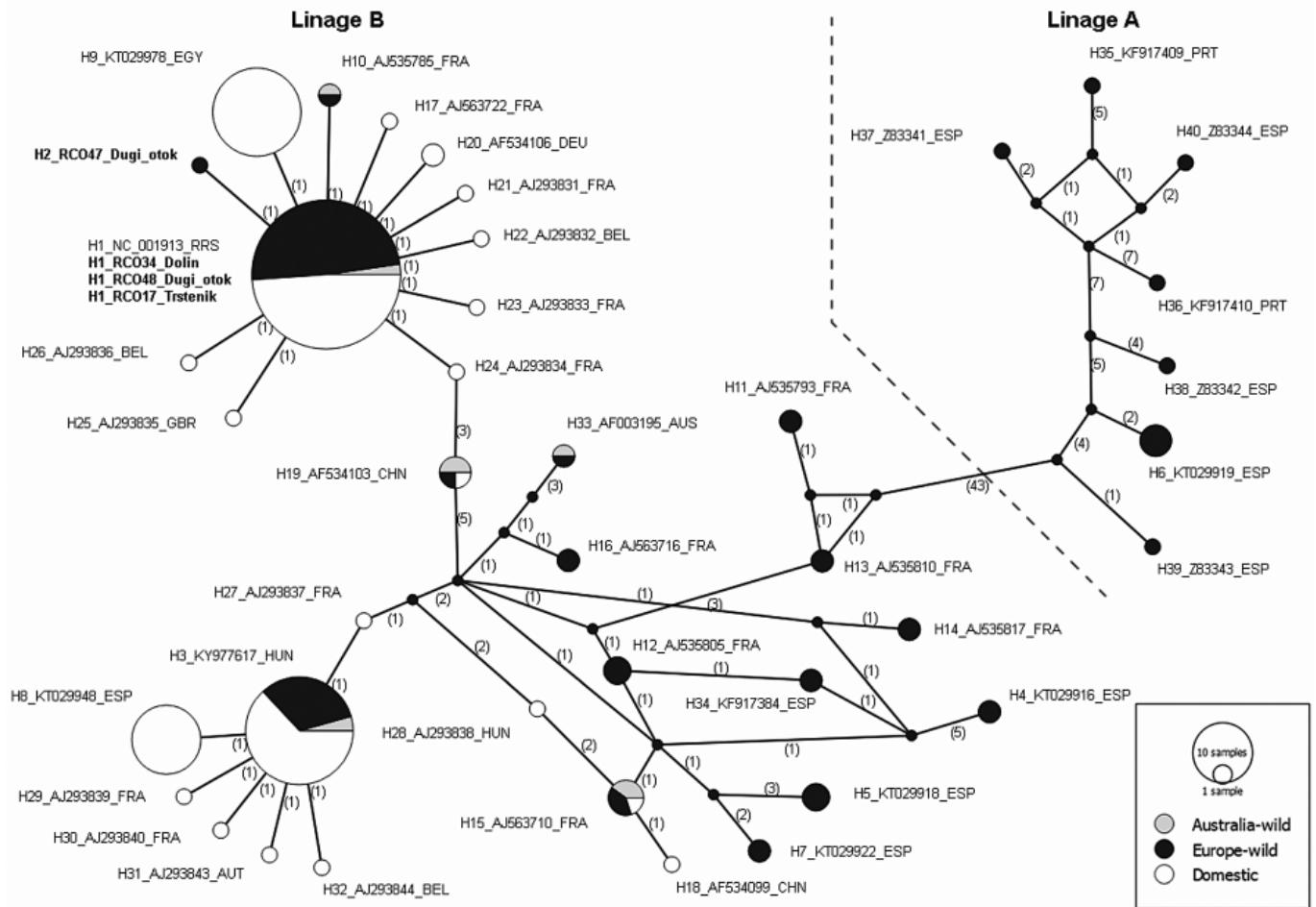


Figure 1. The median-joining (MJ) network for the 317-bp data set showing phylogenetic positions of haplotypes found in Croatian wild rabbits comparing to haplotypes found in other wild and domestic rabbit populations. The names of the haplotypes are displayed on the graph containing haplotype number, accession number and origin (3-digit country code) with the exception of Croatian wild rabbits that are given by the laboratory's ID and island origin (black circle and bold letters). Number of mutations separating nodes are given near branches in parentheses.

**Table 1.** Distribution of haplotype frequencies of the 317-bp fragment of mitochondrial DNA control region in 244 samples of wild and domestic rabbit in Africa, Asia, Australia and Europe. Origin of rabbits is presented using 3-digit country code.

Haplotype	Wild										Domestic						Σ
	Australia	Europe				Africa	Asia		Europe								
		ESP	FRA	HRV	PRT		EGY	CHN	JPN	AUT	BEL	DEU	ESP	FRA	GBR	HUN	
H1	2	7		35		25	9	2			4				3	87	
H2				1												1	
H3	2	15				12					2	13			2	46	
H4		2														2	
H5		3														3	
H6		4														4	
H7		2														2	
H8												19				19	
H9						31										31	
H10	1		1													2	
H11		1	1													2	
H12		2	1													3	
H13			2													2	
H14		1	1													2	
H15	2	1	1										1			5	
H16			2													2	
H17													1			1	
H18							1									1	
H19	2	1					1									4	
H20											2					2	
H21													1			1	
H22											1					1	
H23													1			1	
H24													1			1	
H25														1		1	
H26											1					1	
H27													1			1	
H28															1	1	
H29													1			1	
H30													1			1	
H31									1							1	
H32											1					1	
H33	1	1														2	
H34		2														2	
H35															1	1	
H36															1	1	
H37		1														1	
H38		1														1	
H39		1														1	
H40		1														1	

Singleton haplotypes were removed except for Croatian, Portuguese, and Spanish wild rabbits as well for domestic rabbits.

H19 were shared between wild (Australia, Europe) and domestic (Africa, Asia, Europe) rabbits (Table 1 and Figure 1). In total, 19 unique haplotypes were observed in wild and 17 in domestic rabbits. As shown in this study, the origin of Croatian rabbits is in the same lineage B with other domestic and wild rabbits.

### Conclusion

This study is the first genetic analysis of phylogenetic relationship among wild rabbits in Croatia. The results obtained on a 317-bp fragment of CR-mtDNA show extremely low diversity of Croatian rabbits and indicate their feral origin.

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