

Positive Selection On The Mitochondrial NADH Dehydrogenase Subunit 6 Gene In Hares (*Lepus spp.*)

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INTRODUCTION

Mitochondria are organelles that play a fundamental role in cellular metabolism (energy and/or heat production) via the oxidative phosphorylation (OXPHOS) pathway. The Electron Transport Chain (ETC), which consists of five enzyme complexes, is located at the inner membrane of mitochondria and is responsible for pumping protons into the intermembrane space and therefore forming the electrochemical gradient necessary for the synthesis of ATP. Evolution of mitochondrial DNA (mtDNA) has long been considered neutral, but given the functional importance of some proteins encoded by mtDNA genes for oxidative phosphorylation, some variation in mtDNA may have important fitness implications, and selection pressure may come from conditions that affect metabolic processes, such as adaptations to different environmental conditions (temperature, altitude, precipitation) or coevolution with nuclear genes involved in cellular respiration. Hares (*Lepus spp.*) are considered a particularly suitable group to study the effects of natural selection on mitochondrial OXPHOS genes because hares represent a highly polymorphic group of closely related species that occur in a range of different habitats with varying environmental conditions.

OBJECTIVE

The main objective of this study is to test for presence of natural selection shaping the genetic variability of the mitochondrially encoded gene of NADH dehydrogenase subunit 6 (*MT-ND6*) in hare species occurring in different habitats and to assess the functional implications of the observed amino acid changes in the encoded protein variants.

METHODS

47 publicly available nucleotide sequences of the *MT-ND6* gene from nine hare species were retrieved from the GenBank database. Overall molecular diversity indices (number of haplotypes, polymorphic sites, nonsynonymous and synonymous changes) were calculated using DnaSP v.6. Nucleotide sequences were translated into amino acid sequences using MEGA X. The maximum likelihood (ML) phylogenetic tree was constructed using MEGA X, while the Bayesian phylogenetic tree was constructed using MrBayes on CIPRES online platform. The best evolutionary model (HKY+G) was determined using MEGA X, while the trees were rooted using *Oryctolagus cuniculus* as out group. The median-joining (MJ) network was constructed in Network. The presence of codon-based selection signals was tested by comparing the number of non-synonymous changes per non-synonymous site to the number of synonymous changes per synonymous site. To find codons under the influence of positive selection we used "Datamonkey Adaptive Evolution" web server, specifically tests MEME, FEL, FUBAR and SLAC (individual site) and aBSREL, RELAX (branch site). The potential functional impact of the revealed amino acid substitutions was assessed by evaluating the changes in the physicochemical properties of the substituted amino acids (TreeSAAP - range for radical changes was 6-8, and the $p < 0.001$ - categories that fell within this range were considered to be under positive selection).

RESULTS

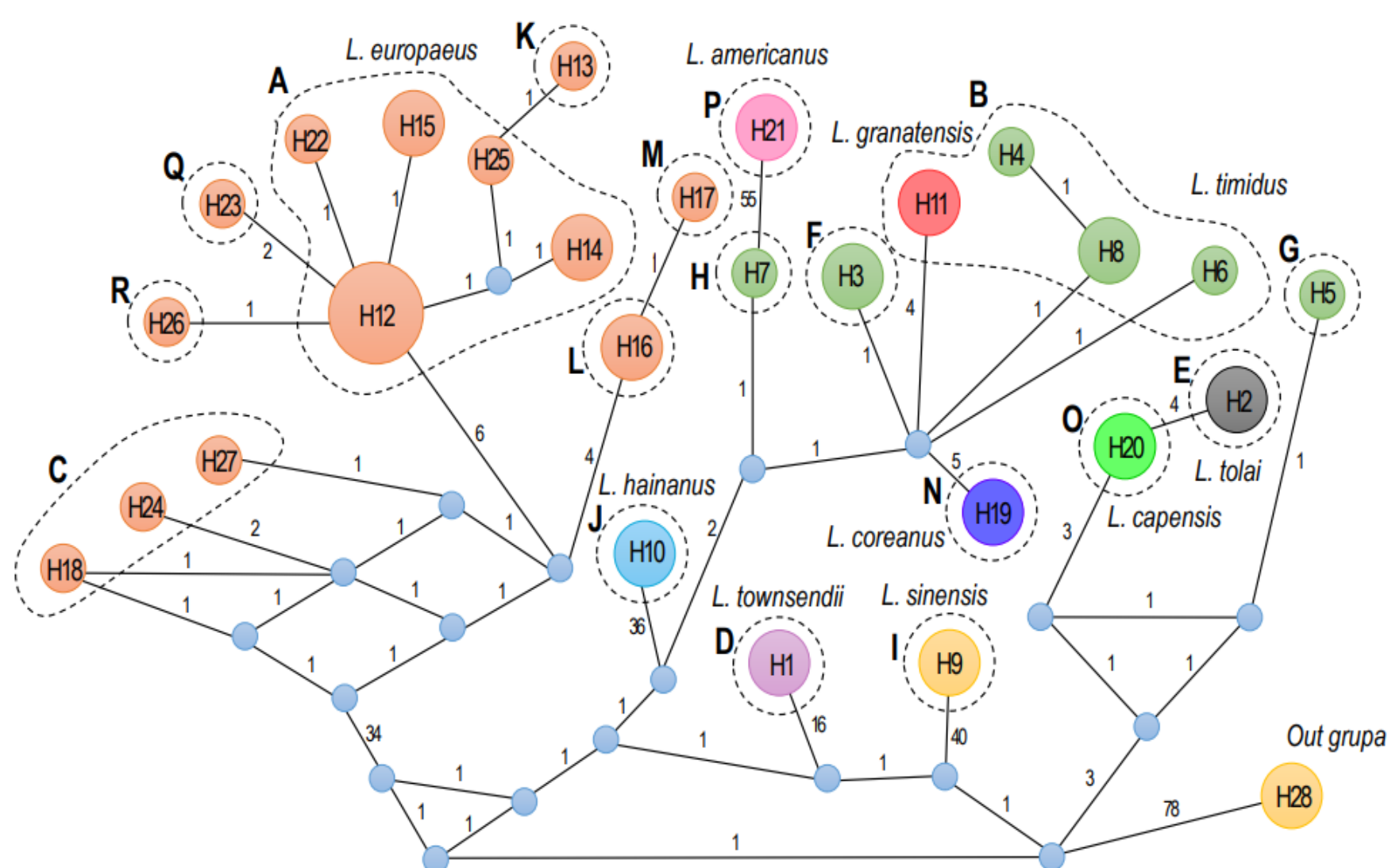
- Analysis of the 47 *MT-ND6* nucleotide sequences based on 110 synonymous and 23 nonsynonymous substitutions revealed 27 haplotypes (H1-H27) and 18 protein variants (A-R) (Table 1.).

Table 1. Distribution of haplotypes and protein variants of the *MT-ND6* gene in studied hare species

protein variant	A	B	C	D	E	F
species: <i>Lepus spp.</i> (haplotype)	<i>L. europaeus</i> (H12, H14, H15, H22, H25)	<i>L. timidus</i> (H4, H6, H8) <i>L. granatensis</i> (H11)	<i>L. europaeus</i> (H18, H24, H27)	<i>L. townsendii</i> (H1)	<i>L. tolai</i> (H2)	<i>L. timidus</i> (H3)
protein variant	G	H	I	J	K	L
species: <i>Lepus spp.</i> (haplotype)	<i>L. timidus</i> (H5)	<i>L. timidus</i> (H7)	<i>L. sinensis</i> (H9)	<i>L. hainanus</i> (H10)	<i>L. europaeus</i> (H13)	<i>L. europaeus</i> (H16)
protein variant	M	N	O	P	Q	R
species: <i>Lepus spp.</i> (haplotype)	<i>L. europaeus</i> (H17)	<i>L. coreanus</i> (H19)	<i>L. capensis</i> (H20)	<i>L. americanus</i> (H21)	<i>L. europaeus</i> (H23)	<i>L. europaeus</i> (H26)

- The ML and Bayesian trees and the MJN network yielded congruent tree topologies, indicating little evolutionary divergence among the species studied and suggesting the possibility of reticulate evolution.
- The evolutionary relationships among the hare species studied are consistent with previous studies. In the case of *L. europaeus*, two clades are observed, one comprising individuals from Asia Minor and the other individuals from Europe, suggesting that the evolution of the *MT-ND6* gene followed neutral expectations, as shown by the analysis of the mtDNA D-loop sequences.

Figure 1. Median-joining network showing the evolutionary relationships among *MT-ND6* haplotypes. (the number of nonsynonymous mutations is shown on the branches; haplotypes with the same amino acid sequence are grouped by the dashed line; each species is represented by the different colour)..

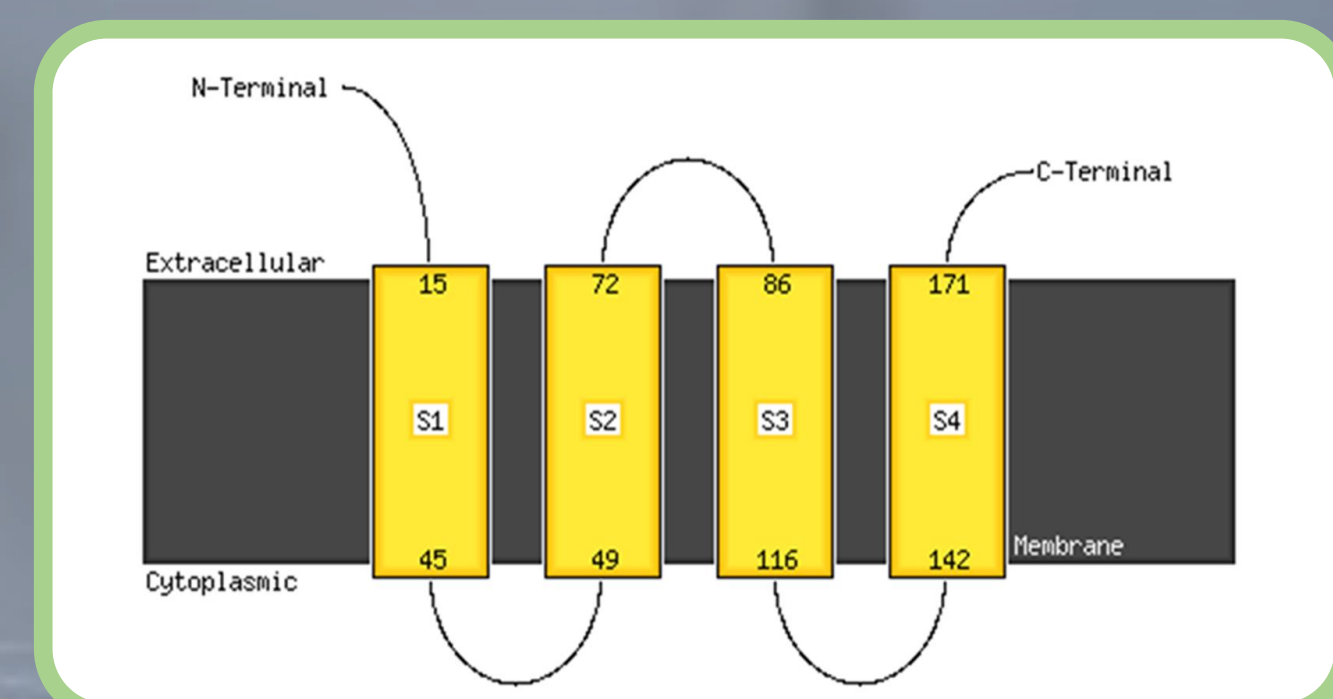


- Based on several selection tests, codons 102, 118, and 148 were shown to be under positive selection, as confirmed by at least one of the selection tests applied (Table 2).

Table 2. Codons under positive selection on *MT-ND6* gene

codon	selection test	Nucleotide positions	amino acid substitution	protein region	haplotype	species
102	individual site (DataMonkey - MEME)	304, 305, 306	Leu→Met	transmembrane	H26	<i>L. europaeus</i>
148		442, 443, 444	Tyr→Ser	transmembrane	H13	<i>L. europaeus</i>
118	branch site (TreeSAAP)	352, 353, 354	Phe→Leu	loop	H1	<i>L. townsendii</i>
					H9	<i>L. sinensis</i>
					H17	<i>L. europaeus</i>

- Codons were predicted to be within the transmembrane domains, while one codon was shown to be within the loop region.



- TreeSAAP analysis showed that the amino acid change in the loop region, codon 118, altered the equilibrium constant (ionization of -COOH) to a moderately radical magnitude (category 8, $p \sim 0.001$).
- Changes in codons 102 and 148 haven't shown any significant effect of the observed amino acid substitutions on the function of the *MT-ND6* protein.

CONSLUSION

Positive selection was observed at three codons in the *MT-ND6* gene in hares, whereas no pronounced effect of amino acid changes in codons under selection on the structure and function of the encoded proteins was detected. Presence of codons evolving under the positive selection suggest that the evolution of *MT-ND6* gene in hares may be shaped by adaptations, with different environmental pressures being the most likely triggers.