



# The Genetics of brown hare or

## How a simple question can result to a 12-year of theoretical and applied research

(including Population and Conservation Genetics,  
Phylogeny, Phylogeography, Evolutionary patterns ...  
etc)



Τμήμα

Βιοχημείας &

Βιοτεχνολογίας

Πανεπιστημίου Θεσσαλίας

Department

of Biochemistry &

Biotechnology

University of Thessaly

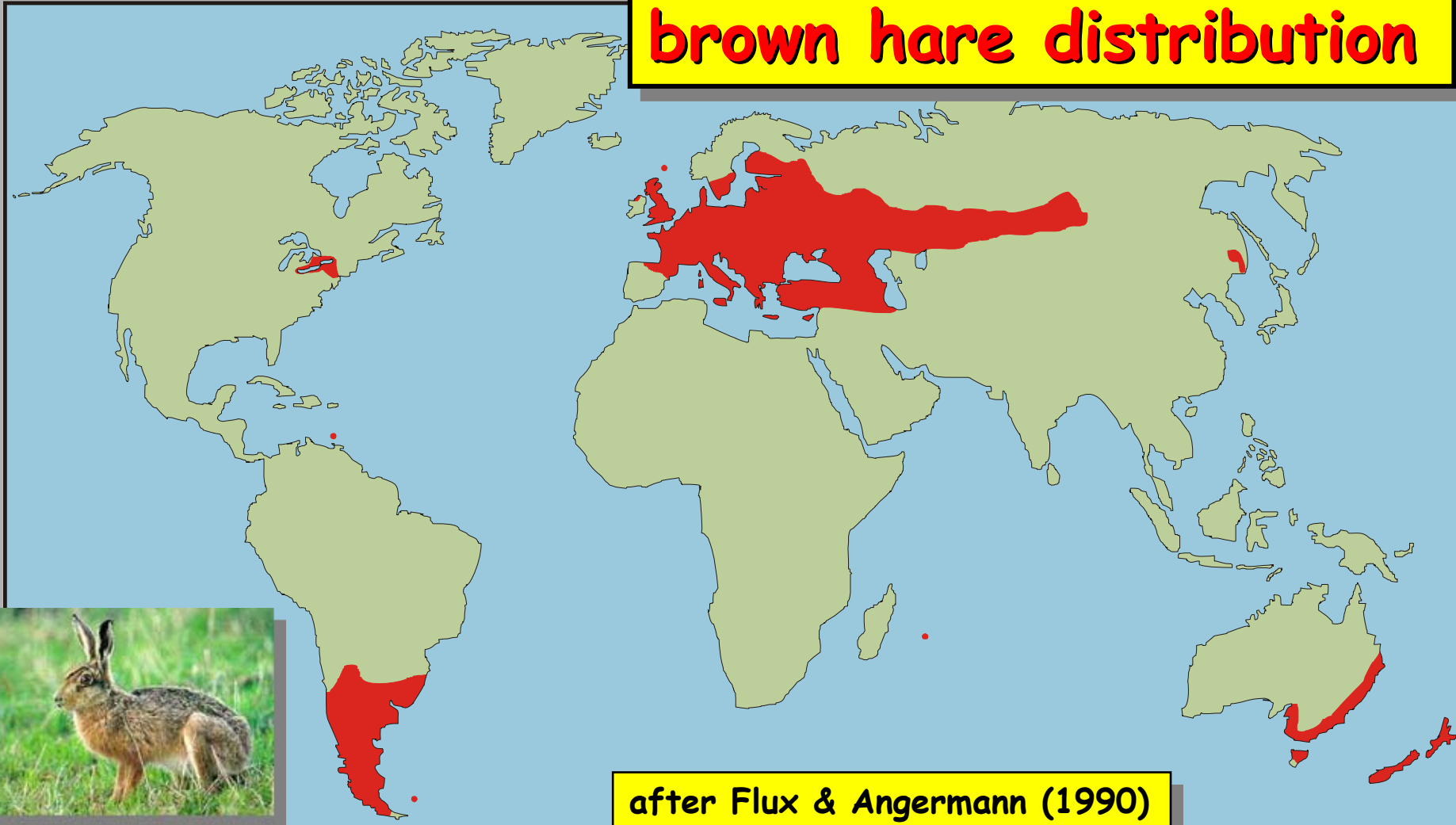
# Why molecular markers in ecology?

- They are available in increasing quantity and quality
- Classic ecological procedures are often time consuming and not easy to apply in all circumstances
- Gene products are not visible by standard ecological methods, but play a significant role in ecology, evolution and behavior of animals
- Molecular markers are:
  - Inherent to the individual (they can't be lost)
  - Inheritable (you can identify progeny)
  - Non-destructive sampling (you don't need to kill the animal -or not always!)

Brown hare *Lepus europaeus* Pallas, 1778 is an important small game species in Europe originated on the open steppe grasslands of Eurasia, adapted very successfully to mixed arable agriculture

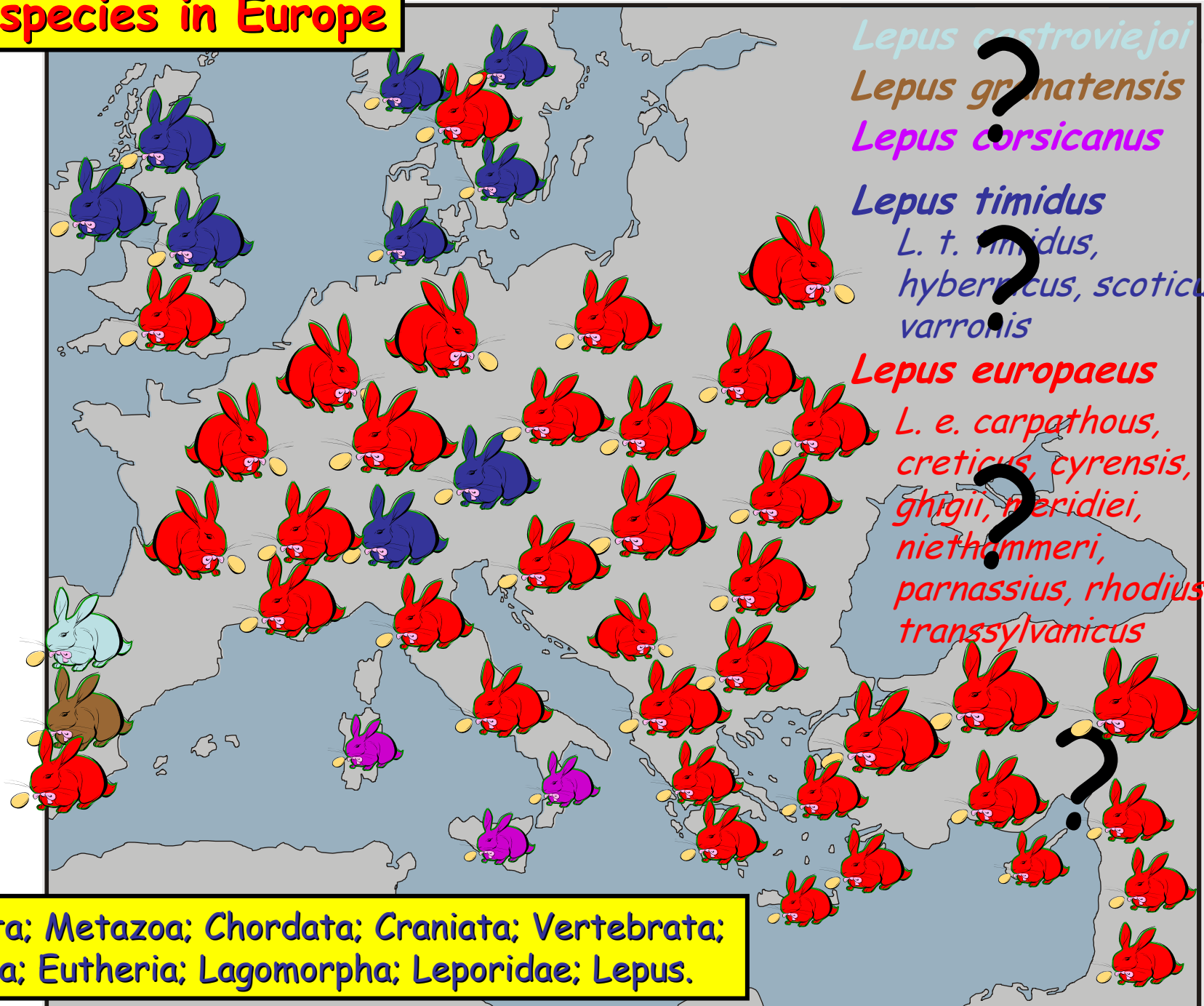
It has been introduced to many parts of the world (Australia, Argentina)

## brown hare distribution



after Flux & Angermann (1990)

# Lepus species in Europe



*Lepus castroviejoi*

*Lepus granatensis*

*Lepus corsicanus*

*Lepus timidus*

*L. t. timidus*,  
*hybernicus*, *scoticus*,  
*varronis*

*Lepus europaeus*

*L. e. carpathous*,  
*creticus*, *cyrensis*,  
*ghigii*, *meridiei*,  
*niethammeri*,  
*parnassius*, *rhodius*,  
*transsylvanicus*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;  
Mammalia; Eutheria; Lagomorpha; Leporidae; Lepus.

# Brown hare populations decline

European hare hunting bags indicate a dramatic decline during the last four decades in several European countries.

The most likely factors responsible for the long-term decline are:

- Changes in agricultural management,
- heavy hunting pressure and
- diseases

As a result, brown hare is protected under Appendix III of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention) and has a Biodiversity Action Plan.

# The problem

To stabilize population decline, restocking programs have been carried out in Greece

In C. Greece, more than 3000 reared individuals were released during the decade 1991-2001 (1.000.000 €)

While simple in concept, restocking as a management tool remains controversial

Typical criticisms focus on:

- ✓ the lack of long-term quantitative information on post-release impact
- ✓ difficulty of establishing success or failure criteria and
- ✓ concerns that extensive gene flow can interfere with local adaptations


## And then on 1998

The Ministry of Agriculture decided to discover the truth about the releases, asking the simple question:

**Is it reasonable to continue with the restocking operations for the brown hare?**

Given the complete absence of data we start studying

1. The Biology (reproduction, growth, feeding etc)
2. The Ecology and
3. The Genetics



# Genetic structure of Greek brown hare populations as revealed by mtDNA RFLP-PCR, RAPD and allozyme analyses: implications for conserving genetic diversity

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# These studies aimed to examine

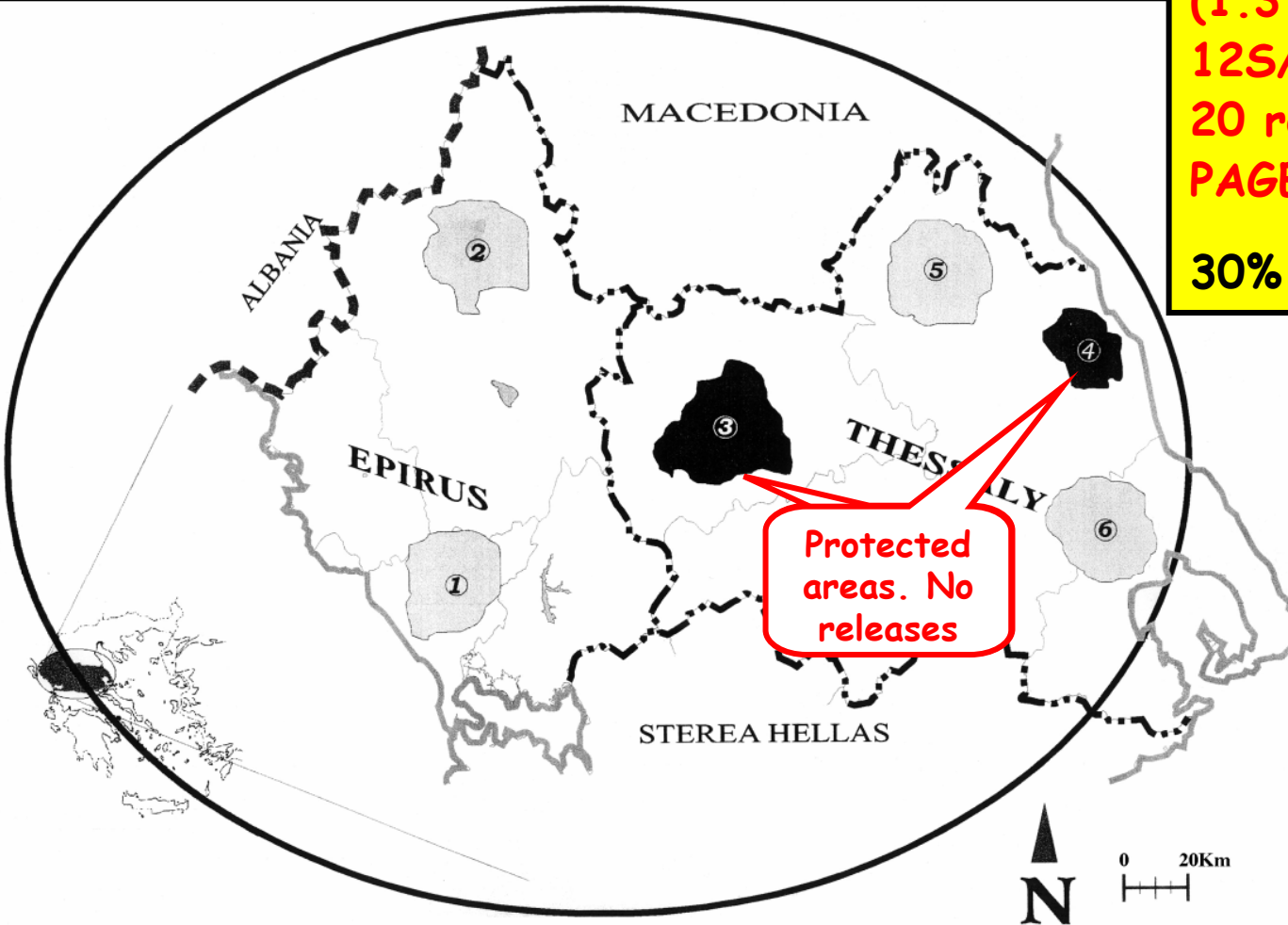
- (1) the phylogenetic status of Greek indigenous brown hares
- (2) the level of genetic diversity within and among populations
- (3) the genetic impact of releases on wild populations
- (4) The detection of genetic markers to identify the reared-released individuals

# mtDNA PCR-RFLP (Mamuris et al. 2001, Biological Conservation, 101, 187-196)

210 brown hares, 24 reared and 186 wild were examined.

CR/cytb (1.8 kb), COI (1.3 kb)  
12S/16S rRNA (2.05 kb)  
20 restriction enzymes,  
PAGE

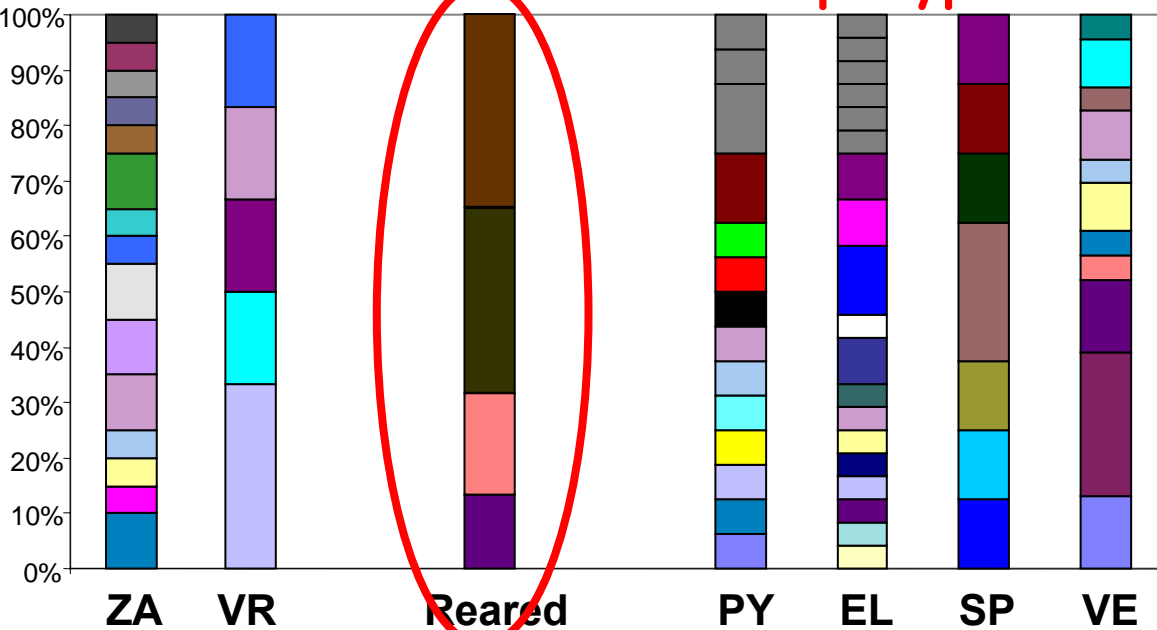
30% of the mtDNA



Brown hare  
sampling sites:

- (1) Zallogo (ZA);
- (2) Vradeto (VR);
- (3) Pyrra (PY);
- (4) Spilia (SP);
- (5) Ellassona (EL);
- (6) Velestino (VE).

# Distribution of 56 haplotypes. Each color represents a different haplotype



- 56 different haplotypes

- Very high genetic mtDNA diversity  
42 haplotypes were unique, i.e. observed only in one population, and 14 were common between populations

- Reared = same species

The most interesting finding:  
Haplotypes of the reared hares were genetically different from the "Greek" haplotypes found in the wild



# RAPD (Mamuris et al. 2002, Biochemical Genetics, 40, 323-338)

Brown hare sampling sites within Greece: (1) Zallogo (2) Vradeto (3) Pyrra (4) Spilia (5) Ellassona (6) Velestino (VE).

327 brown hares, 26 reared and 301 wild were examined.

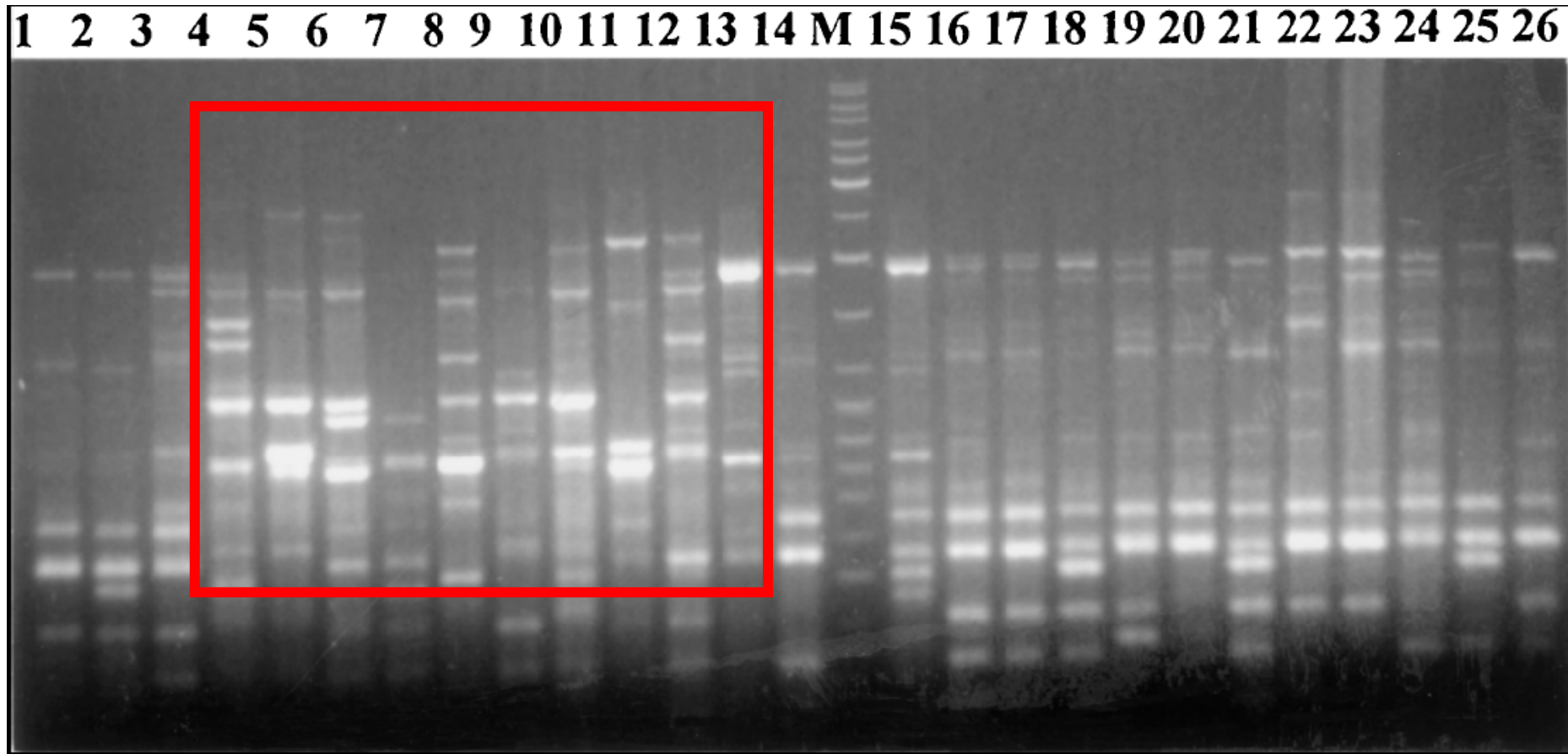
172 belonged to central Greece (Epirus and Thessaly)



The remaining 129 wild hares came from Austria, Poland, Germany, France, and Bulgaria

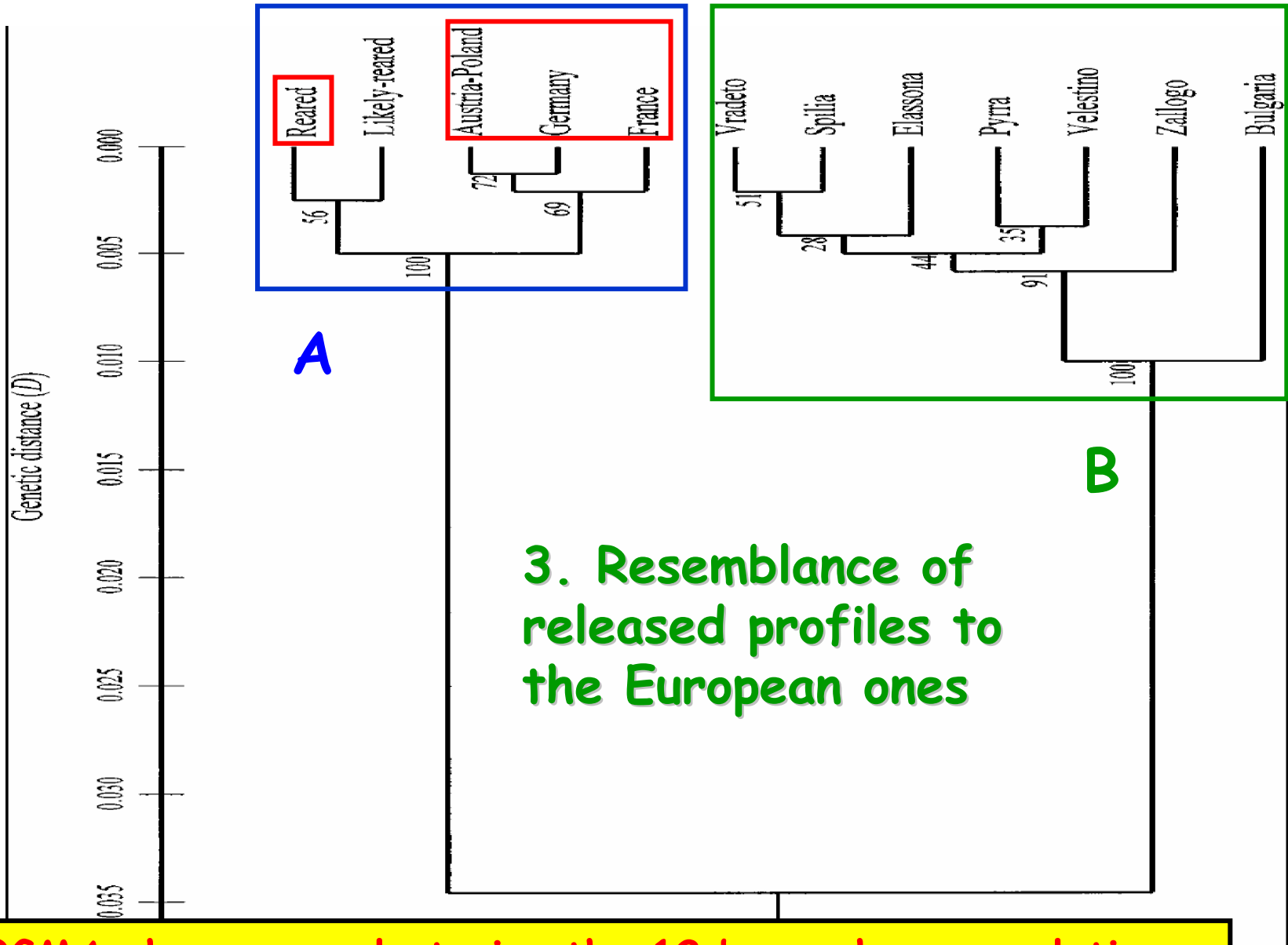
Forty decamer primers were used

# Three major findings: 1. Existence of deviant profiles



RAPD profiles after amplification with the primer OPA-02. 1, 2, 3, and 14-26, common RAPD profile of some individuals from all populations. 4-13, individuals with "deviant" profiles from reared (4, 5), "likely-reared" (6, 7, 8, 9), and EL (10, 11, 12, 13) populations. *M* = 100 bp ladder

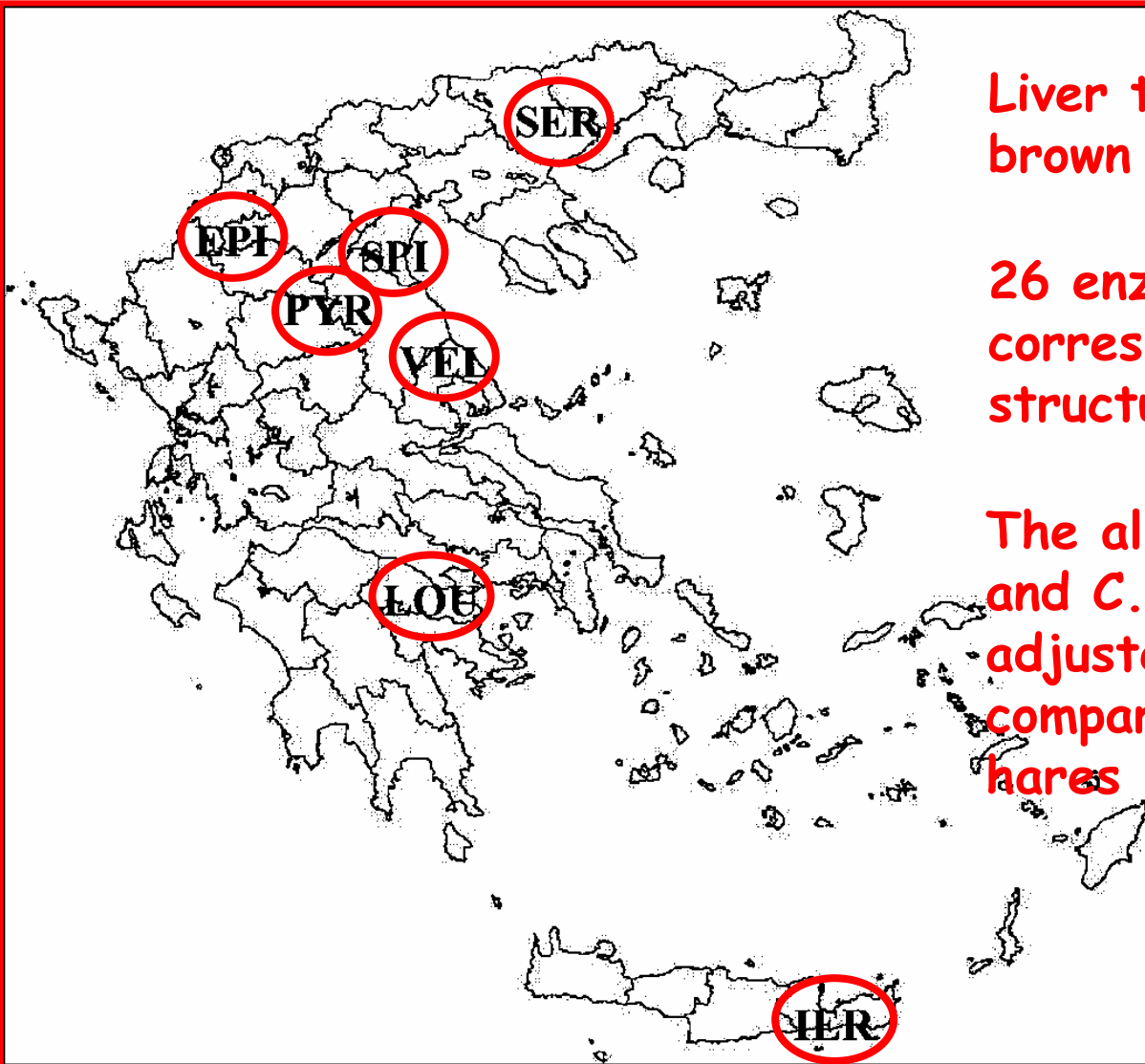
## 2. Genetic Differences between C. Europe and Greece



UPGMA phenogram clustering the 12 brown hare populations

# Allozymes (Suchentrunk et al. 2003, Biochemical Genetics, 41,127-140)

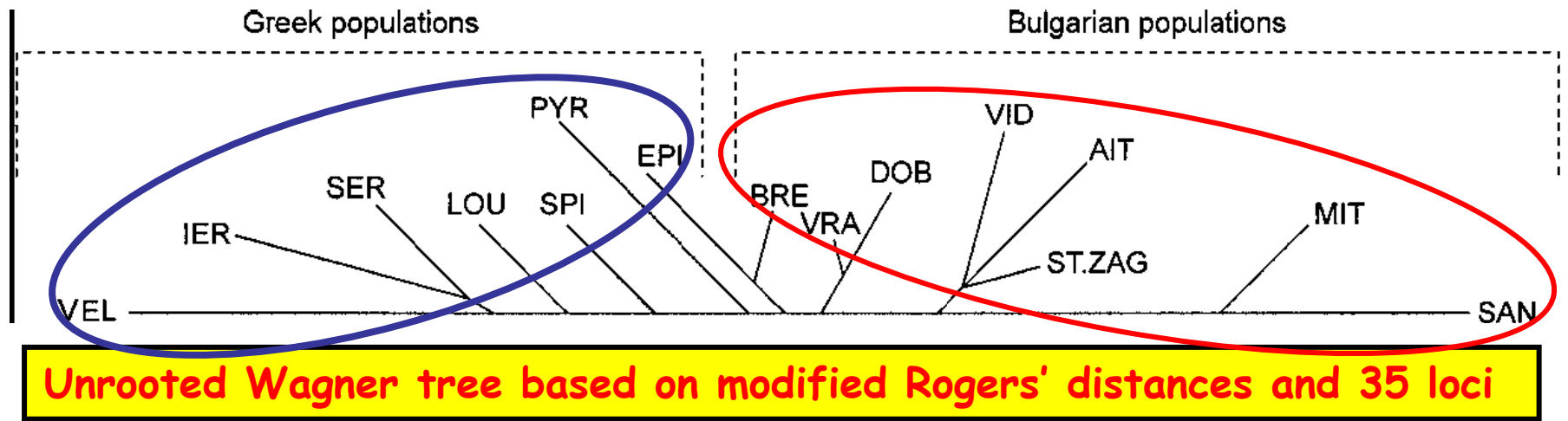
Locations of study populations of brown hares in Greece: *EPI* = Epirus, *IER* = Ierapetra, *LOU* = Loutraki, *PYR* = Pyrra, *SER* = Serres, *SPI* = Spilia, *VEL* = Velestino.



Liver tissue samples of 91 brown hares

26 enzymes/enzyme systems corresponding to 35 putative structural gene loci

The allozyme data of Bulgarian and C. European hares were adjusted to the 35 loci and compared with the Greek hares



- ✓ Three alleles, *Es-1*<sup>162</sup>, *Pep-2*<sup>114</sup> and *Mp*<sup>88</sup>, detected in the Greek hares with low frequencies, were absent in brown hares from C. and S. Europe.
- ✓ On the other hand, the alleles *Sdh*<sup>300</sup>, *Ldh-2*<sup>83</sup>, and *Pep-2*<sup>94</sup> are present in Bulgaria and C. Europe at low frequencies, respectively, but absent in Greece
- ✓ This distributional pattern of alleles indicates some gene pool differentiation both between Greek and Bulgarian hares and between Greek and C. European populations



# General Conclusions

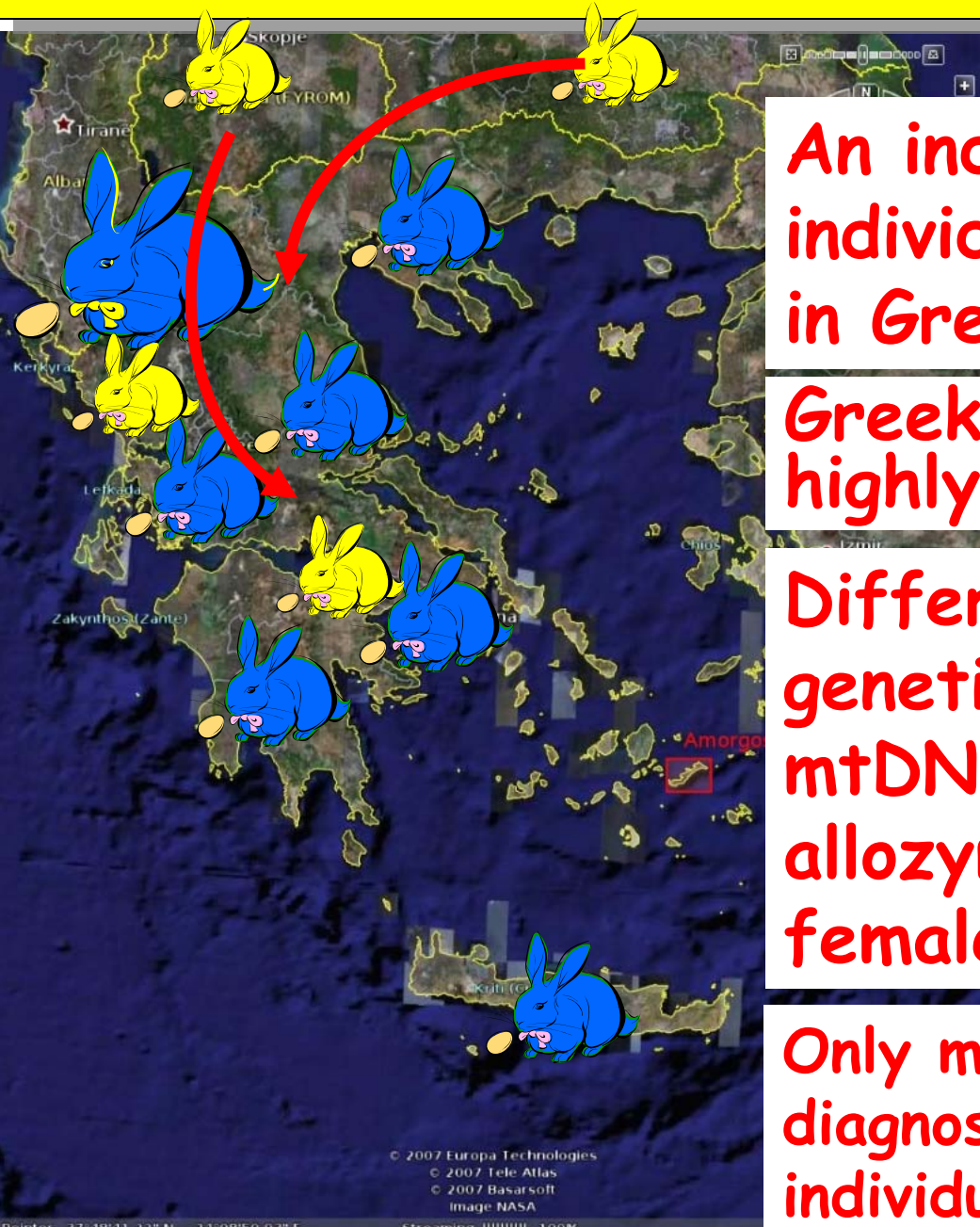
Brown hares in Greece form one species

An indication that reared individuals were introduced in Greece from abroad

Greek brown hares are highly polymorphic

Different patterns of genetic distribution between mtDNA and nDNA (RAPD, allozymes) probably due to female philopatry

Only mtDNA could provide diagnostic markers to identify individuals from different regions



## The next step

- The study of brown hare genetic stocks for the whole of Greece to determine native genotypes and to record the present level of polymorphism
- The study of the level of genetic divergence and variability between the Greek, European and Anatolian hares to determine the interaction between populations and to identify the source of the reared individuals

Collection of samples from Greece, Europe, Turkey and Israel

However, in 2001 the Ministry of Agriculture, based on our report, officially (...and unexpectedly) suspended the restocking operations in Greece

..... and based on the same report the Ministry suspended also the releases of the wild boar!!!!!!



Phylogeography of European brown hares, *Lepus europaeus*, in Europe: a legacy of southeastern Mediterranean refugia?

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Stamatis et al., 2008, *Journal of Biogeography*, 36: 515-528

**926 BROWN HARES**  
from  
**25 SAMPLE REGIONS**

**mtDNA PCR-RFLP:**

CR/cytb (1.8 kb), COI (1.3 kb)

12S/16S rRNA (2.05 kb)

20 4-, 5-, 6-base cutters, PAGE

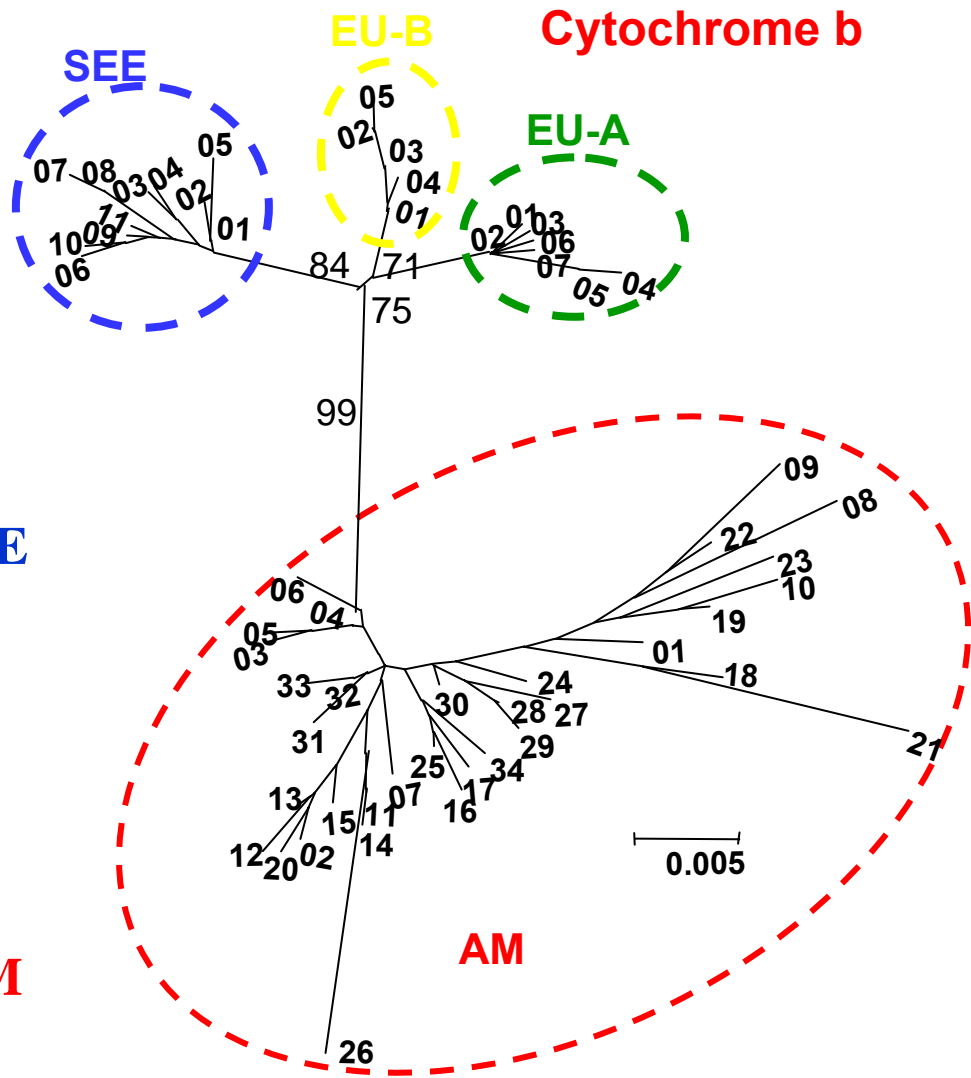
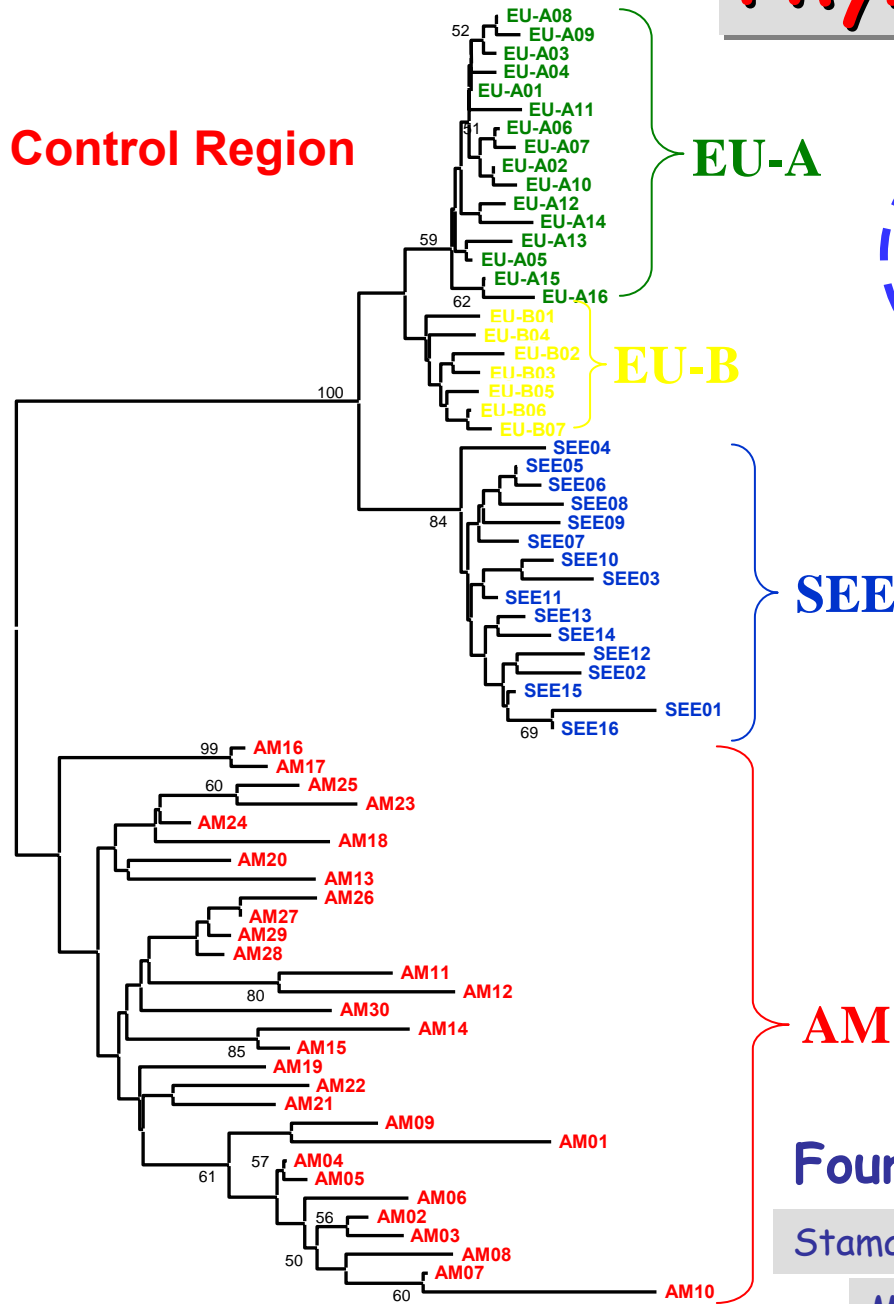
**Direct sequencing of the CRI**

**75 individuals from all areas**

- **Our study, 926 hares**
- ★ **Kasapidis et al. 2005**  
72 seq, 98 hares
- ★ **Pierpaoli et al. 1999**  
22 seq, 41 hares
- ★ **Fickel et al. (direct submission)**  
42 seq



# Phylogenetics of mtDNA



Four phylogenetic groups are formed

Stamatis et al., 2008, *Journal of Biogeography*

Mamuris Z. et al. 2008, *Mammalian Biology*

# Network of the 112 haplotypes of PCR mtDNA-RFLPs

Intermediate Bulgarian haplogroup

Southeastern European haplogroup

Anatolia/Middle East

SEE

AM

Central European haplogroup

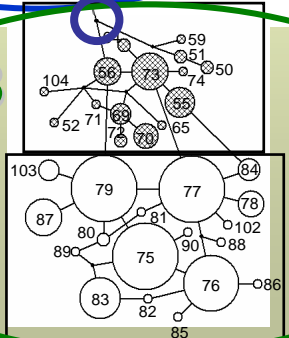
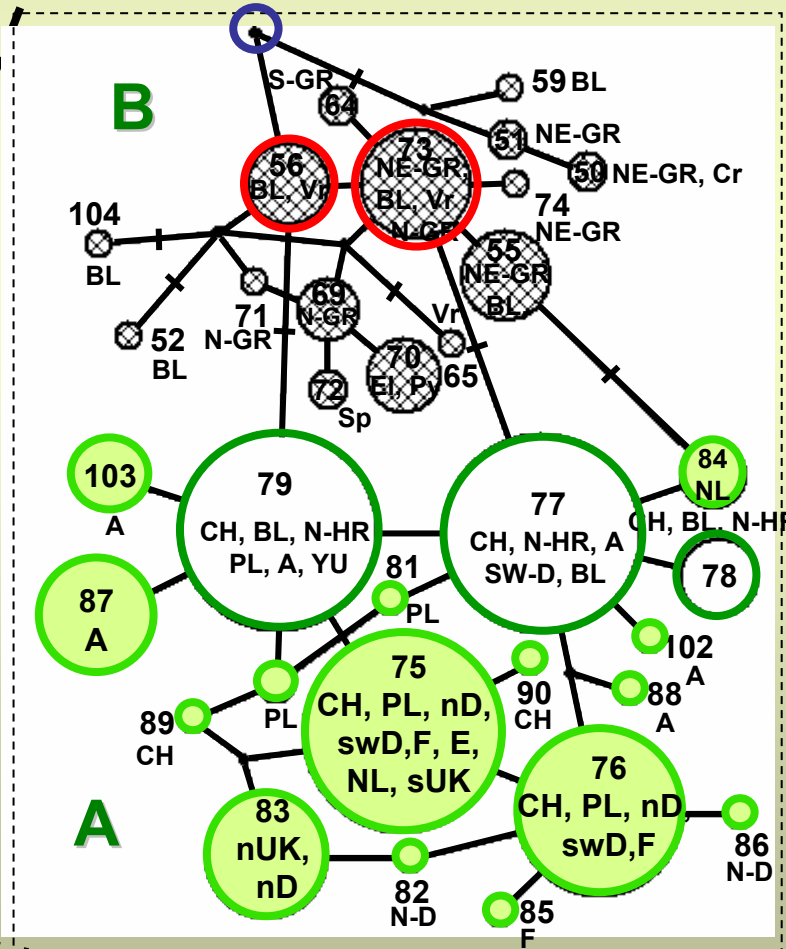
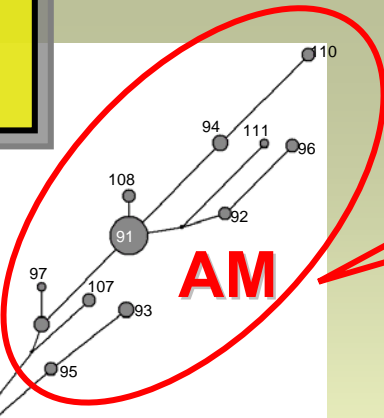
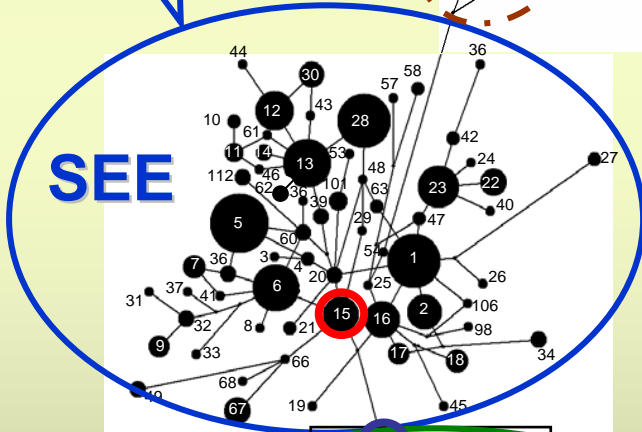
B

EU

A

B

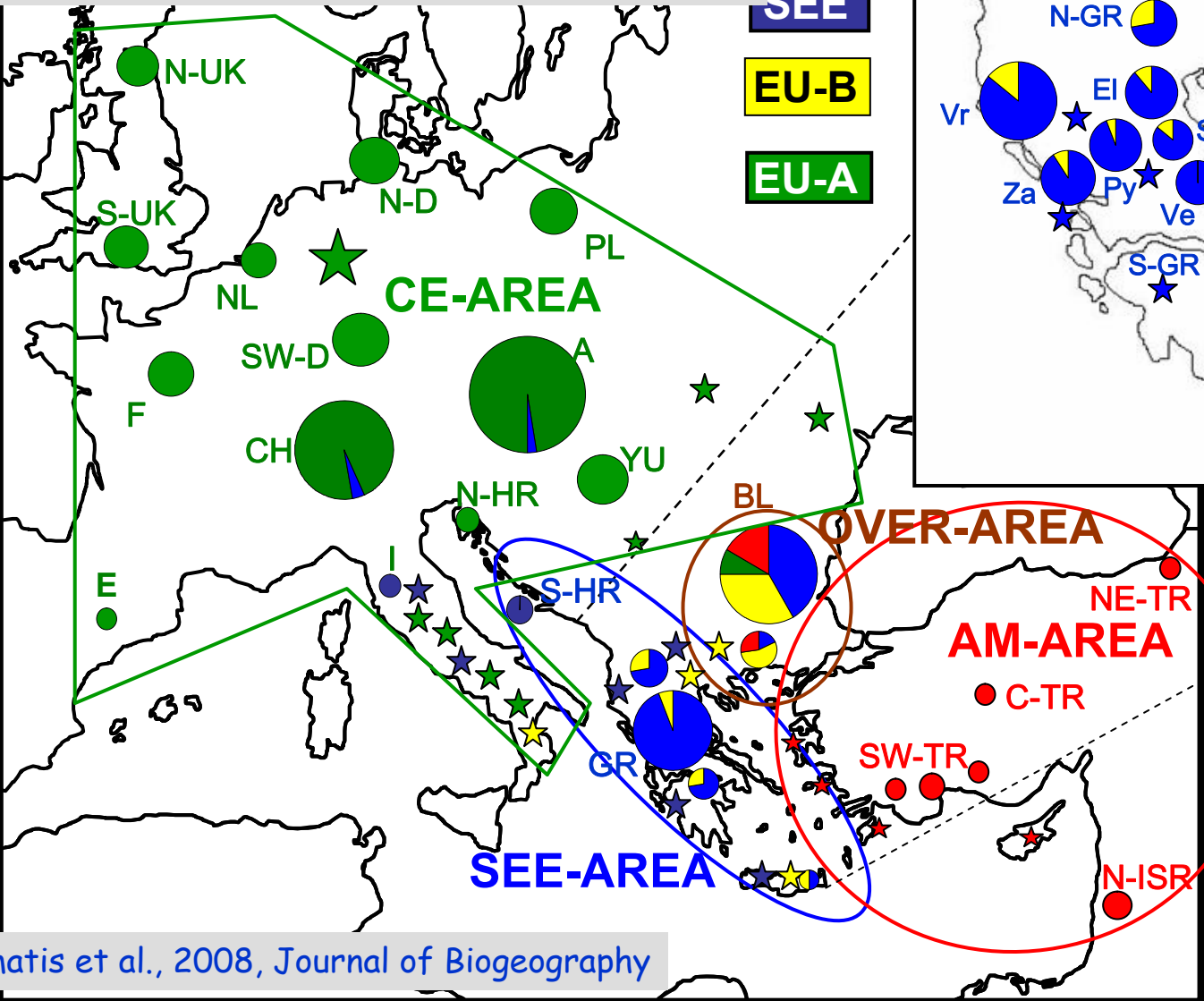
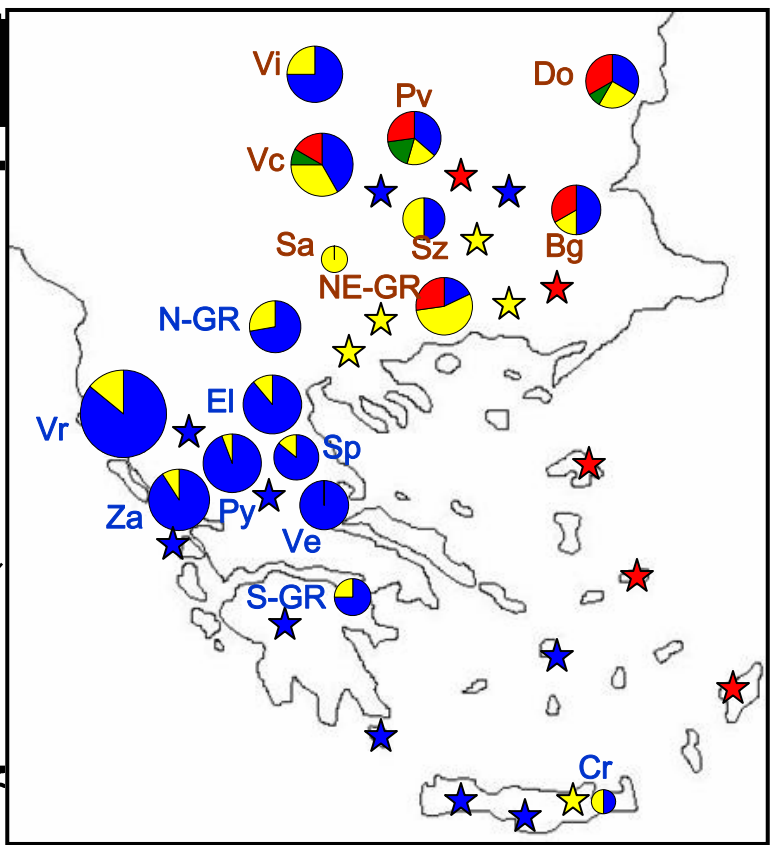
A



# Brown hare haplotype distribution

Pierpaoli et al. 1999, *Mol Ecol*, 8, 1805-1817  
 Fickel et al. 1999, *Zeit Jagdwiss*, 45, 230-237  
 Kasapidis et al. 2005, *Mol Phyl Evol*, 34, 55-66

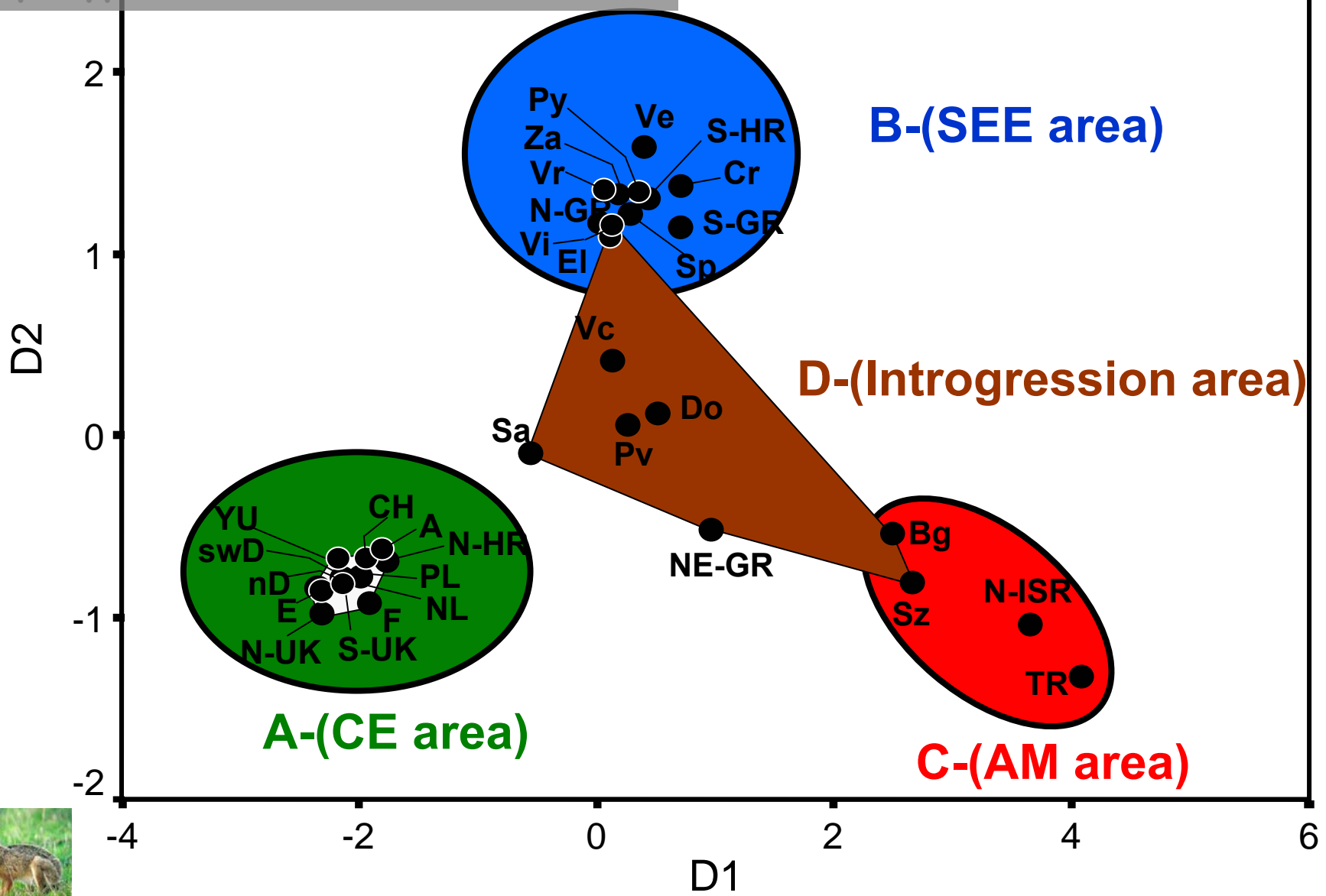
**AM**  
**SEE**  
**EU-B**  
**EU-A**



Stamatis et al., 2008, *Journal of Biogeography*

Multidimensional Scaling of the matrix of pairwise distances based on nucleotide divergence of the 112 haplotypes

stress = 0.02885, RSQ = 0.99656







Recruitment of mitochondrial tRNA  
genes as auxiliary variability  
markers for both intra- and inter-  
species analysis: The paradigm of  
brown hare (*Lepus europaeus*)

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**H. Sert**

Akdeniz Universitesi, Antalya, Turkey

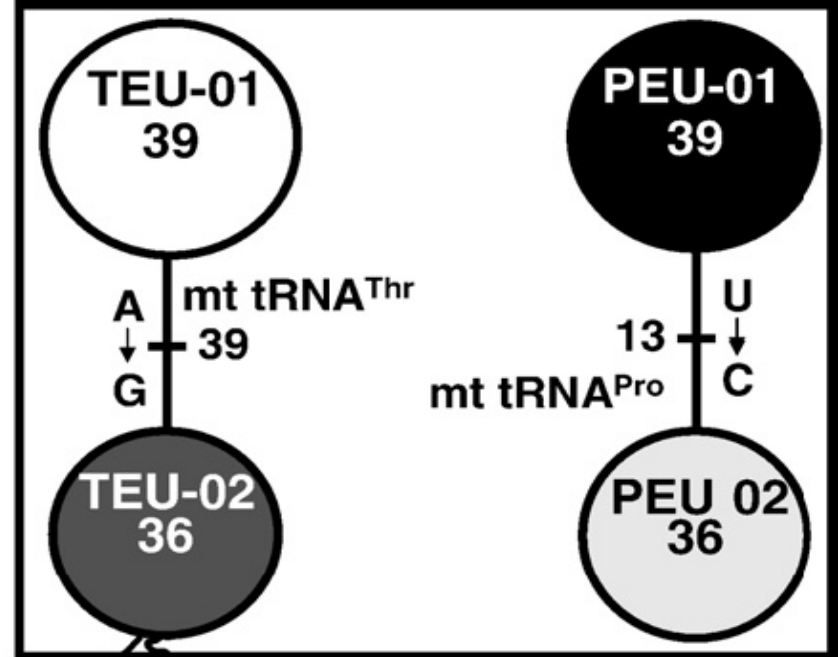
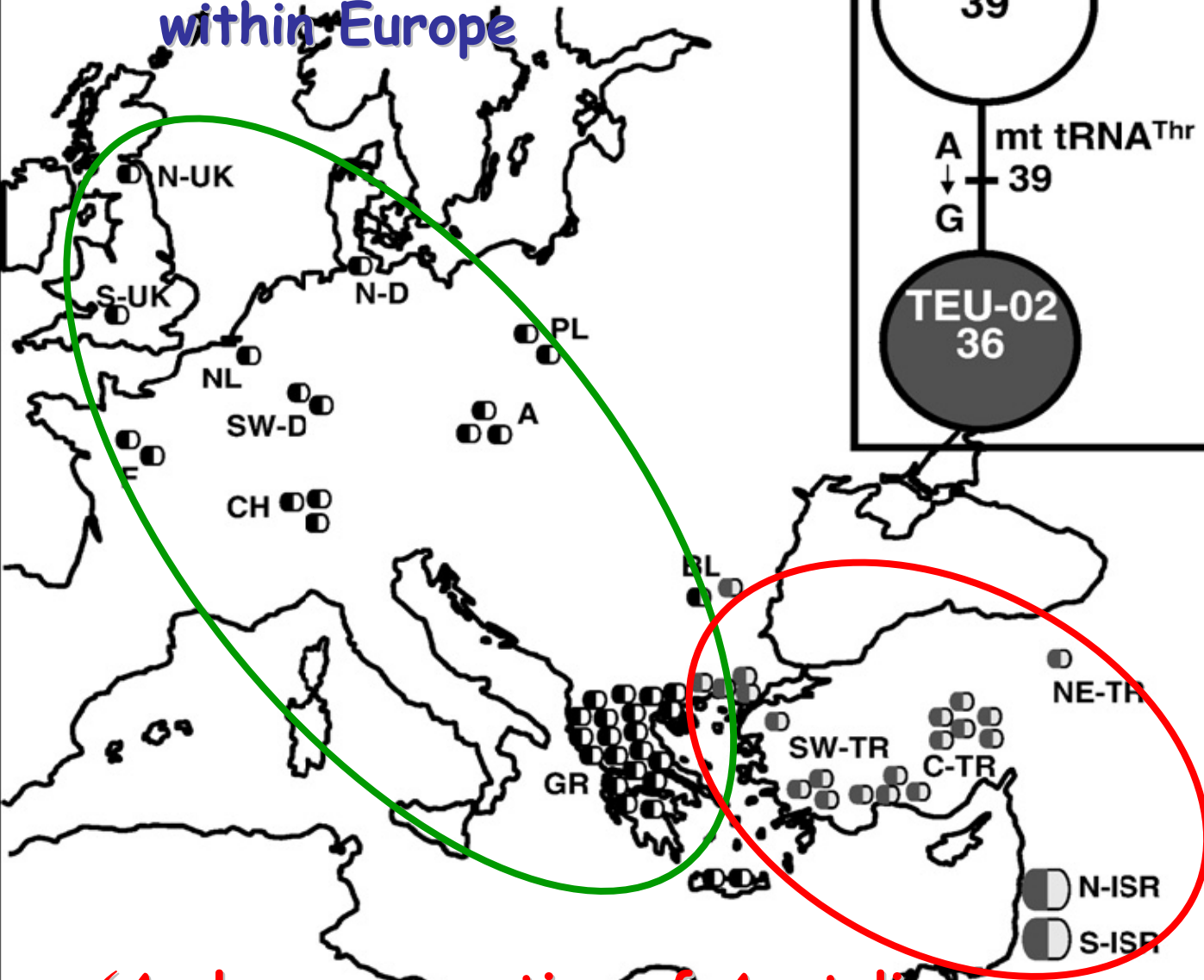
**F. Suchentrunk**

Research Institute of Wildlife Ecology, Vienna Veterinary  
University, Austria

(Stamatis et al., 2008, *Gene* 410 154-164)

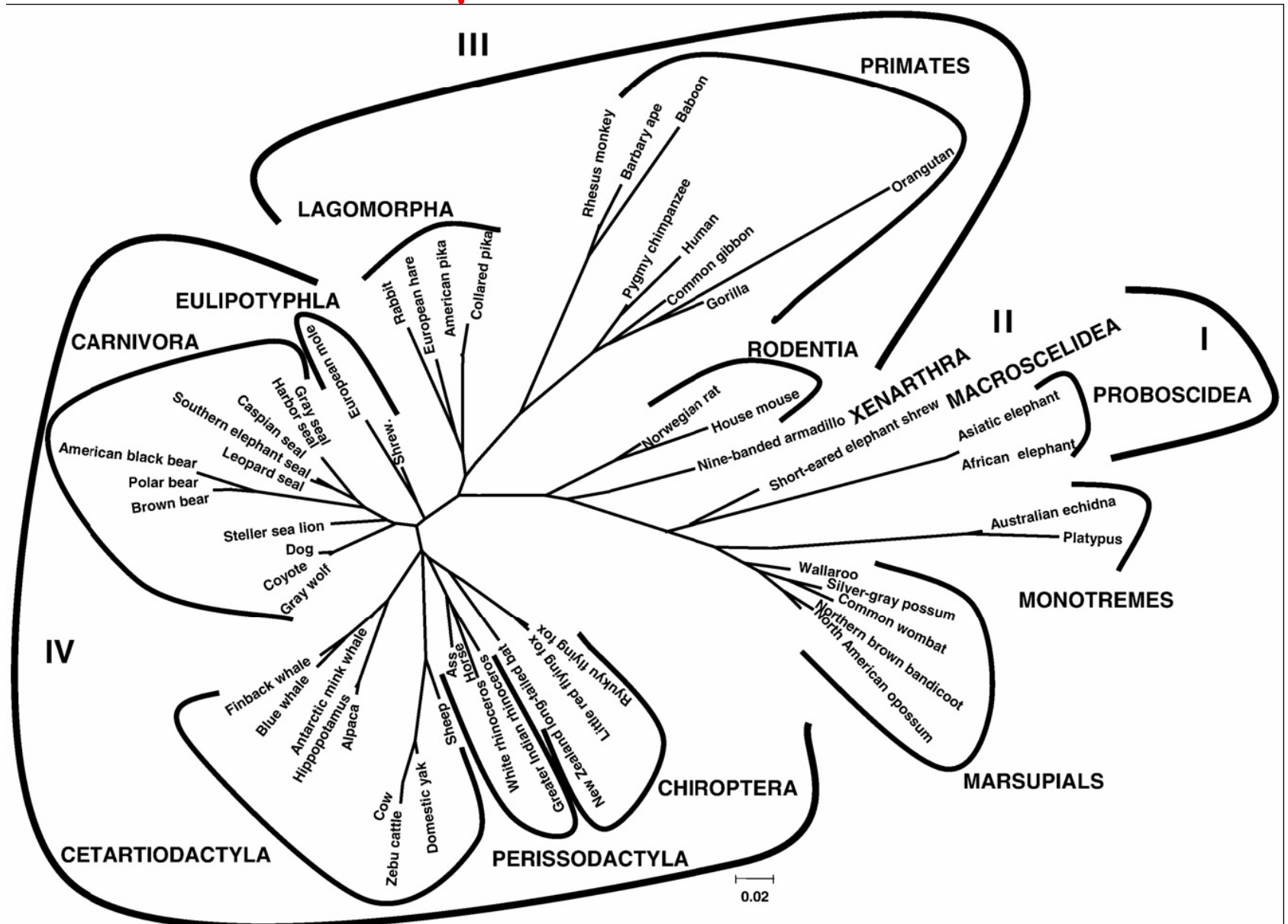


✓ No divergent lineages within Europe



✓ A clear separation of Anatolian and European lineages

# Phylogenetic tree based on Bayesian analysis of the combined sequences of the tRNAThr and tRNAPro genes from 55 different species retrieved in the GenBank



However, our mtDNA analysis based on five segments, each one digested with 20 restriction enzymes, was time- and money-consuming to continue monitoring Greek regions

Therefore, we identified diagnostic RFLP markers on the mtDNA, introducing a quick and inexpensive method of distinguishing different hare haplogroups.

Such a method provides the potential of a time- and money-wise monitoring scheme with a depth of time and a large number of individuals.

A photograph of a brown hare (Lepus europaeus) in a grassy field. The hare is the central focus, with its long ears and brown fur clearly visible. The background is a soft-focus green field.

# Genetic evidence for survival of released captive-bred brown hares (*Lepus europaeus*) during restocking operations in Greece

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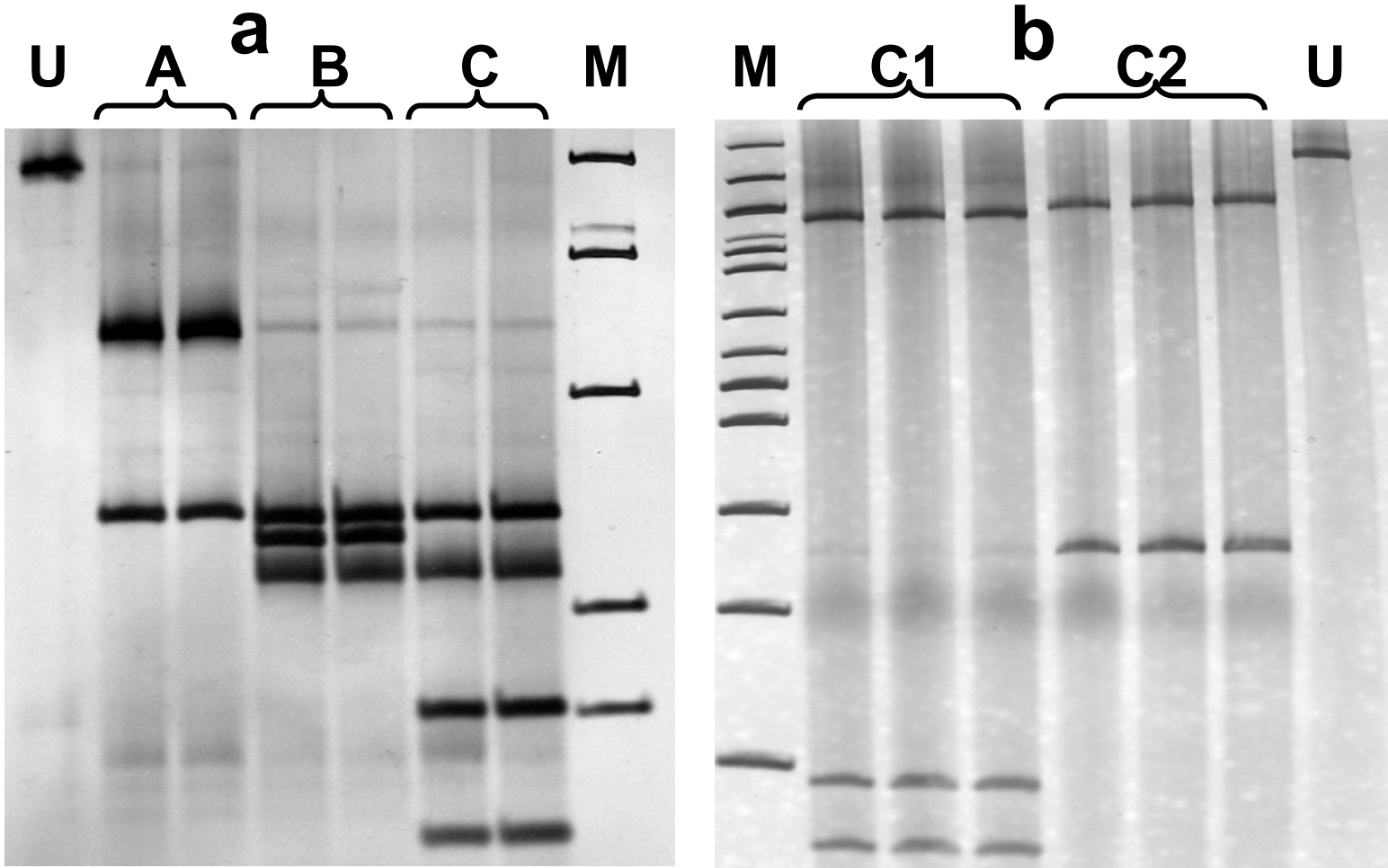
Hakan Sert

Akdeniz Universitesi, Antalya, Turkey

Costas Triantaphyllidis

School of Biology, Aristotle University of Thessaloniki, Greece

(Stamatis et al., 2007, *Oryx*, 41: 548-551)



A=AM

B=SEE

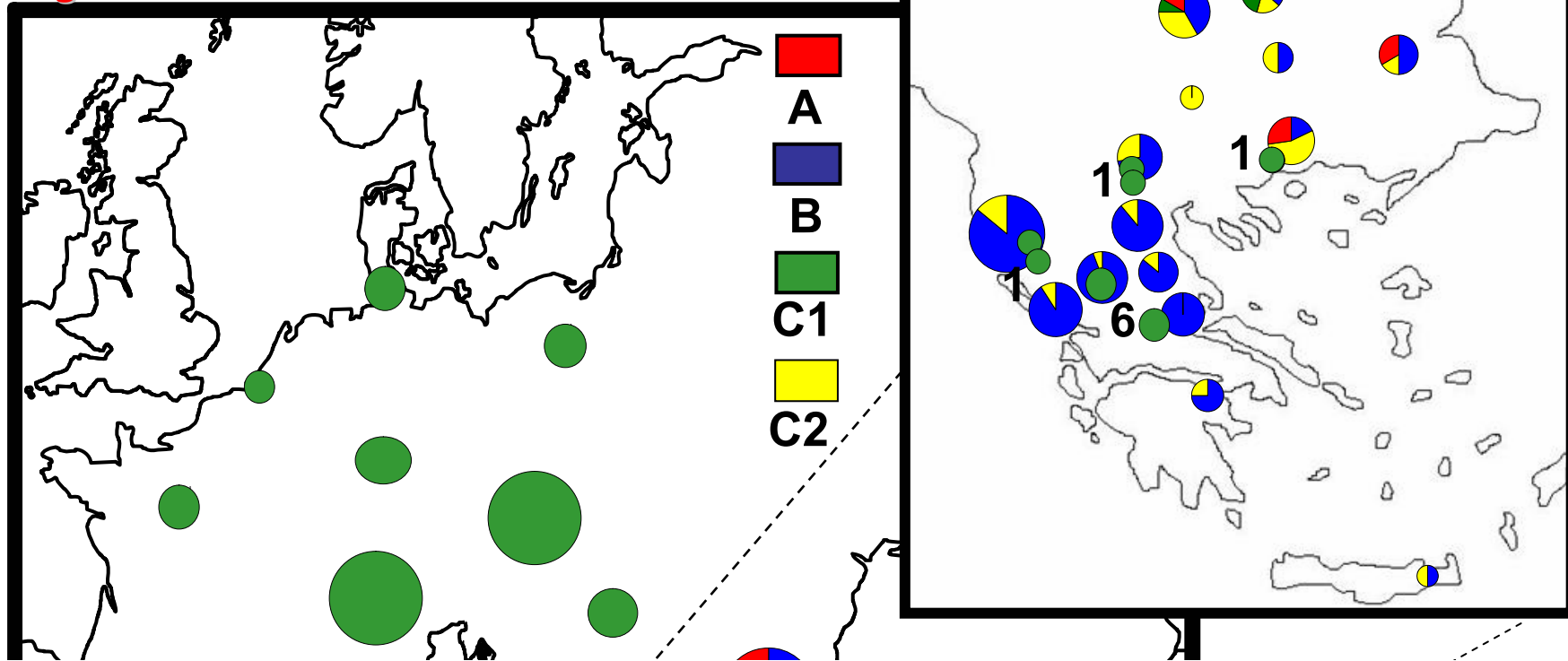
C1=EU-A

C2=EU-B

(A) Digestion of Cytb segment with *AluI* and *HinfI* produced three different profiles referred to as A, B and C. (B) Individuals with profile C was further differentiated in C1 and C2 after the digestion of COI segment with *HhaI*. U=undigested DNA, M=100bp ladder.

To validate the introduced method, both analyses were used in a large proportion of the samples to confirm the results. The diagnostic enzymes permitted the allocation of individuals safely in the different haplogroups.

The sites sampled and the occurrence of the mtDNA profiles, throughout sampling area.




Until 2003, after the analysis of more than 400 individuals, we had never detected in the wild brown hares bearing the profile C1, even in regions intensively surveyed genetically (central Greece).

However, during the monitoring of the years 2003 and 2004 we detected nine brown hares (four females, one male and four of unidentified sex) mainly from central (seven) and northern Greece (two), having the profile C1.



But surprisingly, studying brown hares from Switzerland we realized that few genotypes did not correspond to brown hare

Therefore, we decided to check more careful this mismatch, analyzing also the only other hare species found in Switzerland, the mountain hare, *Lepus timidus*



**Bi-directional introgressive hybridization between Alpine mountain hares, *Lepus timidus varronis*, and brown hares, *L. europaeus***

by **Z. Mamuris & C. Stamatis**  (Larissa, GR),  
Department of Biochemistry and Biotechnology, University of Thessaly

**F. Suchentrunk**  (Vienna, A),  
Research Institute of Wildlife Ecology, Vienna Veterinary University

in collaboration with  
**M. Giacometti & G. Haehrer**  
(Berne, Stampa, CH)



# Range of brown hares and mountain hares in Switzerland (from Hausser 1995: Swiss Mammals Atlas)

Distribution  
*L. europaeus*



Distribution  
*L. timidus*

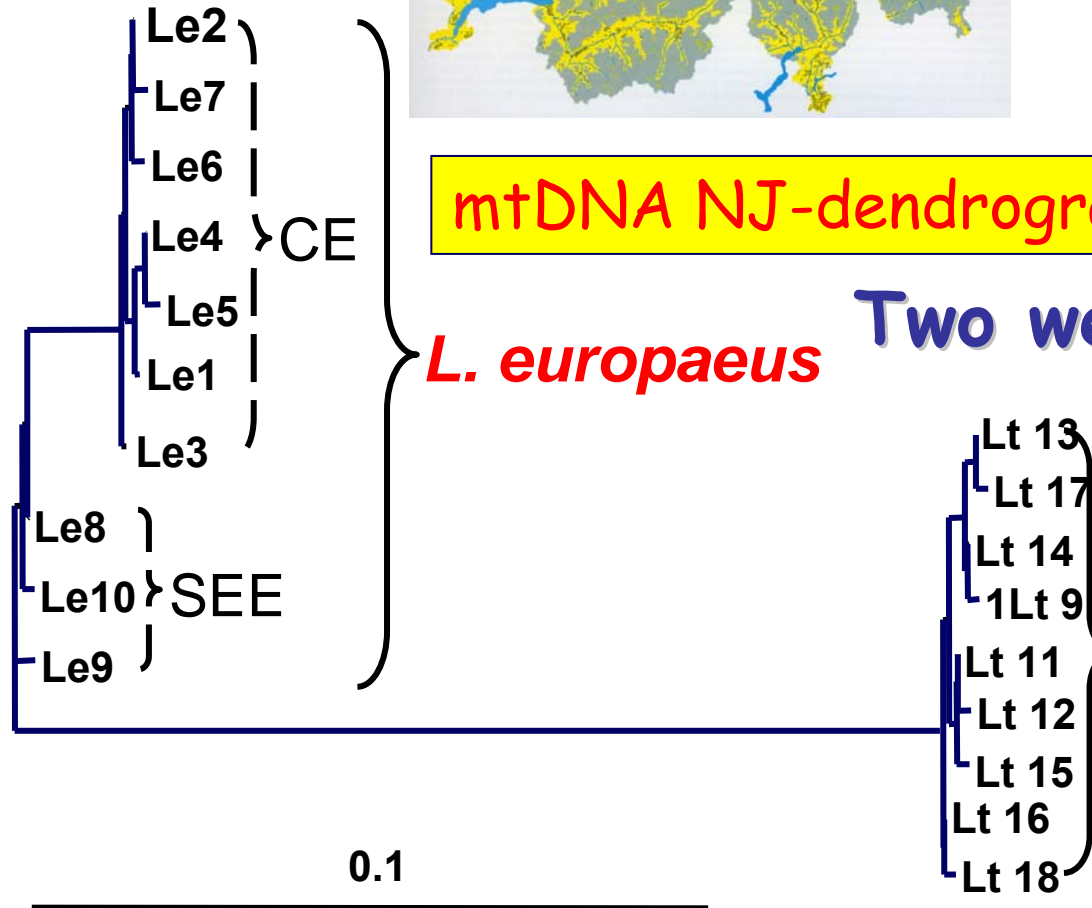


mtDNA NJ-dendrogram

Two well established species

*L. europaeus*

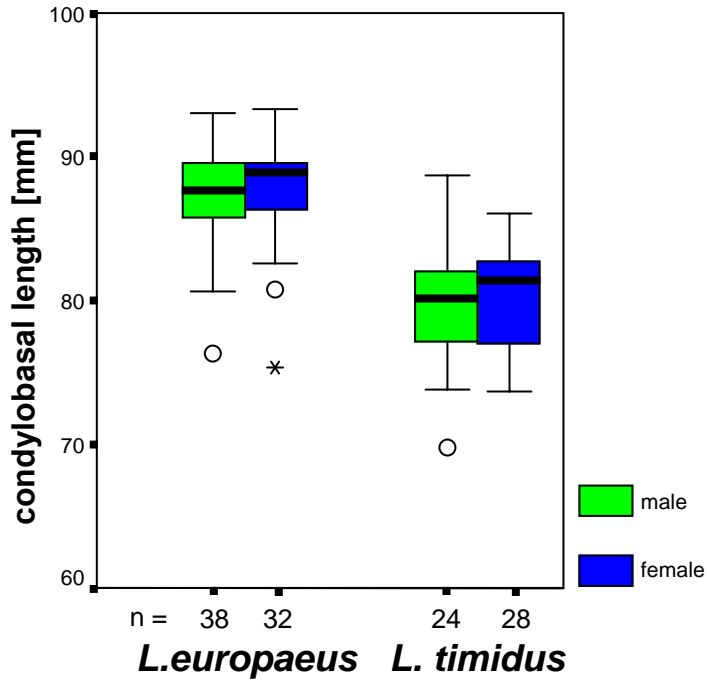
*L. timidus*



# Phenotypes of brown hares & mountain hares from Switzerland

bigger body size in brown hares

relatively longer ears in brown hares



GLM, type III sum of squares, full factorial for CBL (AD hares only)

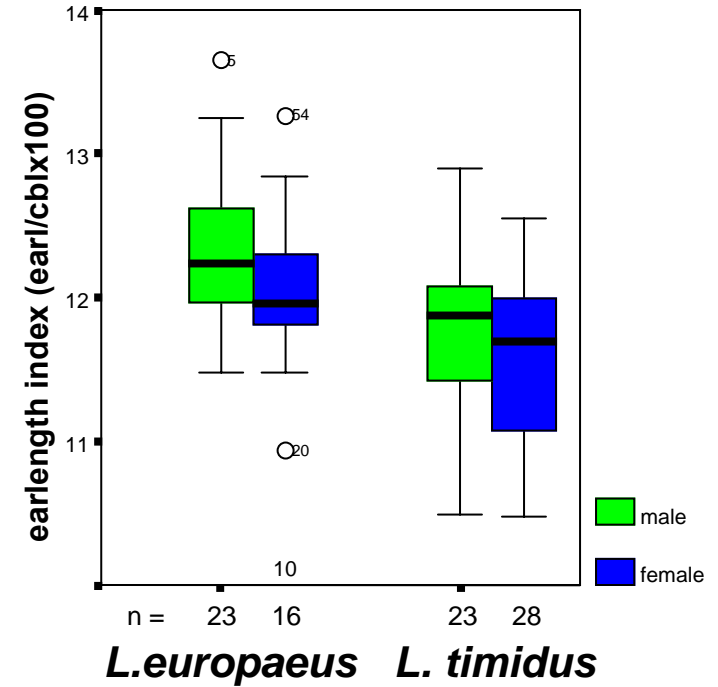
Tests of Between-Subjects Effects

Dependent Variable: condylobasal length

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power <sup>a</sup>
Corrected Model	1010,734 <sup>b</sup>	3	336,911	58,964	,000	176,891	1,000
Intercept	619377,573	1	619377,573	108398,8	,000	108398,840	1,000
<b>SPECIES</b>	<b>1005,655</b>	<b>1</b>	<b>1005,655</b>	<b>176,002</b>	<b>,000</b>	<b>176,002</b>	<b>1,000</b>
SEX	14,198	1	14,198	2,485	,118	2,485	,345
SPECIES * SEX	,439	1	,439	,077	,782	,077	,059
Error	514,249	90	5,714				
Total	699484,749	94					
Corrected Total	1524,983	93					

a. Computed using alpha = ,05

b. R Squared = ,663 (Adjusted R Squared = ,652)



GLM, type III sum of squares, full factorial for ear length index (SA & AD hares)

Tests of Between-Subjects Effects

Dependent Variable: earlength index (earl/cblx100)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9,248 <sup>a</sup>	3	3,083	8,514	,000
Intercept	12797,697	1	12797,697	35344,255	,000
<b>SPECIES</b>	<b>6,960</b>	<b>1</b>	<b>6,960</b>	<b>19,221</b>	<b>,000</b>
SEX	1,281	1	1,281	3,539	,063
SPECIES * SEX	5,830E-05	1	5,830E-05	,000	,990
Error	31,864	88	,362		
Total	13202,407	92			
Corrected Total	41,112	91			

a. R Squared = ,225 (Adjusted R Squared = ,199)

## molecular techniques:

### mtDNA PCR-RFLP:



CR/cyt b (c. 1.8 kb),

COI (c.1.3 kb)

12S / 16S rRNA (c. 2.05 kb)

20 4-, 5-, 6-base cutters,

PAGE

### Useful markers to check:

- a) The occurrence of the hybridization and
- b) The direction of the hybridization

### allozyme marker for nuclear DNA

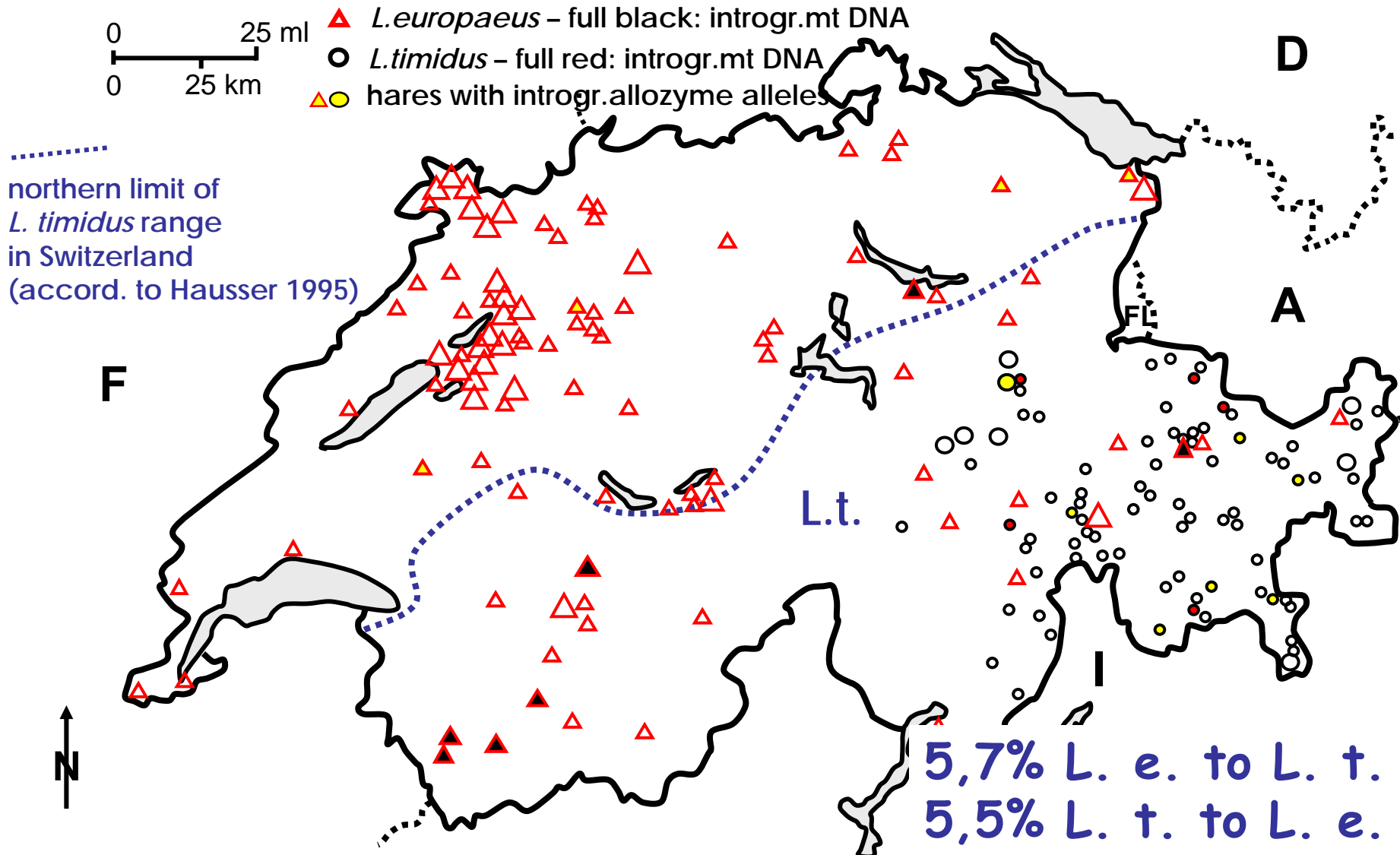


Sdh & Acp-1: diagnostic for the two species, HSGE

### Useful markers to check:

- a) The occurrence of the hybridization and
- b) The generation of the hybrids

# distribution of pure & introgressed hares



We demonstrated for the first time bi-directional hybridization between mountain and brown hares

# Y DNA and Mitochondrial lineages in European and Asian populations of the brown hare (*Lepus europaeus*).

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SARAFIDOU,  
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F. SUCHENTRUNK  
Research Institute of Wildlife Ecology, Vienna Veterinary  
University, Austria

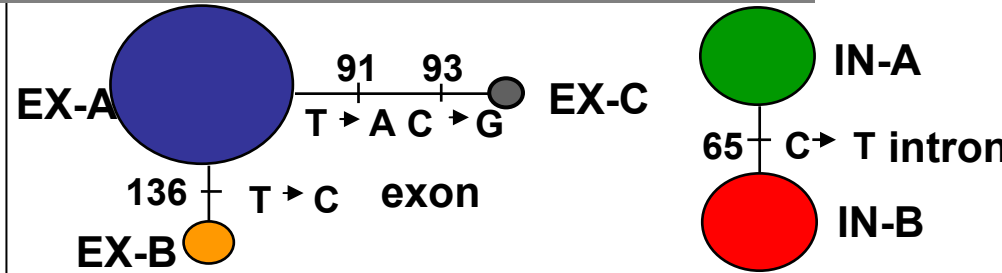
Mamuris Z. et al. (2008) *Mammalian Biology*



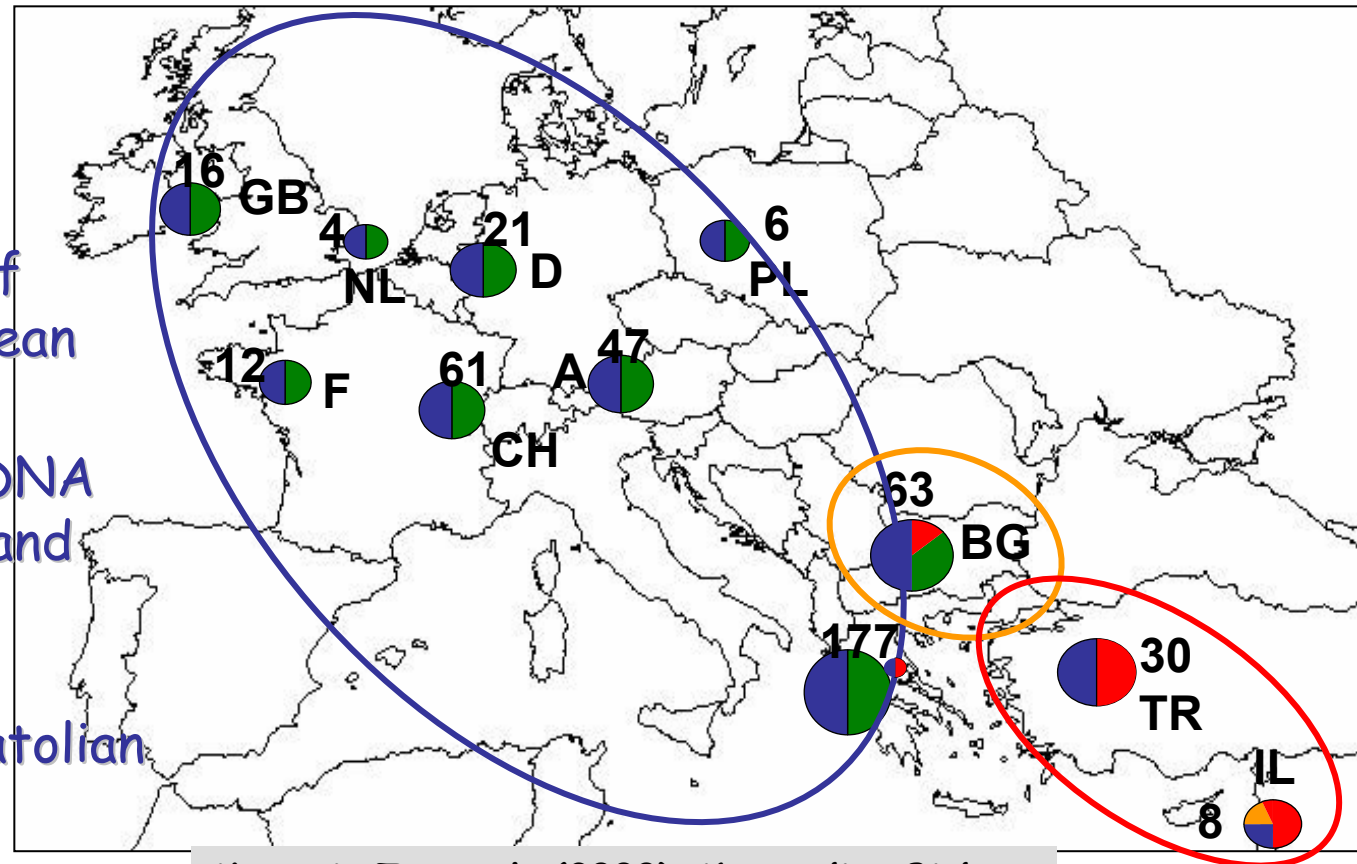
# Two networks showing the mutational relationships for Y DNA

## Y DNA variation

489 brown hares



## Distribution of sampling sites and the composition of each population for Y DNA



Mamuris Z. et al. (2008) *Mammalian Biology*

- ✓ Y markers are mostly well conserved
- ✓ No divergent lineages within Europe
- ✓ Divergent lineages within Anatolia
- ✓ A deep separation of Anatolian and European lineages
- ✓ An admixture of Y DNA lineages in Bulgaria and NE Greece
- ✓ A more expanded introgression of Anatolian lineage in Greece, compared to mtDNA

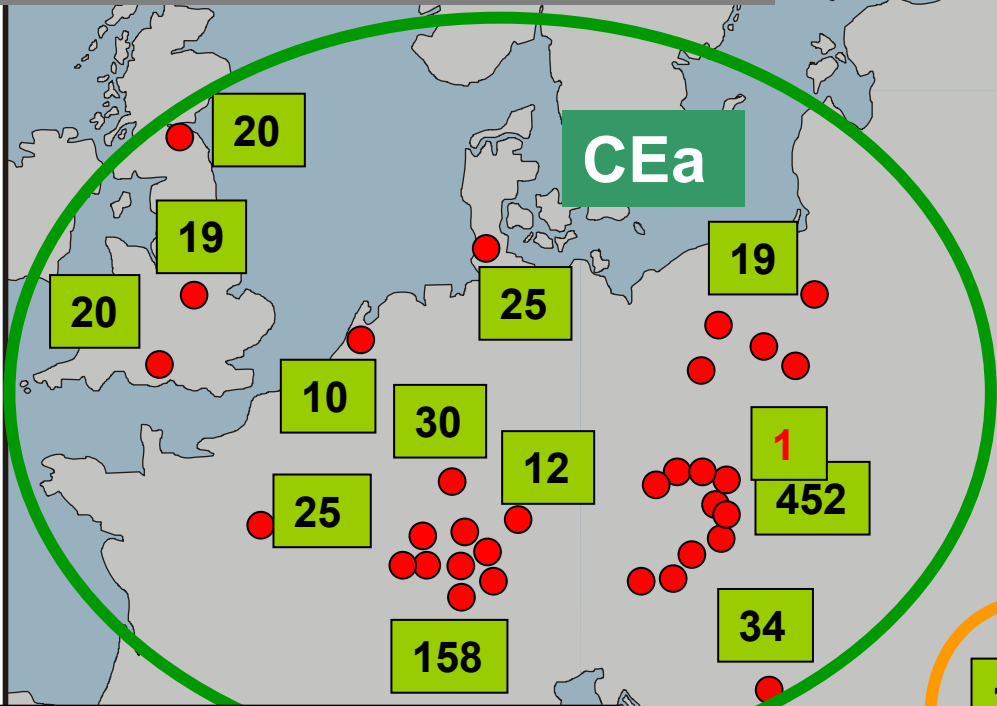


# Analysis of allozymes

1119 BROWN HARES  
from  
46 SAMPLE REGIONS

nuclear evidence:  
28 allozyme loci, h SGE

number of private alleles



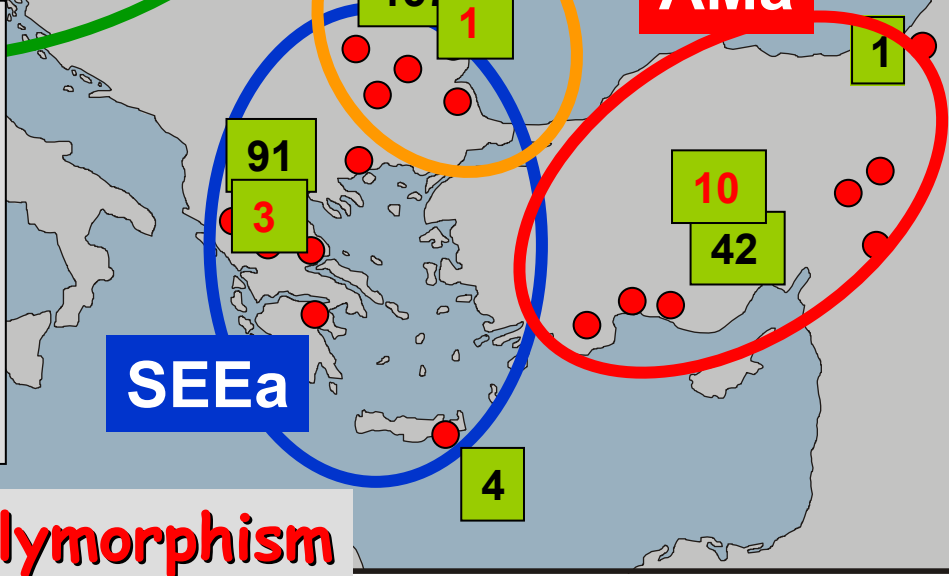
OVERa

AMa

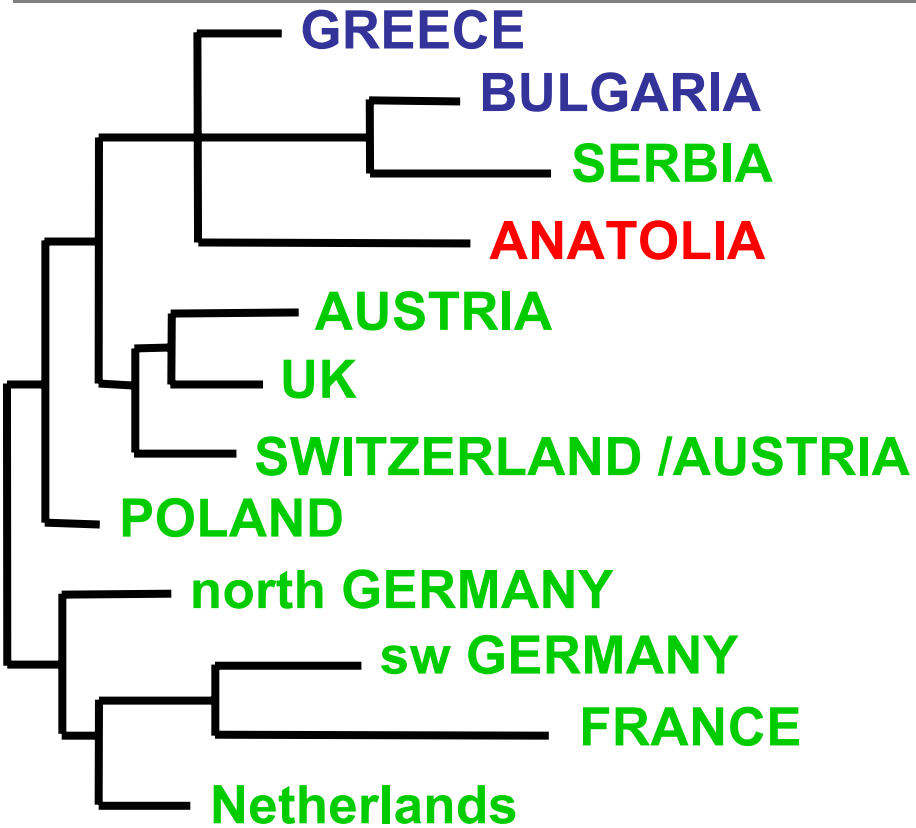
SEEa

allozyme results:  
Overall rate of polymorphism: 46.4%  
rate of private alleles: 9.6%  
rate of private alleles (CE/SEE/Anatolia): 21.2%

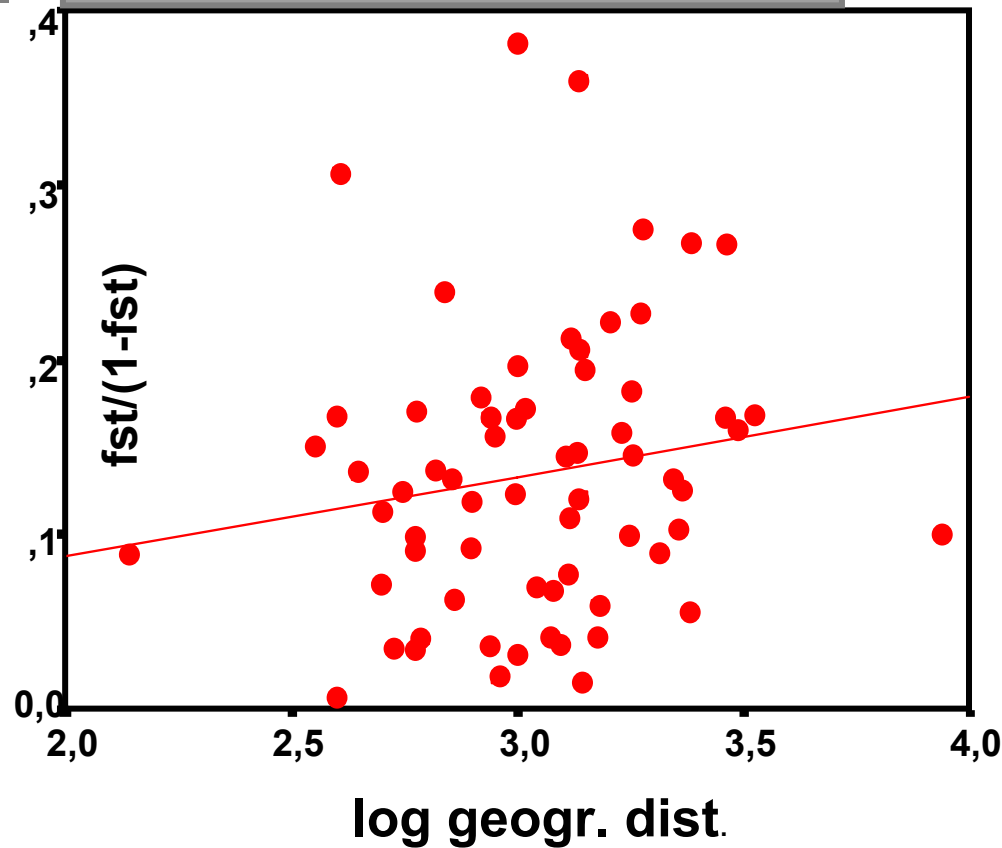
An increased genetic polymorphism



No clear phylogeographic separation



No genetic isolation by geographical distance



Quite high gene flow over long geographic distances probably due to a higher rate of male dispersal

Low level of sub structuring of nuclear gene pools in Europe, with no distinct pattern of spatial changes of genetic diversity

But, however, a slight divergence between Europe and Anatolia

# Bayesian analyses of microsatellite variation in brown hares (*Lepus europaeus*) from Greece



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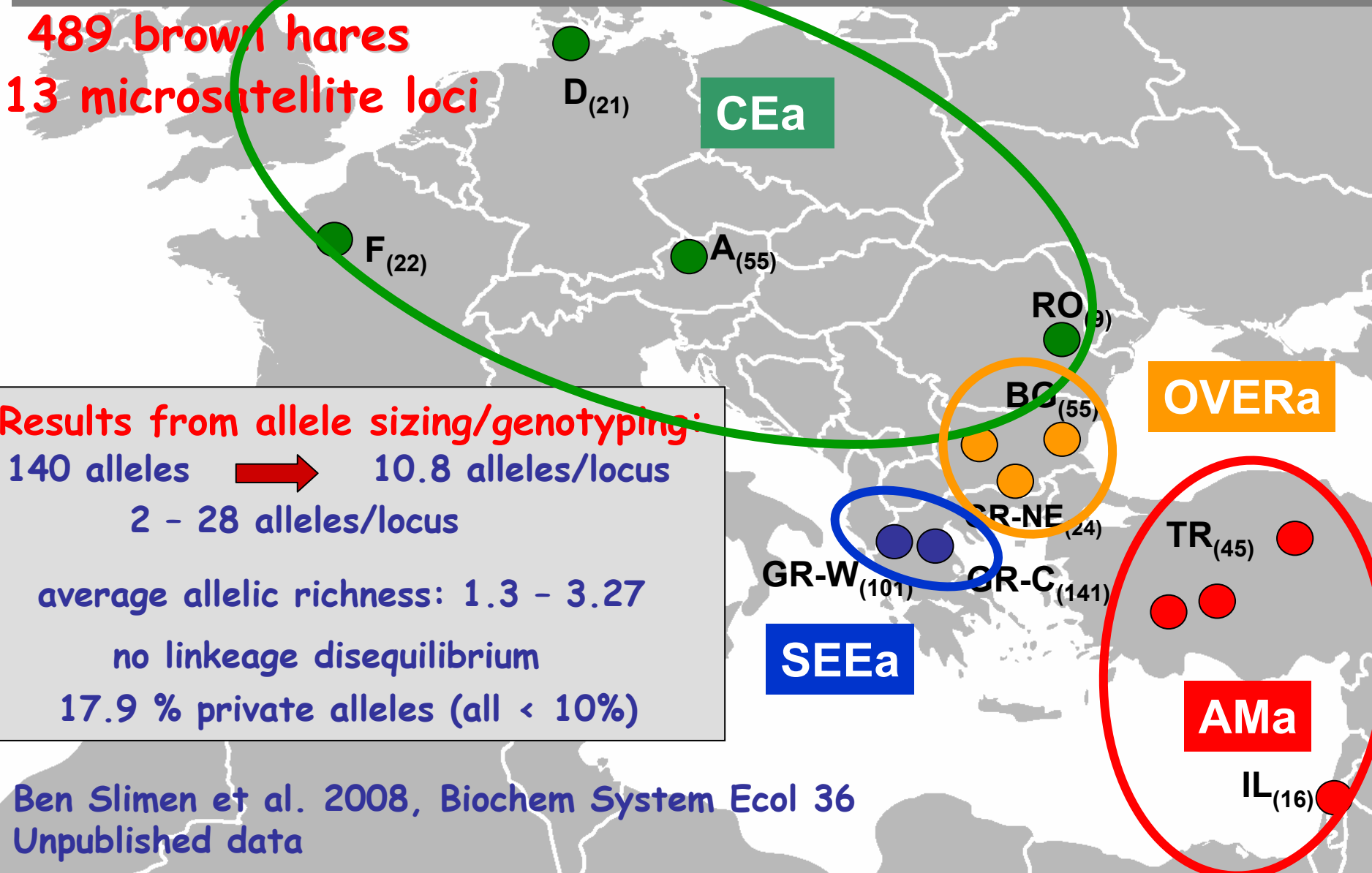
Laboratoire de Génétique Moléculaire,  
Immunologie et Biotechnologie  
Universitaire El Manar, Tunis

Dept. of Biochemistry and Biotechnology,  
University of Thessaly, Larissa, Greece

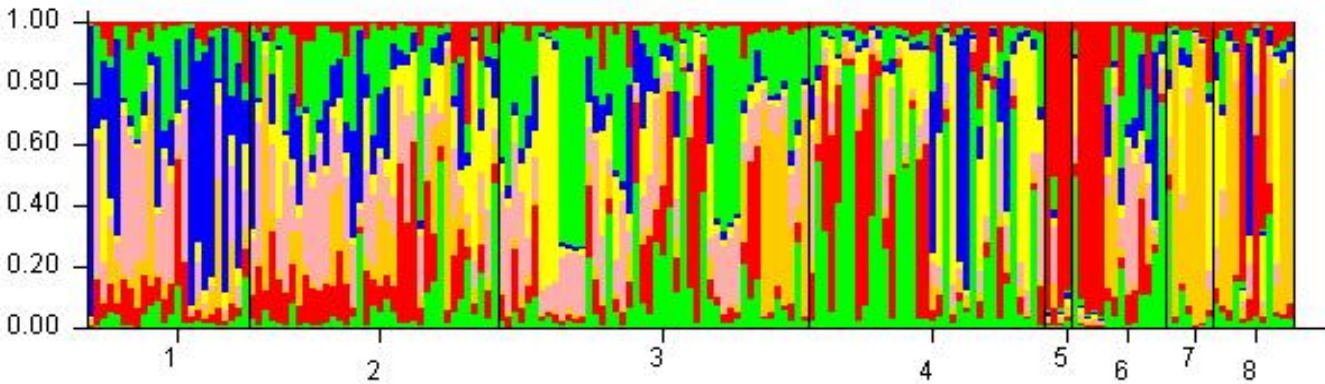
# Microsatellite variation in brown hares from Europe and Anatolia

489 brown hares  
13 microsatellite loci

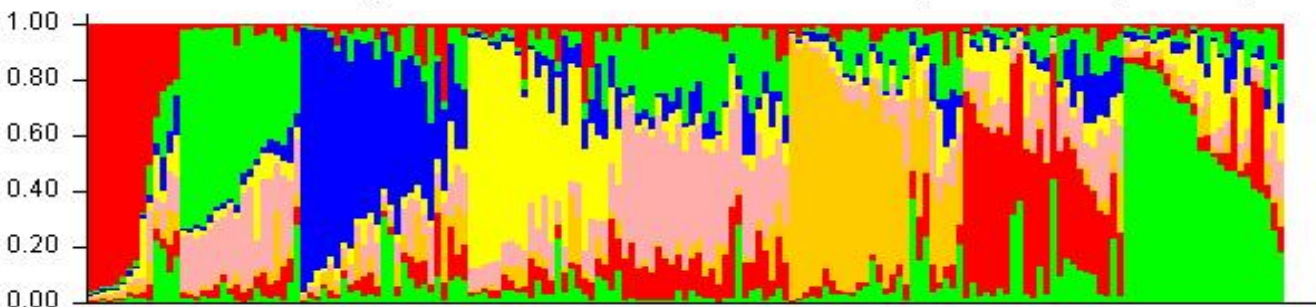
Results from allele sizing/genotyping:  
140 alleles → 10.8 alleles/locus  
2 - 28 alleles/locus  
average allelic richness: 1.3 - 3.27  
no linkage disequilibrium  
17.9 % private alleles (all < 10%)



**Bayesian admixture models** (K=8-12, 5 runs per K; i.e. 25 models)  
Burn-in period = 150.000  
repetitions = 150.000  
(STRUCTURE software)



original order of  
individuals and orig.  
population  
membership



individuals  
rearranged according  
to most probabilistic  
population assignment

High nuclear gene pool diversity

Little geographic structuring within Europe

An increased divergence of Anatolian hares from other regions

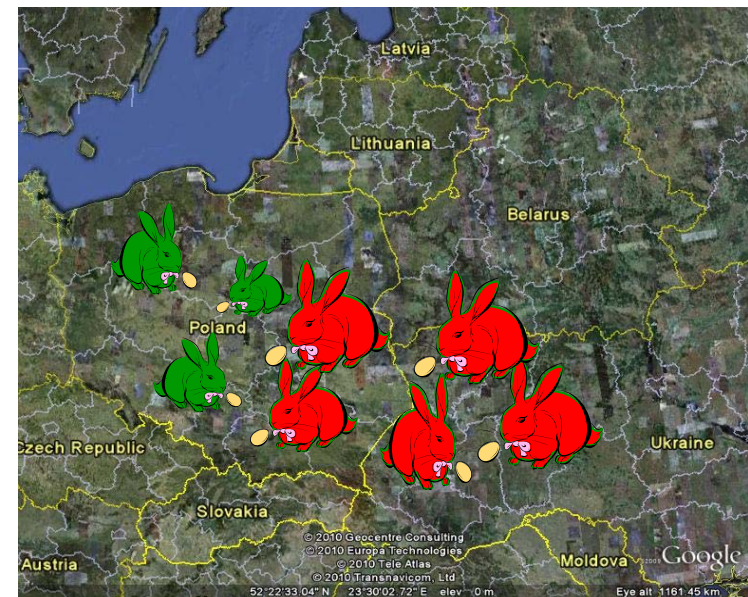
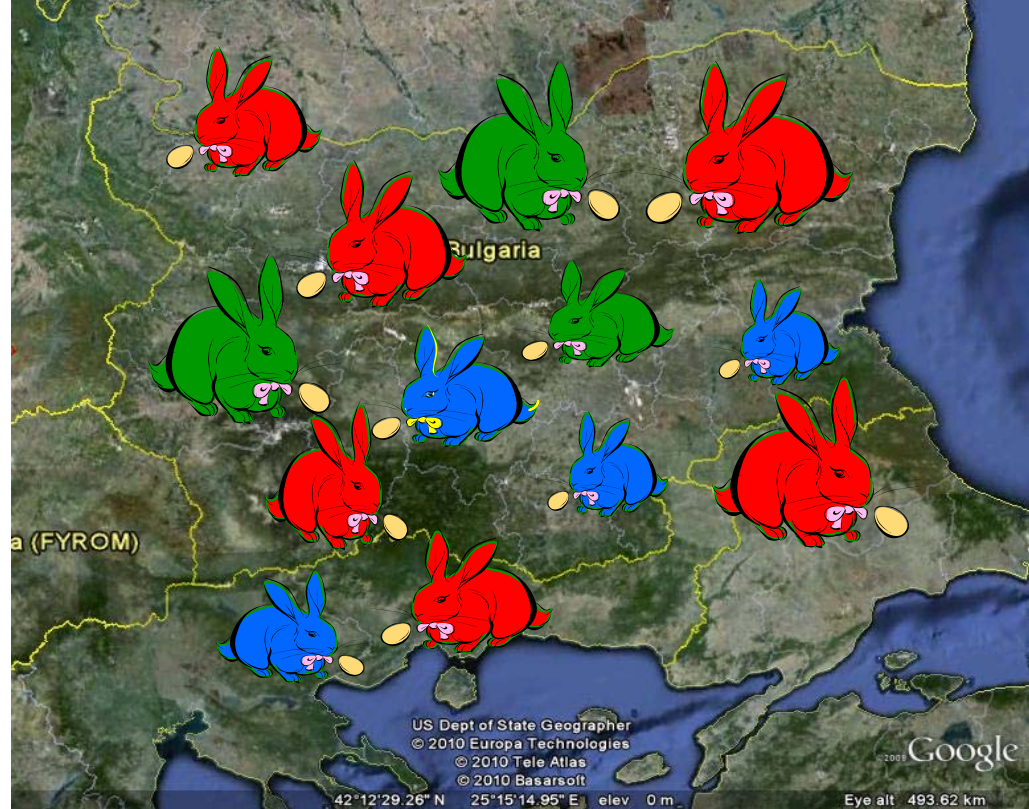
Higher nuclear gene flow compared to mtDNA

# Conclusions

## Bulgaria and northeastern Greece

In Bulgaria and northeastern Greece all haplogroups were present, forming a large introgression zone

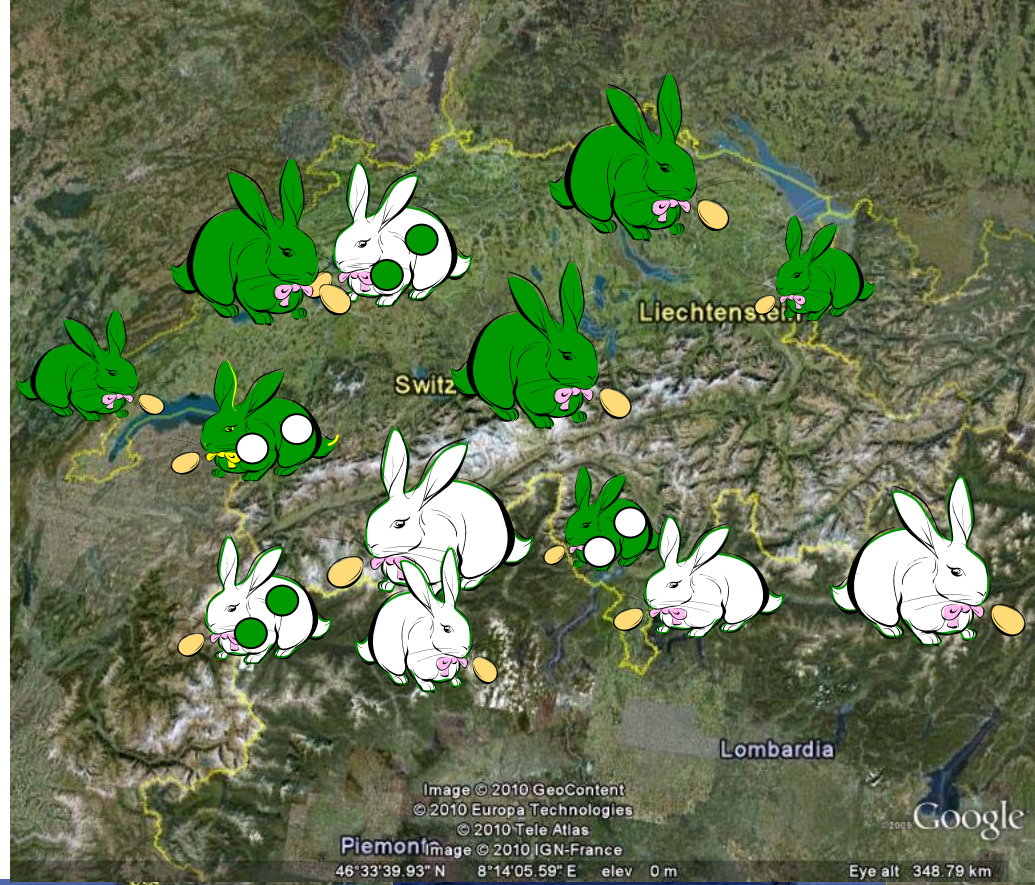
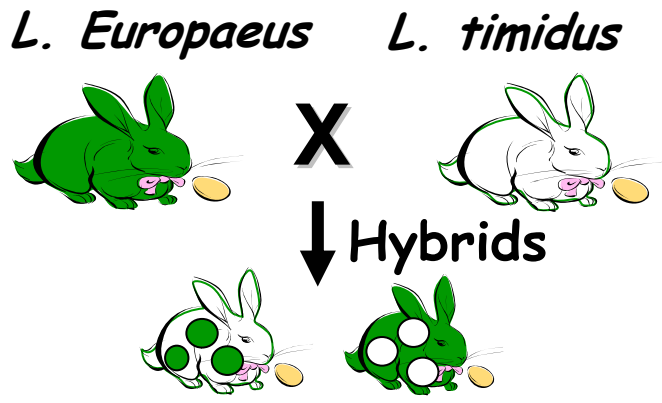
A very recent study revealed a similar situation in Poland



# Conclusions

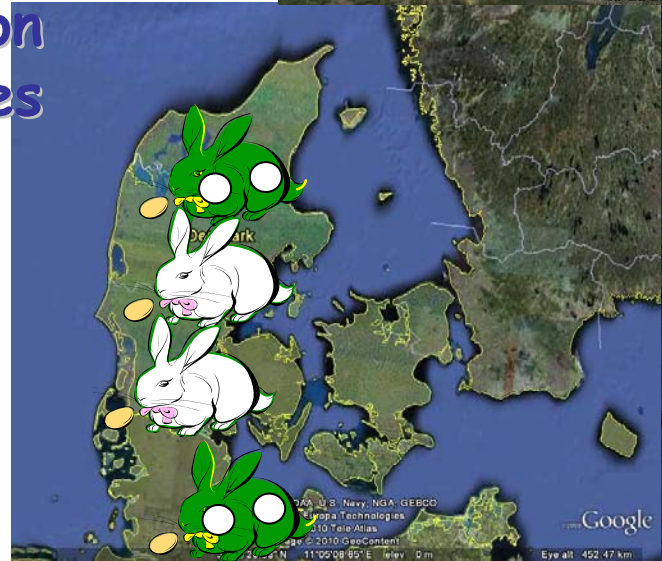
## Switzerland

Bi-directional hybridisation between mountain and brown hares



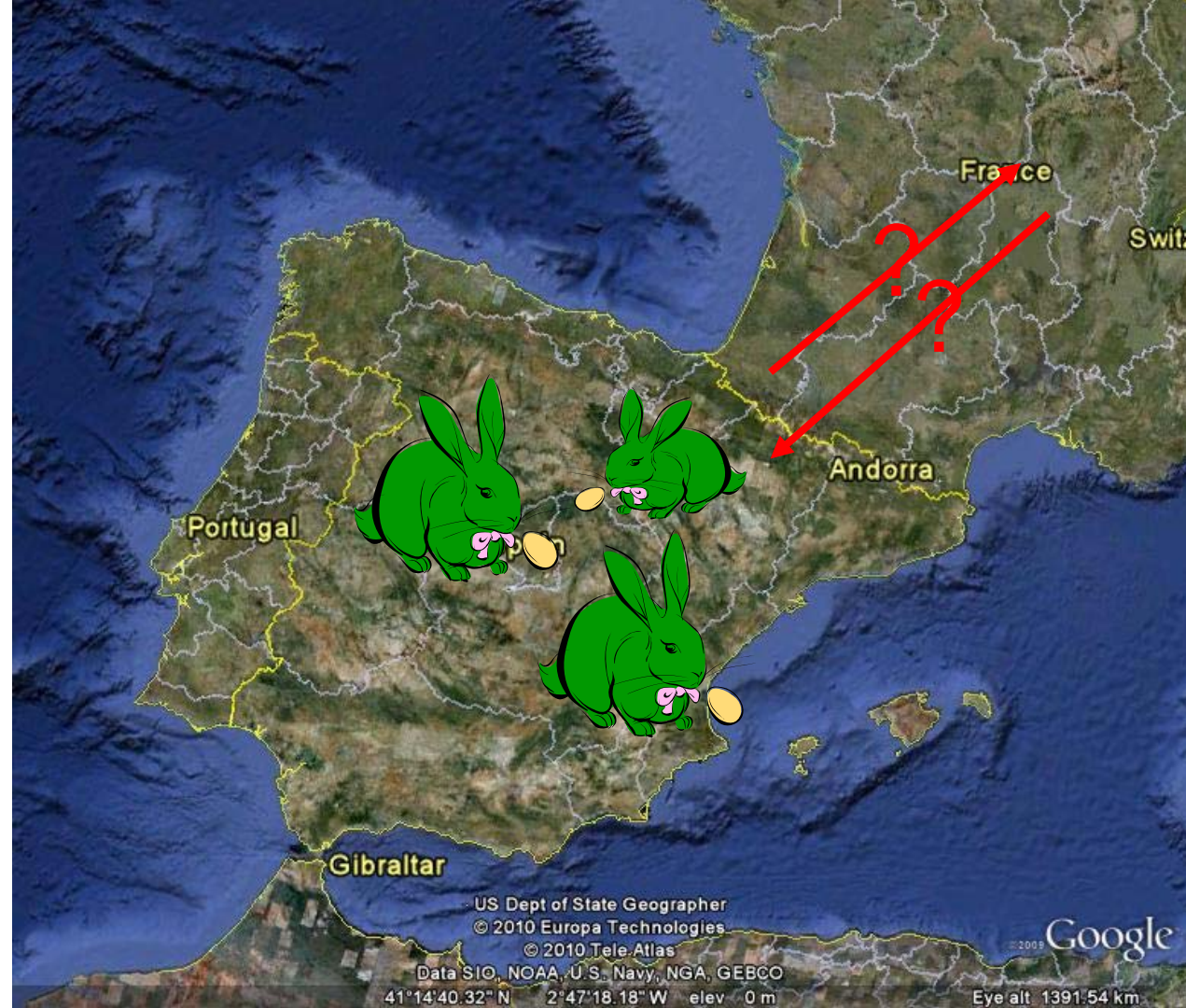
A common phenomenon between *Lepus* species in Europe

However, nuclear markers (MC1R, MHC, allozymes) showed ancient introgression



# Conclusions

## Iberia



So far, mtDNA of non-introgressed brown hares from Iberia have standard central European haplotypes,

**Existence or not of a late Pleistocene refuge in Iberia**



# British Isles

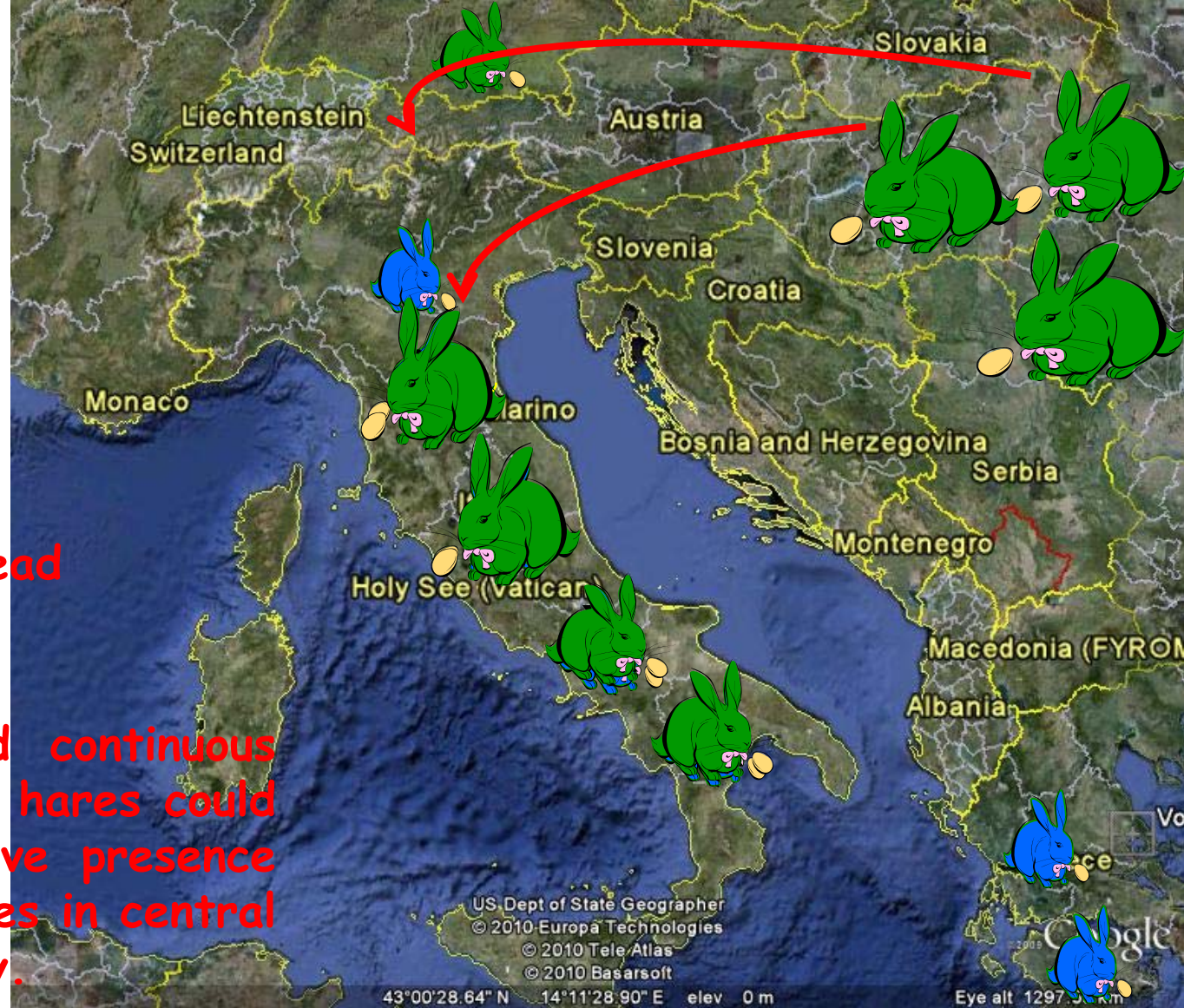


- ✓ Brown hares from the British Isles had very low mtDNA polymorphism, with only two haplotypes
  - ✓ They are not a separate subspecies (*L. e. occidentalis*).
- Northern Germany could be a possible source region of the current populations

# Italy

Originally widespread  
SEE haplotypes

Over hunting and continuous  
releases of brown hares could  
explain the massive presence  
of EU-A haplotypes in central  
and northern Italy.



This practice might have replaced the native and possibly originally widespread SEE haplotypes. Our data contradict the idea of the existence of the subspecies *L. e. meridiei* in the Italian peninsula

# Greece - Anatolia



Gene flow from Anatolia to Greece, across the late Pleistocene land bridges, was detected

Interestingly, there is a deep break in haplotype transition to Greece

## Conclusions

Allozymes and microsats indicate a quite high gene flow over long geographic distances; a higher rate of male dispersal may cause high nuclear gene flow

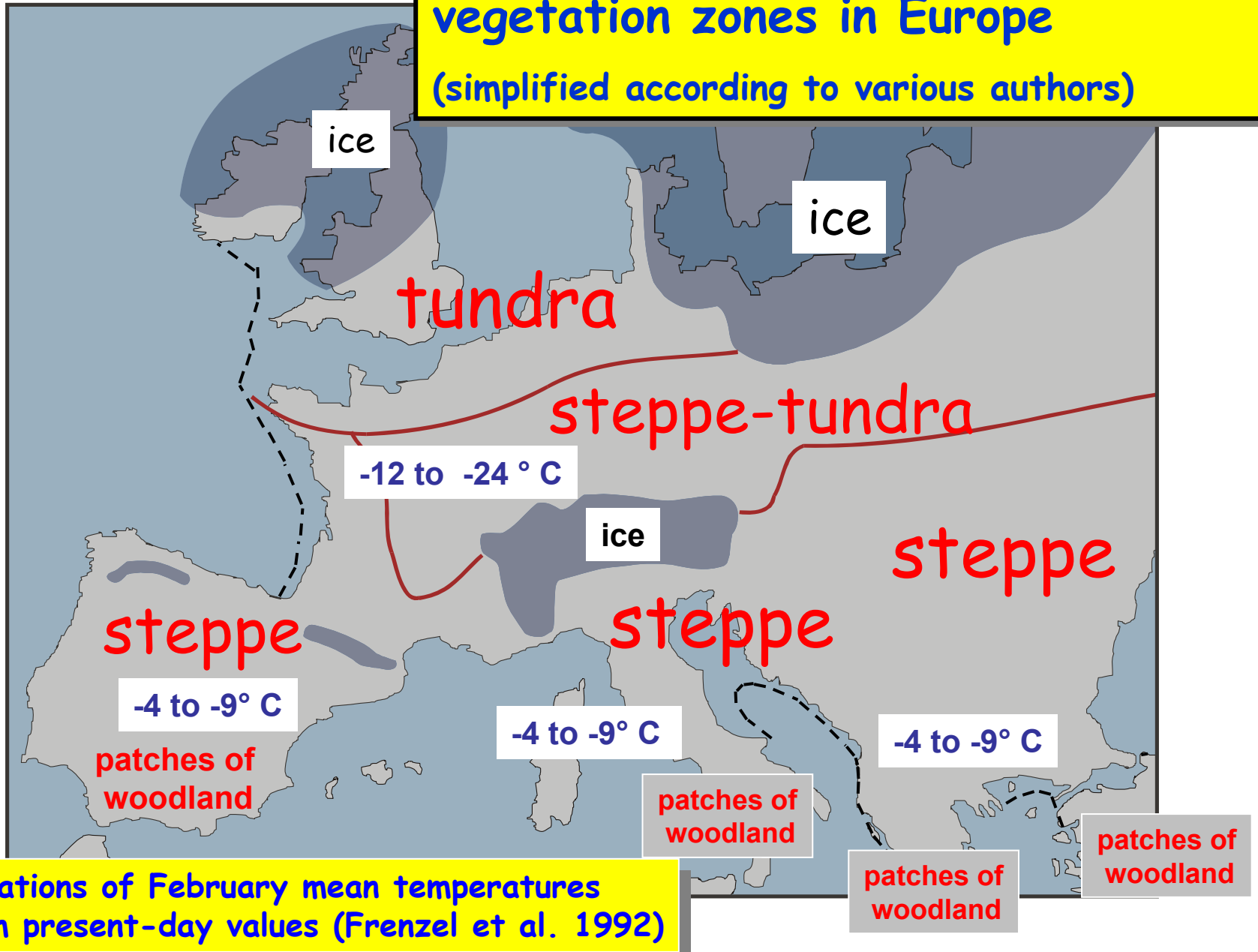
this means a low level of sub structuring of nuclear gene pools in large parts of central Europe,

and it is associated with no distinct pattern of spatial changes of genetic diversity in continental Europe.

However, new data based on the study of the Major Histocompatibility Complex (MHC) indicate differentiation of nuclear gene pools between the different regions

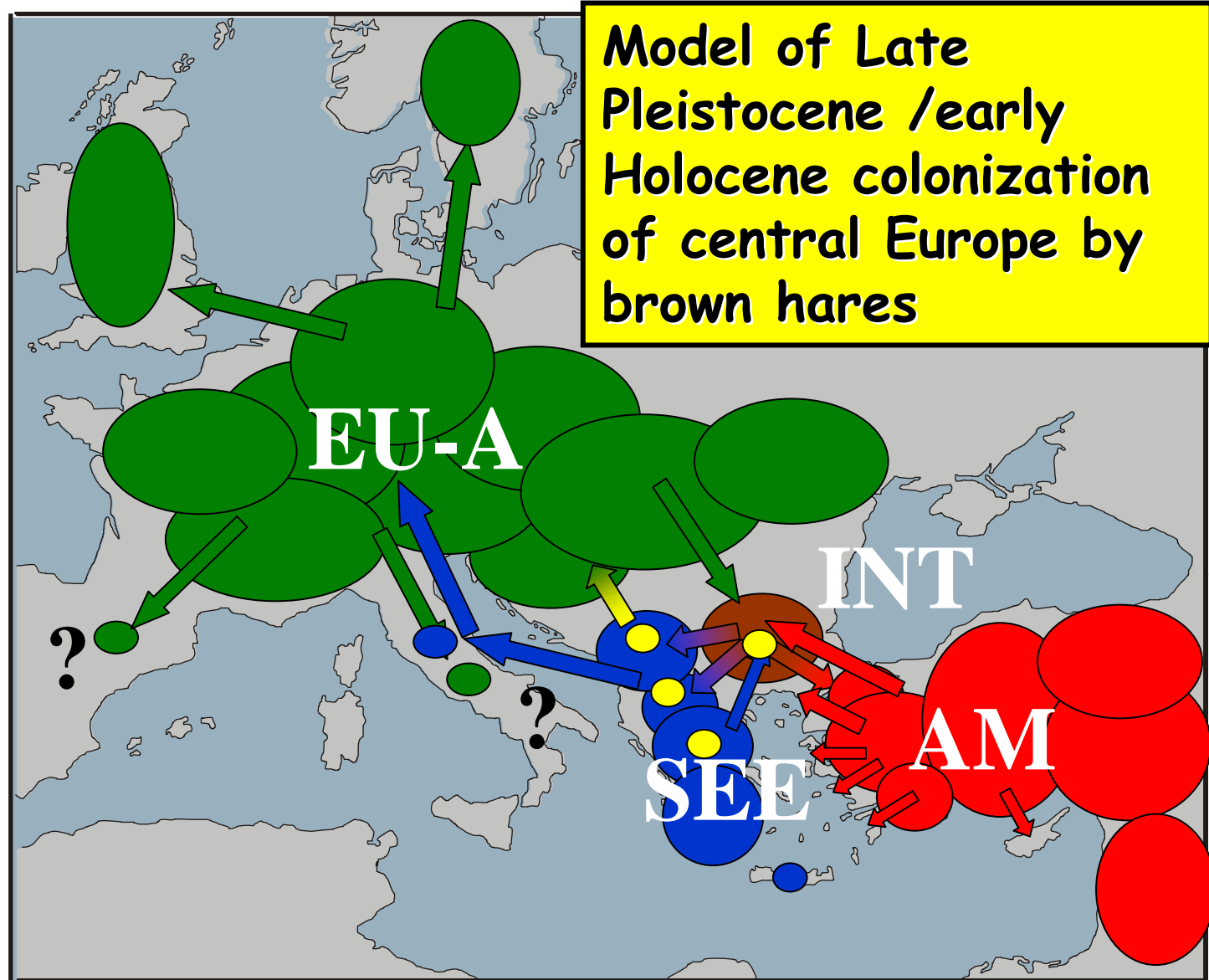
Evolutionary adaptation or phylogeographic signal?

Late Pleistocene (c. 20,000 yr. BP)  
vegetation zones in Europe  
(simplified according to various authors)



# Conclusions

Model of Late Pleistocene /early Holocene colonization of central Europe by brown hares



Although various restocking operations could be partly responsible for the presence of unexpected haplotypes in certain areas, a strong phylogeographic signal occurs throughout all regions under study.

- To estimate genetic differentiation and variability of natural populations, population genetics are mostly based on markers

neutral to natural selection

- However, neutral loci cannot provide information on the adaptive polymorphism, the contribution of different evolutionary processes, or the action of natural selection

Therefore, to understand how selection can act to maintain adaptive polymorphism in natural populations we need a substantial amount of genetic population data based on functional genes

# Major histocompatibility complex variation at class II DQA locus in the brown hare (*Lepus europaeus*)



Evagelia A. Koutsogiannouli, Katerina A. Moutou,  
Theologia Sarafidou, Costas Stamatis, Zissis  
Mamuris

Department of Biochemistry and Biotechnology, University of  
Thessaly, Greece

Vassiliki Spyrou

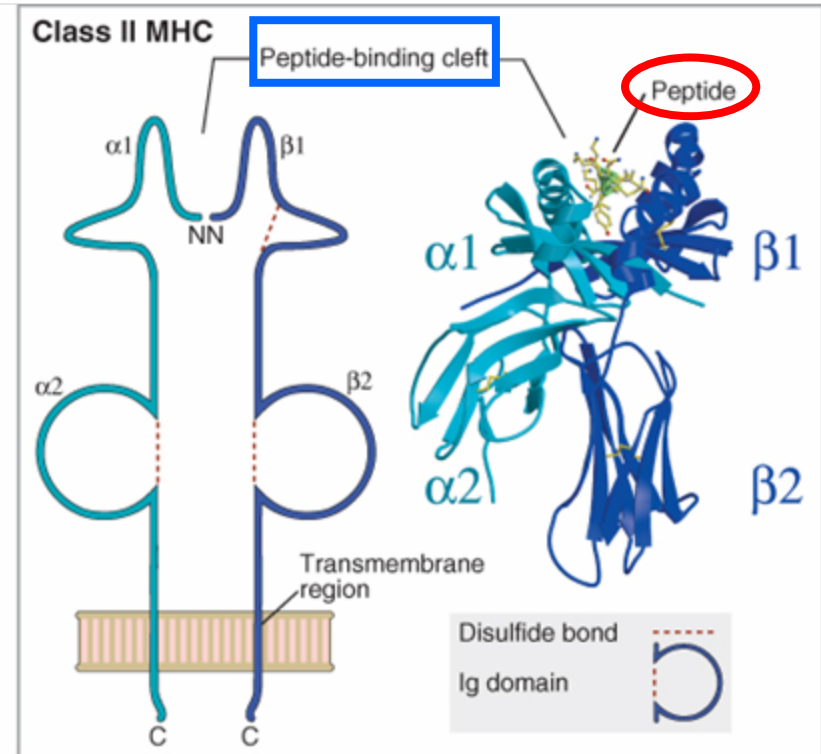
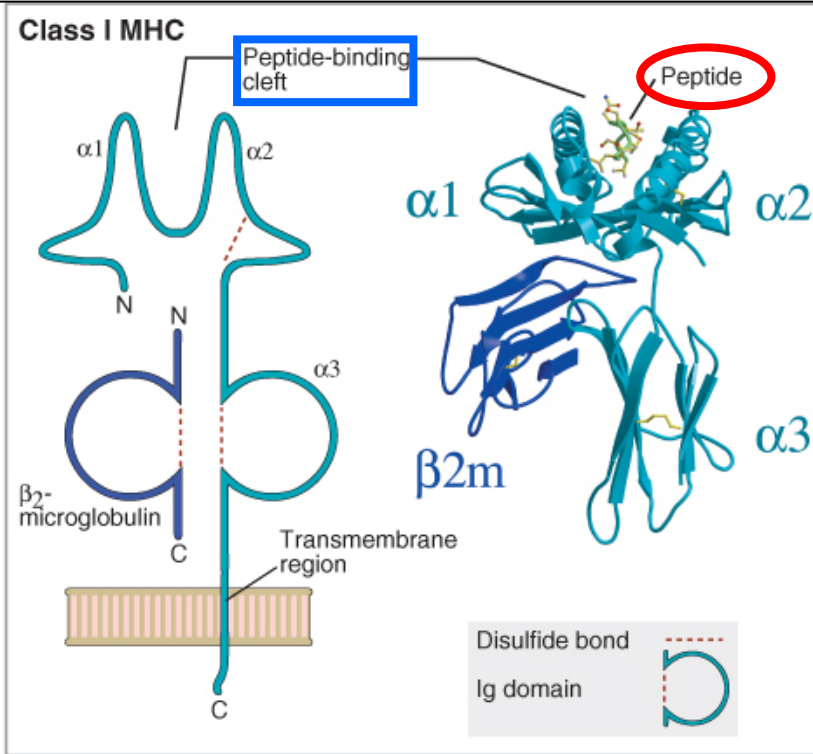
Department of Animal Production, Technological Educational Institute,  
Larissa, Greece

Koutsogiannouli E.A. et al. *Molecular Ecology* 2010



# MHC structure - Class I and II

MHC is a multigene family encoding receptors that bind and present antigenic peptides to T-cell receptors



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Class I molecules-  
found in all nucleated  
cells

Class II molecules- found in  
antigen presenting cells (B-  
lymphocytes, macrophages, etc.)

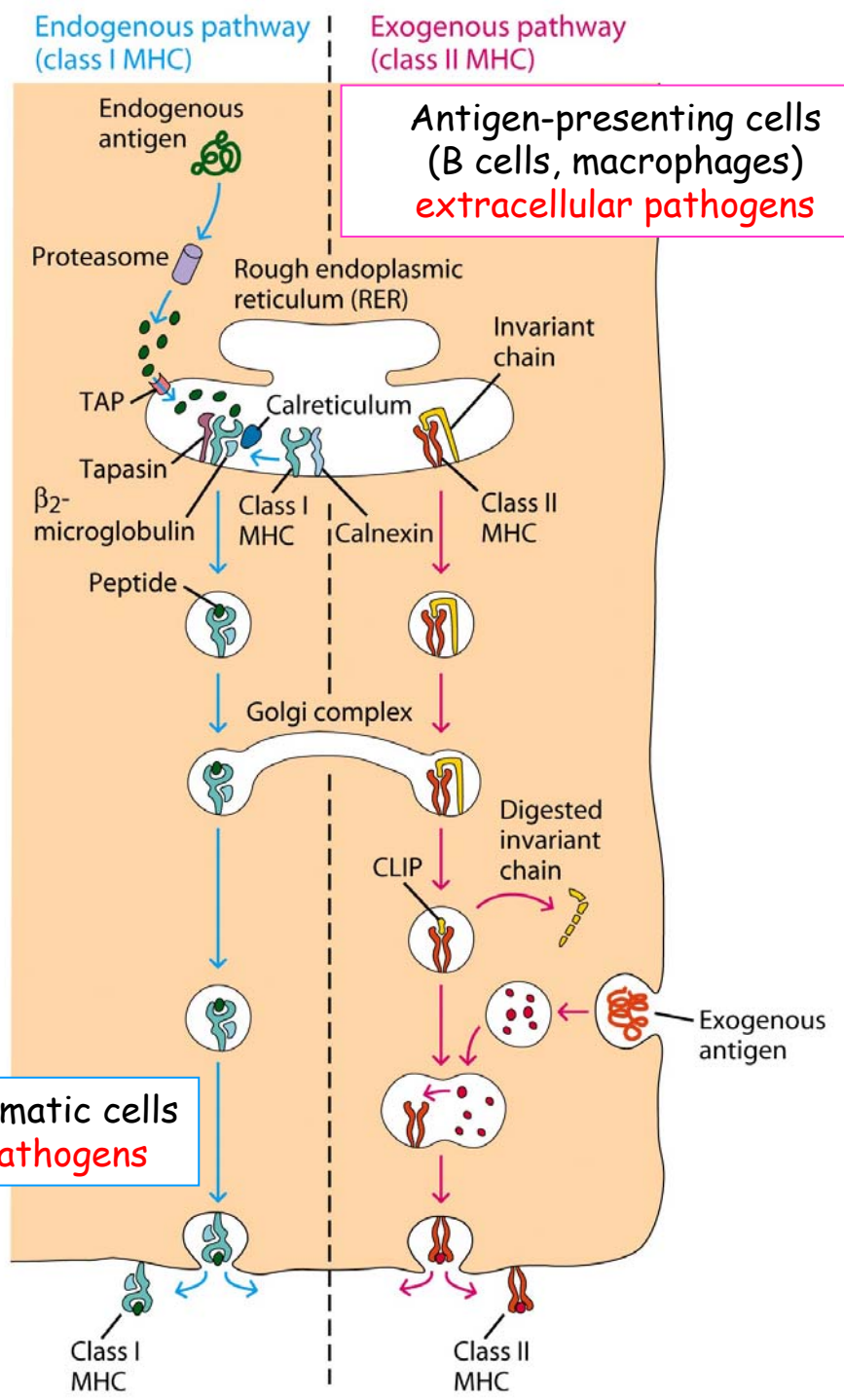
# MHC function

## antigen presentation

MHC receptors bind fragments of antigenic proteins within cells and then transport them to the surface of the cell membrane.

The complex is recognized by T-cell receptors (TCRs), which can initiate the cascade of immune responses

All nucleated somatic cells  
intracellular pathogens



## The peptide-binding region (PBR)

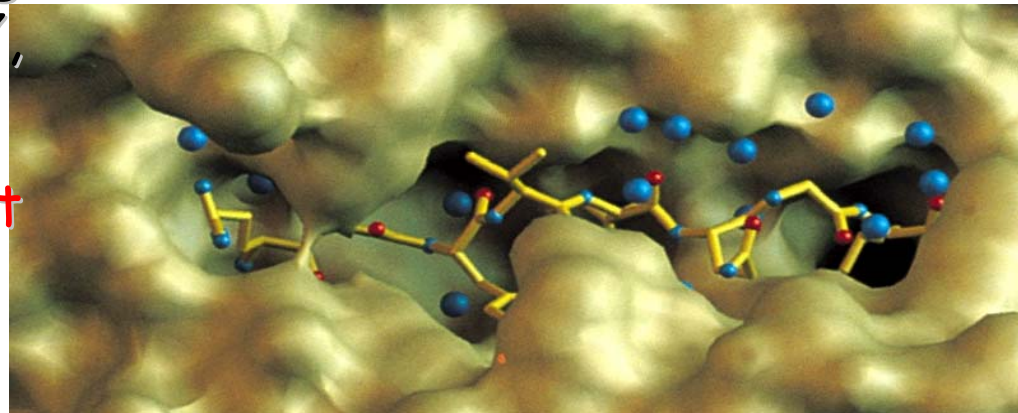
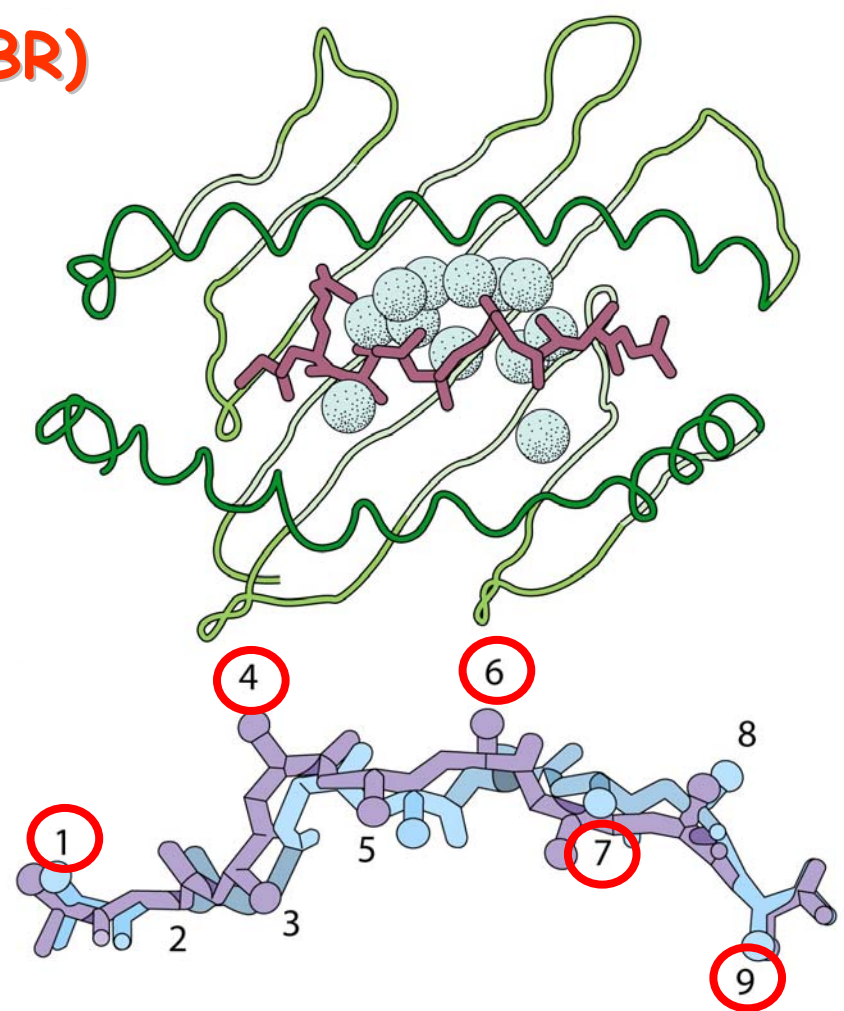
The peptide-binding region (PBR) is responsible for antigen recognition, binding and presentation

A match between the PBR, antigenic peptide and TCR is required to initiate an immune cascade

The peptide is bound specifically via interactions of its residues at relative positions (p) 1, 4, 6, 7, and 9 with residues forming the **binding pockets** P1, P4, P7, P6, P9) of the PBR

The PBR exhibits the **highest** levels of **polymorphism**

PBR = antigen recognition site (ARS)

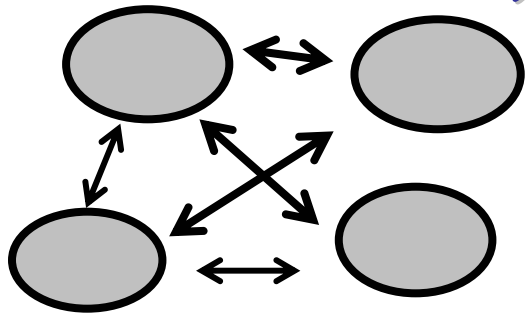


# Allelic polymorphism in MHC is supposed to be maintained by

- ✓ Disease based mechanisms (host-parasite interactions)
  - overdominance hypothesis (=heterozygote advantage) supports that heterozygotes resist a broader array of pathogens than homozygotes
  - negative frequency dependent selection hypothesis (=rare allele advantage) supports the cycling of fitness values of different genotypes in both hosts and pathogens
- ✓ Reproductive mechanisms (sexual selection)

# Aims

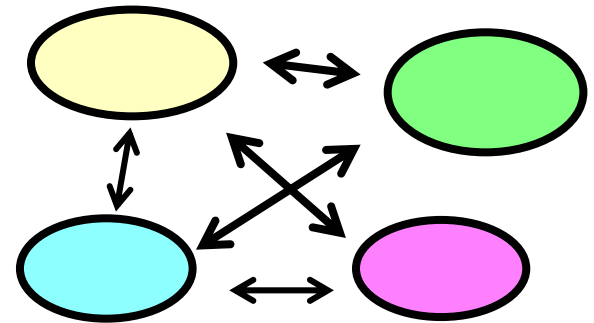
- ✓ To assess the level of MHC genetic diversity, in brown hare (*Lepus europaeus*)
- ✓ To correlate the results obtained with genetic variability and phylogenetic status estimated from maternally (mtDNA) paternally (Y) and biparentally (allozymes, microsatellites) inherited loci.



Comparison of neutral and MHC variation  
- another way to detect contemporary selection

## neutral variation (microsatellites)

- random genetic drift
- migration (gene flow)



## MHC variation

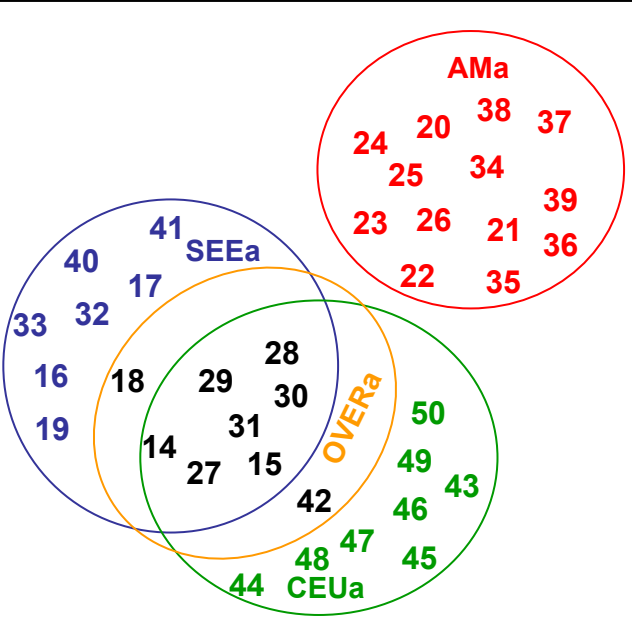
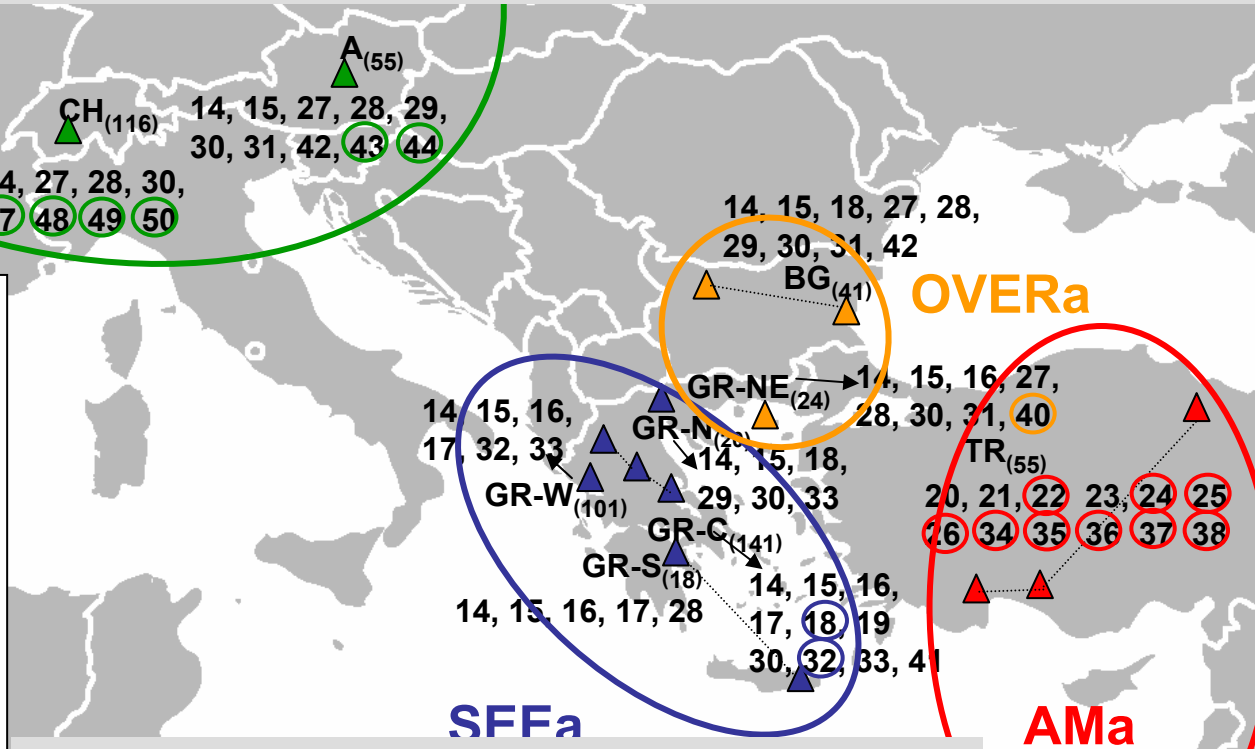
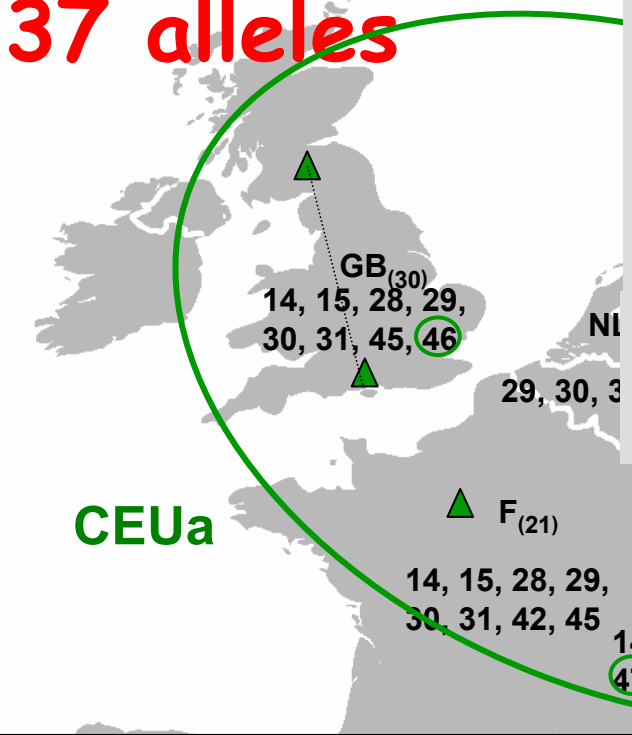
- random genetic drift
- migration (gene flow)
- selection

- ✓ To understand how polymorphism in the PBR affects variation within the binding pocket, and the functional implications of this polymorphism across populations.

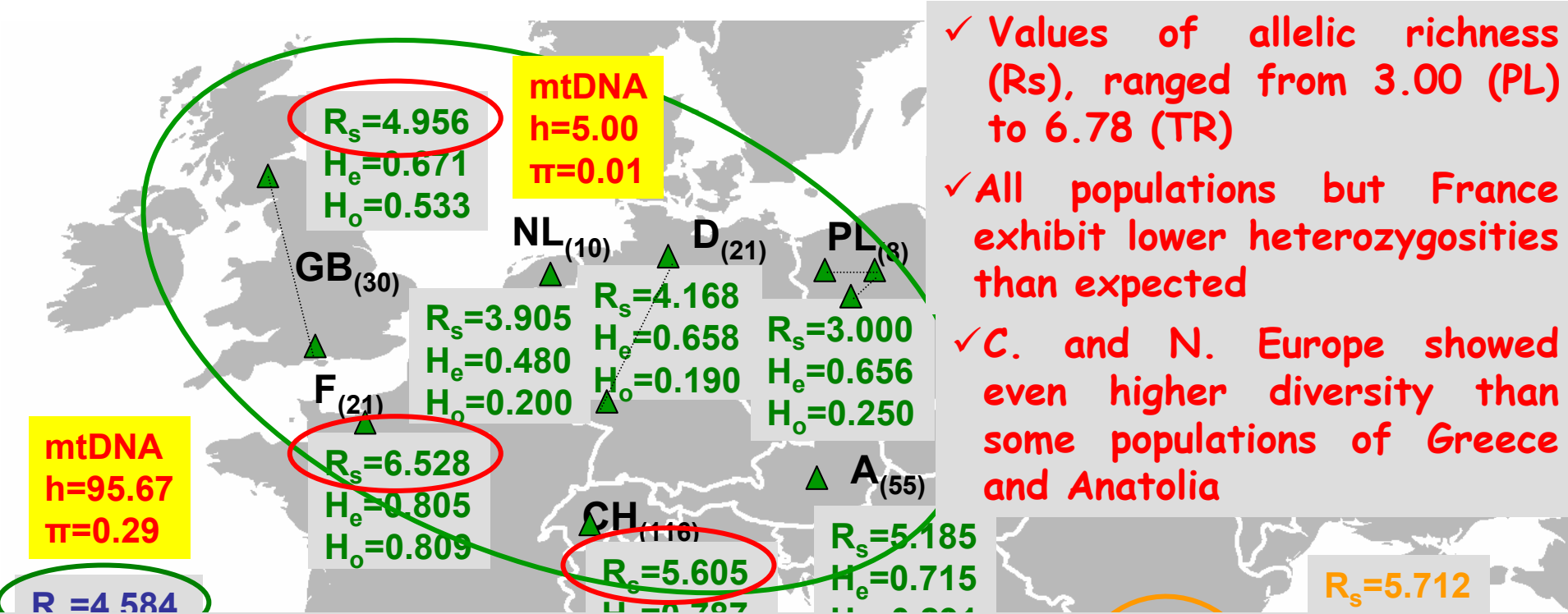


# 37 alleles

- ✓ Greece shared seven alleles with the rest of Europe
- ✓ However, several alleles appeared only in Greece or only in Europe
- ✓ No common alleles between Europe and Turkey-Israel



✓ Several private alleles within each population



✓ Values of allelic richness (R<sub>s</sub>), ranged from 3.00 (PL) to 6.78 (TR)

✓ All populations but France exhibit lower heterozygosities than expected

✓ C. and N. Europe showed even higher diversity than some populations of Greece and Anatolia

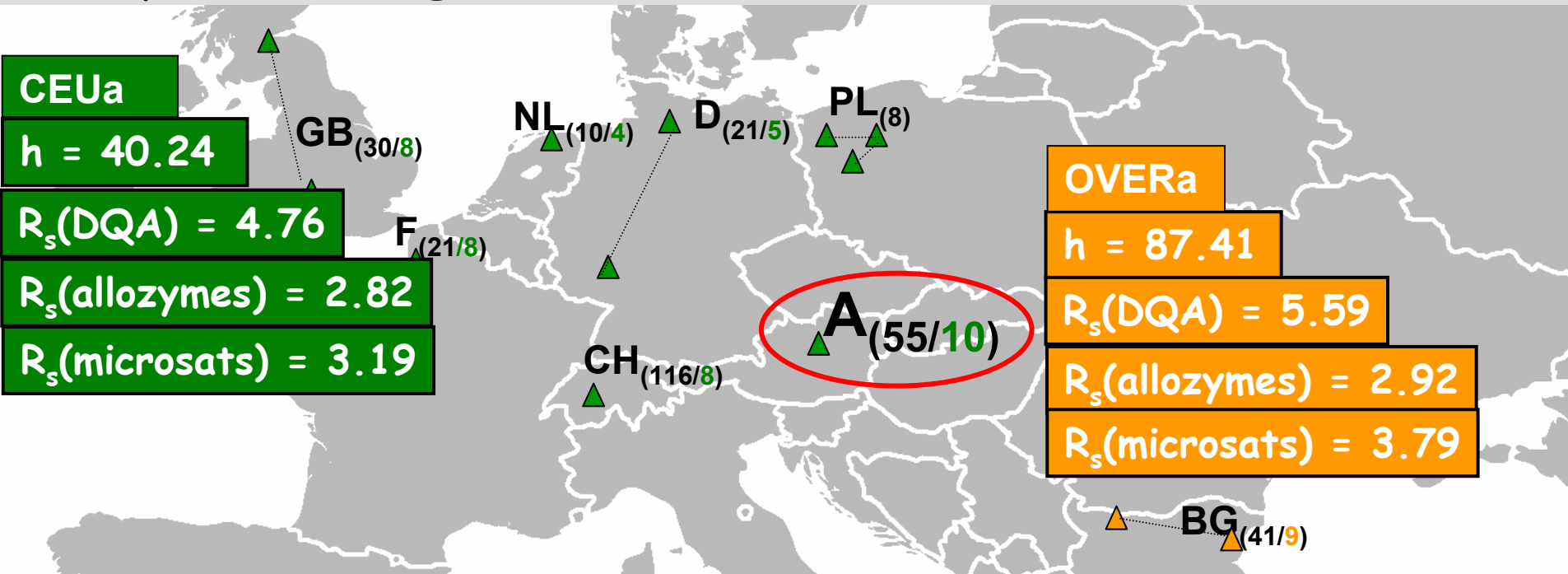
✓ MtDNA showed a post-glacial colonization of C. and N. Europe with reduced genetic diversity due to the founder effect.

✓ DQA diversity contrasts this scenario

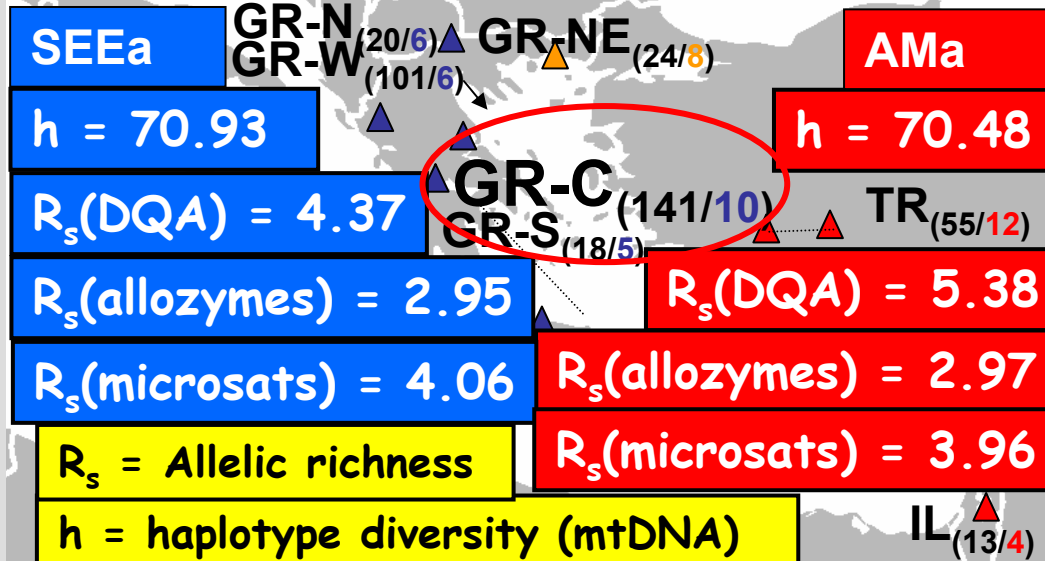
✓ These findings suggest high rates of mutation and recombination, combined with a complex evolutionary mode that extends beyond historical demography and biogeography of the species



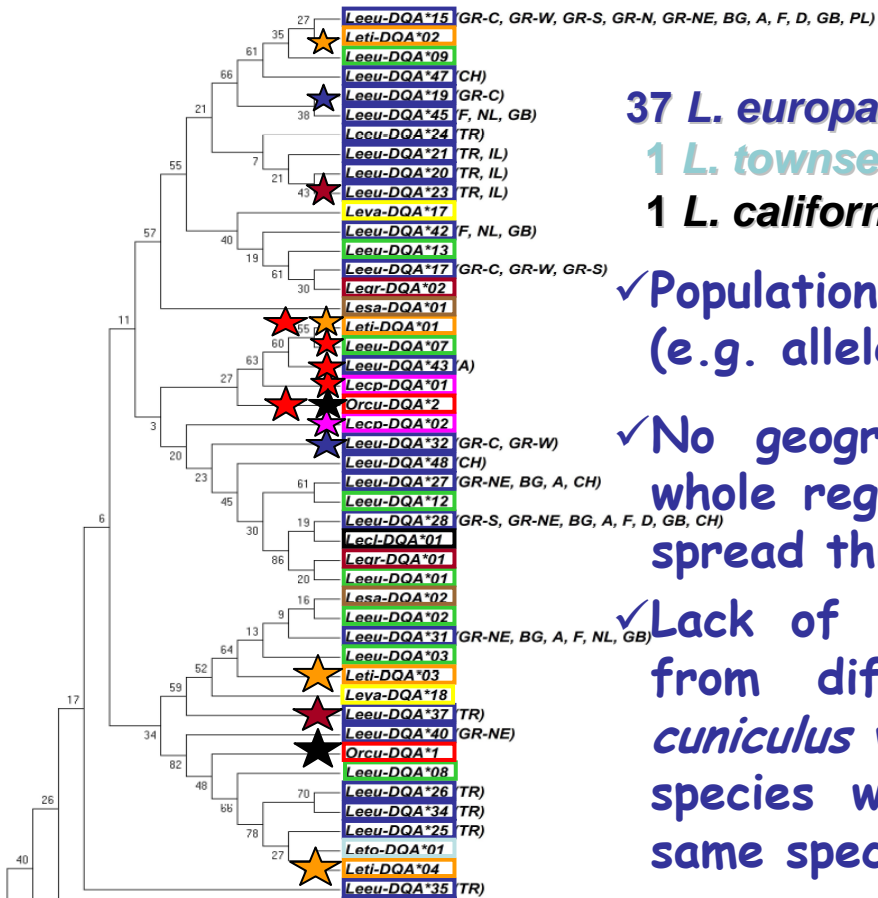
✓ Numbers of alleles did not increase proportionally with sample sizes (e.g. GR-C, and A)



This implies that in a given, restricted area there is a limited number of DQA alleles and sampling of more individuals would not increase the quantity of alleles



# Phylogeny of the DQA alleles



37 *L. europaeus* 13 *L. europaeus* 5 *L. timidus* 3 *L. granatensis*  
 1 *L. townsendii* 2 *L. saxatilis* 3 *L. capensis* 3 *L. yarkandensis*  
 1 *L. californicus* 2 *O. cuniculus*

✓ Population-specific alleles did not cluster together (e.g. alleles from GR-C)

✓ No geographic separation of alleles even for a whole region (e.g. alleles specific to Anatolia were spread throughout the tree)

✓ Lack of phylogenetic signal even when sequences from different *Lepus* species, and from *O. cuniculus* were included (e.g. alleles from different species were more related than those from the same species)

✓ This contradicts the data from mtDNA, which demonstrated a strong phylogeography throughout all regions

✓ Sharing of alleles among populations and across species are characteristic features of some MHC loci probably resulting from balancing selection

✓ Pathogen recognition might provide the selection pressure required to maintain particular MHC sequences, and the observation that *Lepus* and *Oryctolagus* share similar alleles may be evidence of the need for a specific immune response to a common pathogen.

# Non-synonymous ( $d_N$ ) and synonymous ( $d_S$ ) substitutions: Z test for positive selection

	N	$d_S$	$d_N$	$d_N/d_S$	P
<b>PBR</b>	16	0.062 (0.036)	0.153 (0.051)	2.46	0.041*
<b>Non-PBR</b>	54	0.088 (0.027)	0.062 (0.015)	0.70	0.084**
<b>exon 2</b>	70	0.073 (0.019)	0.067 (0.013)	0.92	0.397**

P: probability of rejecting the null hypothesis of strict neutrality ( $d_N = d_S$ ) in favor of the alternative hypothesis ( $d_N > d_S$ , \*) or ( $d_N < d_S$ , \*\*)

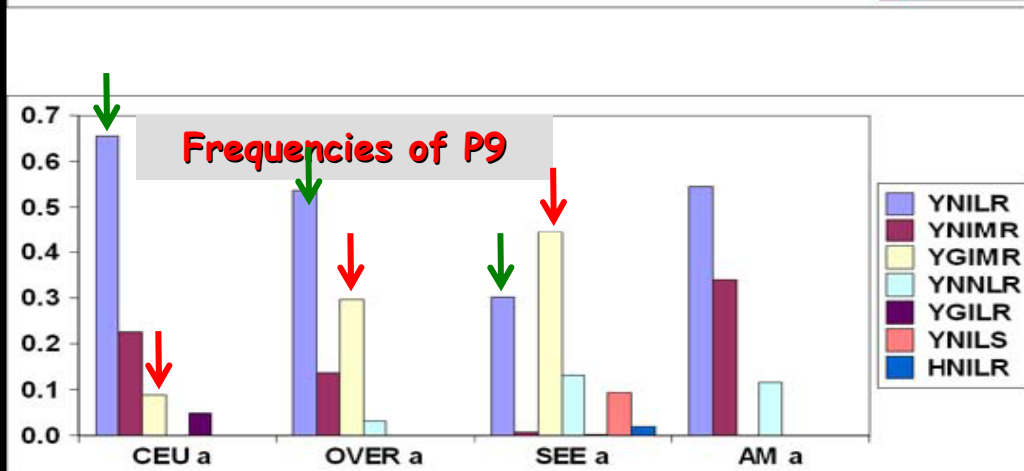
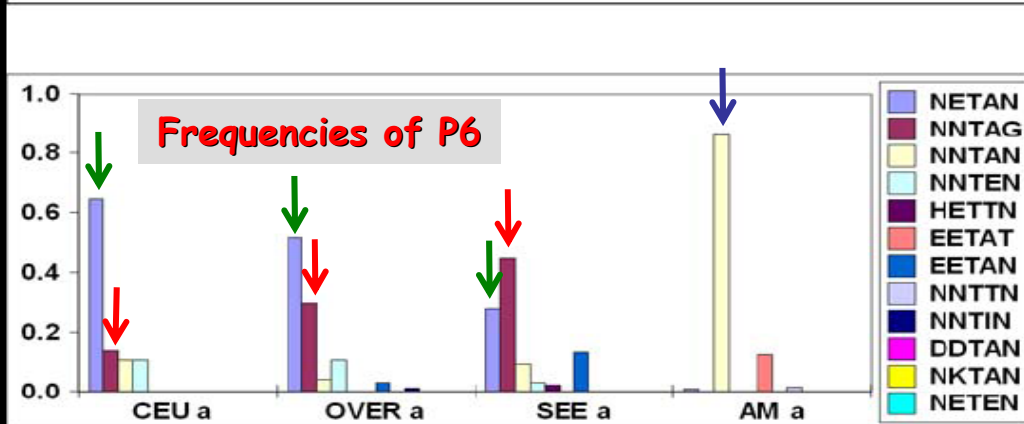
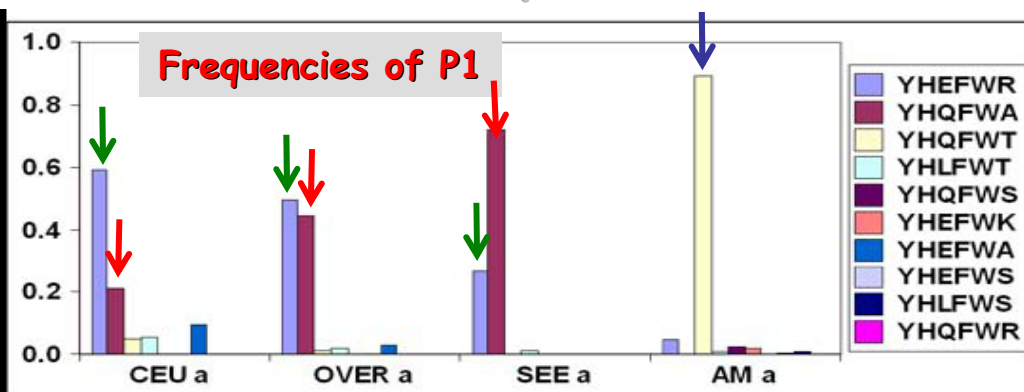
✓ Within PBR the number of non-synonymous substitutions was significantly higher than that of synonymous, indicating

● Positive selection

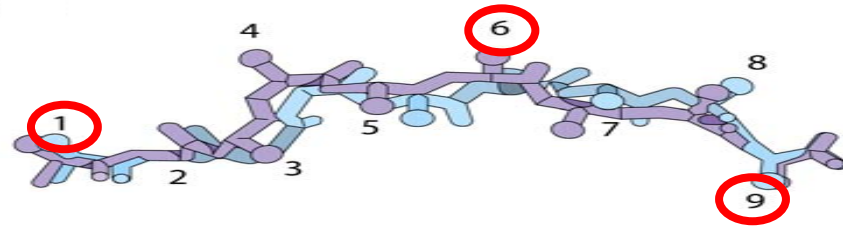
✓ Within the two other regions the ratio of synonymous vs non-synonymous substitutions was reversed, indicating

● Purifying selection

# Distribution of pocket variants varied within areas



Any combination of amino acids in a binding pocket is considered a pocket variant



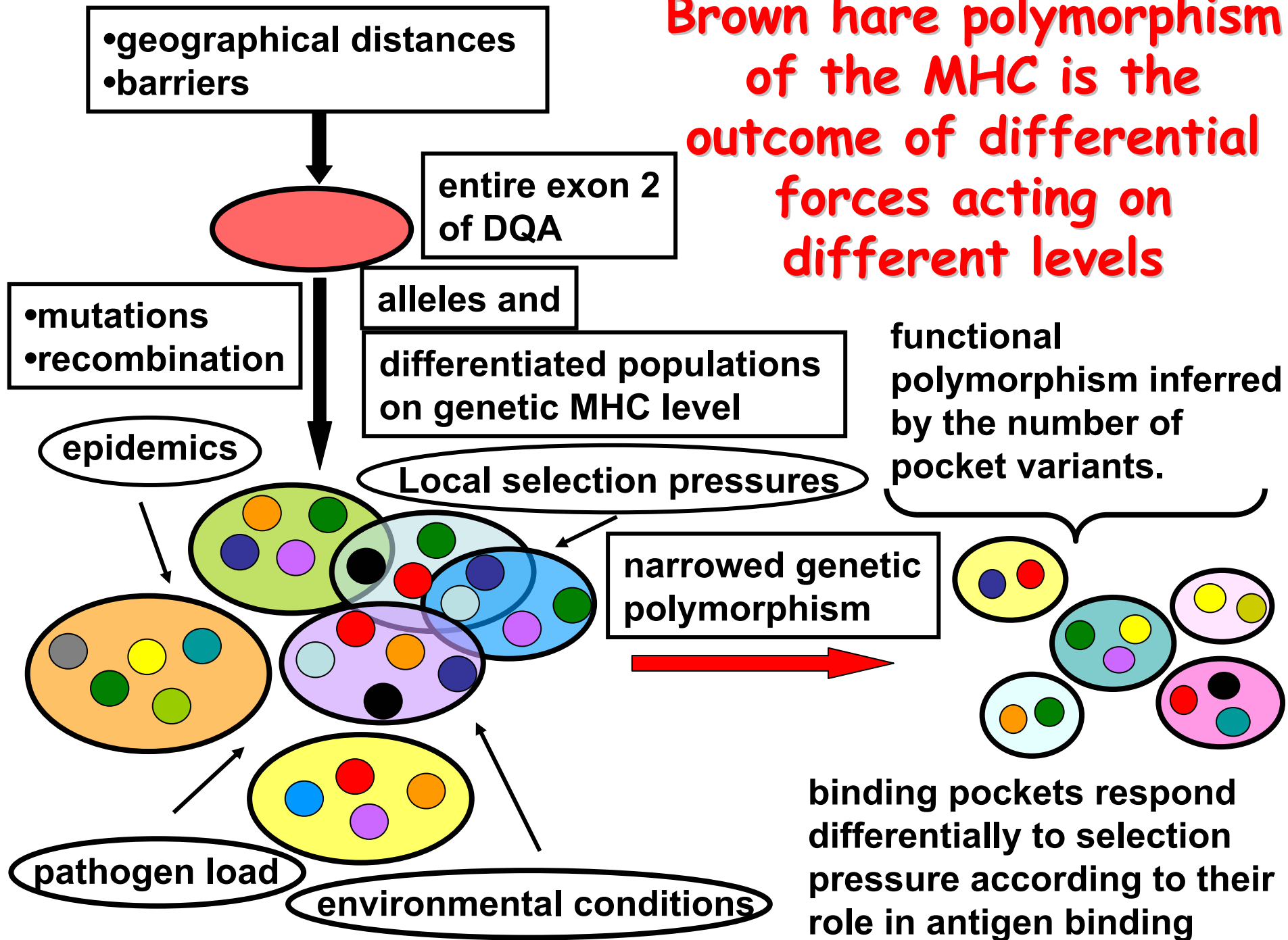
The variants (P1, YHQFWA; P6, NNTAG; P9, YGIMR) dominated in Greece decreasing northwards.

The variants (P1, YHEFWR; P6, NETAN; P9, YNILR) dominated in C. Europe decreasing southwards.

The variants (P1, YHQFWT; and P6, NNTAN) were present in TR and IL with high frequencies

Surprisingly, Greek variants were not present in Anatolia but the European ones were

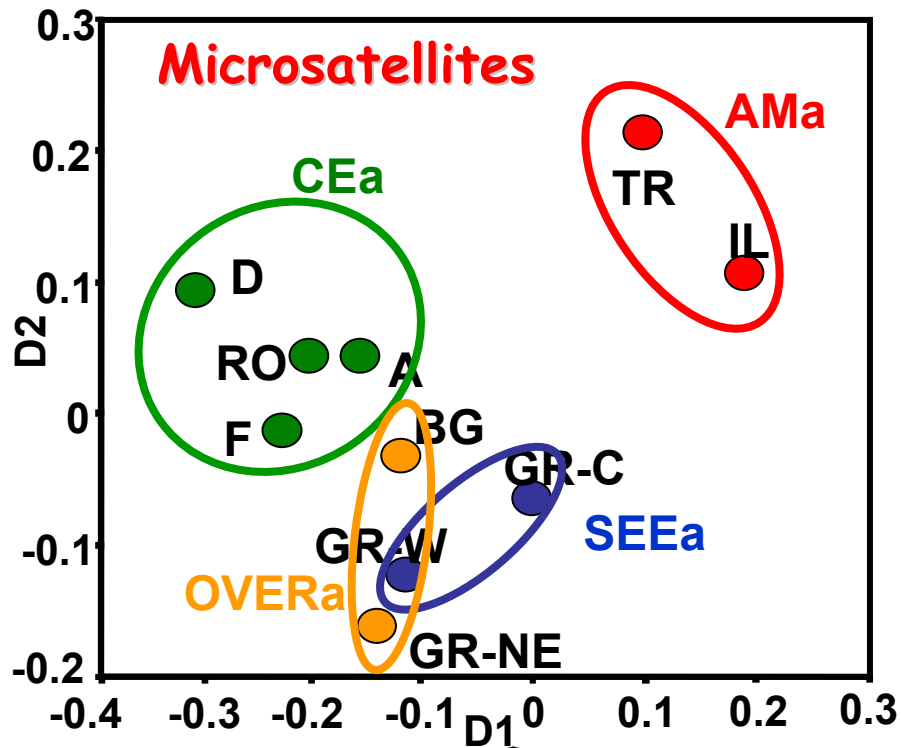
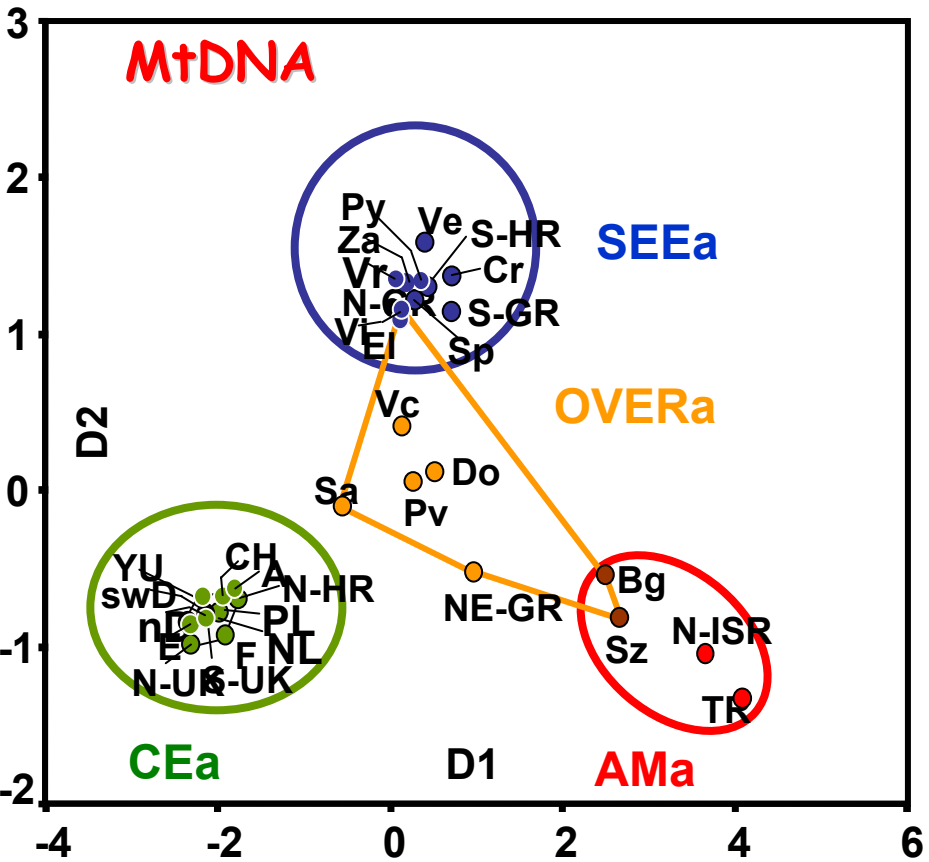
# Brown hare polymorphism of the MHC is the outcome of differential forces acting on different levels



The background of the slide is a photograph of two brown hares standing on their hind legs in a grassy field. They are facing each other, with their front paws raised towards each other's heads, as if in a social interaction or fight. The hares have brown fur with some lighter patches on their chests and faces. The background is a soft-focus green field.

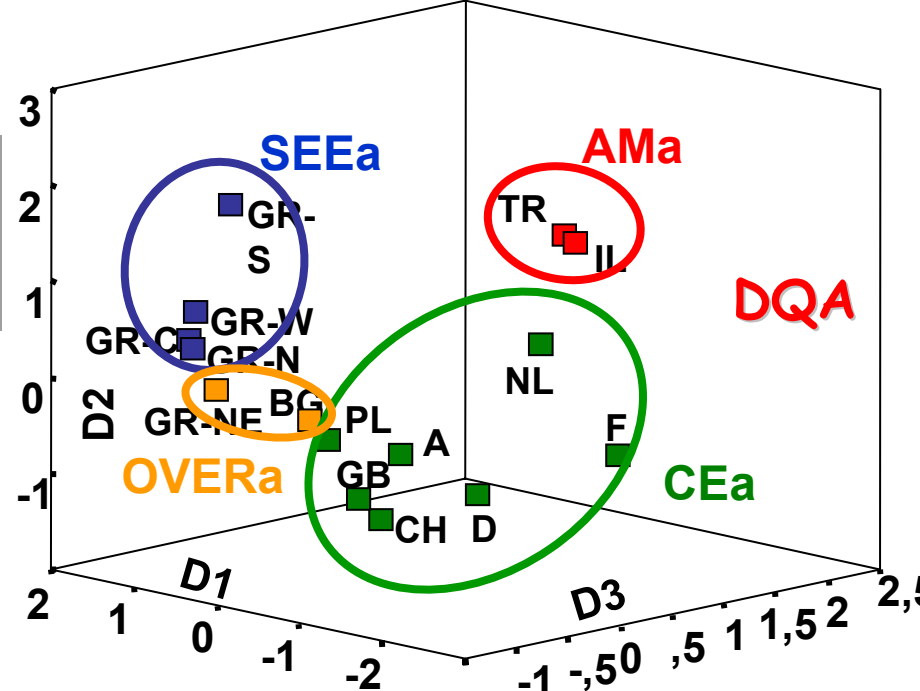
# Introducing mother's curse: low male fertility associated with mtDNA mutations in brown hares

Zissis Mamuris, Costas Stamatis, Evagelia A. Koutsogiannouli, Katerina A. Moutou, Theologia Sarafidou  
Department of Biochemistry and Biotechnology,  
University of Thessaly, Greece



**Populations' Differentiation**  
 Multidimensional Scaling of the matrix of pairwise distances

- ✓ A clear differentiation between Anatolia and Europe.
- ✓ A smaller, but significant differentiation between Greece and the rest of Europe.

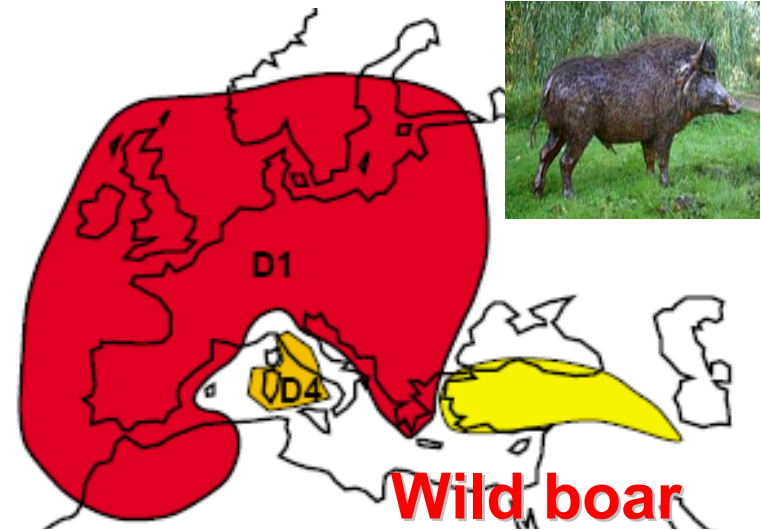


# Several mammals show a "Europe-Anatolia" break



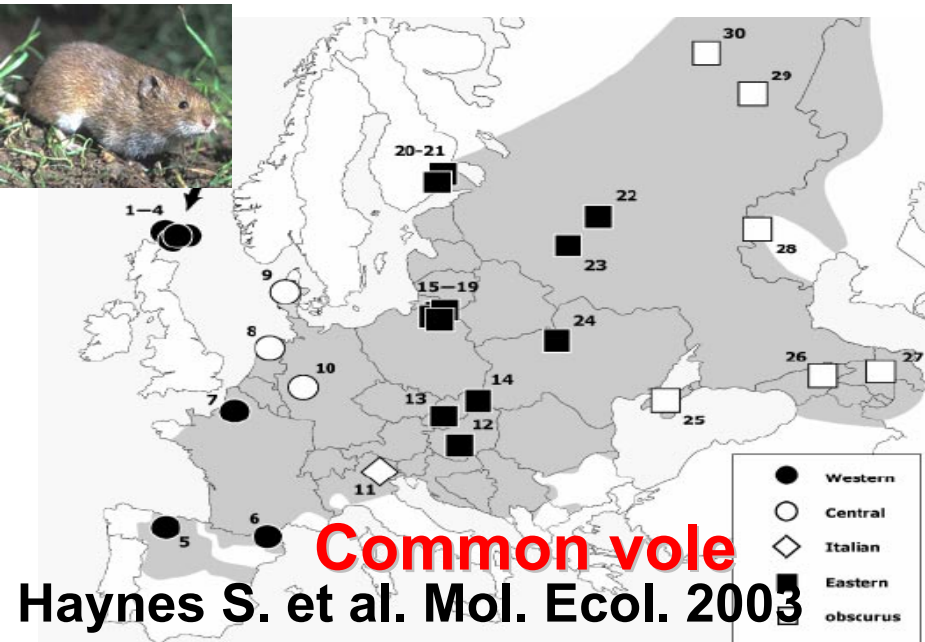
**Horseshoe bat**

Flanders J. et al. Mol. Ecol. 2009



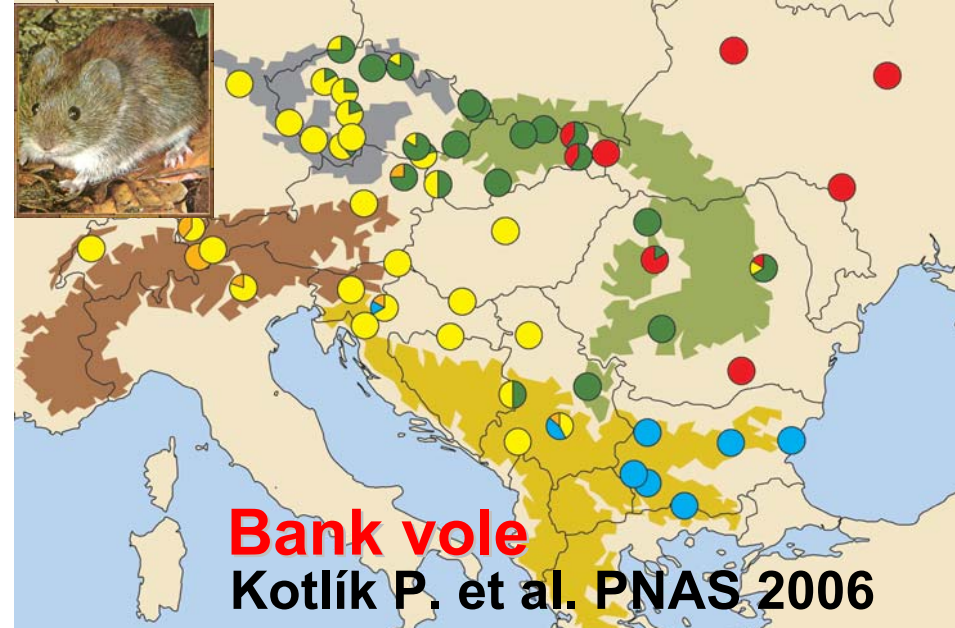
**Wild boar**

Larson G. et al. Science 2005



**Common vole**

Haynes S. et al. Mol. Ecol. 2003



**Bank vole**

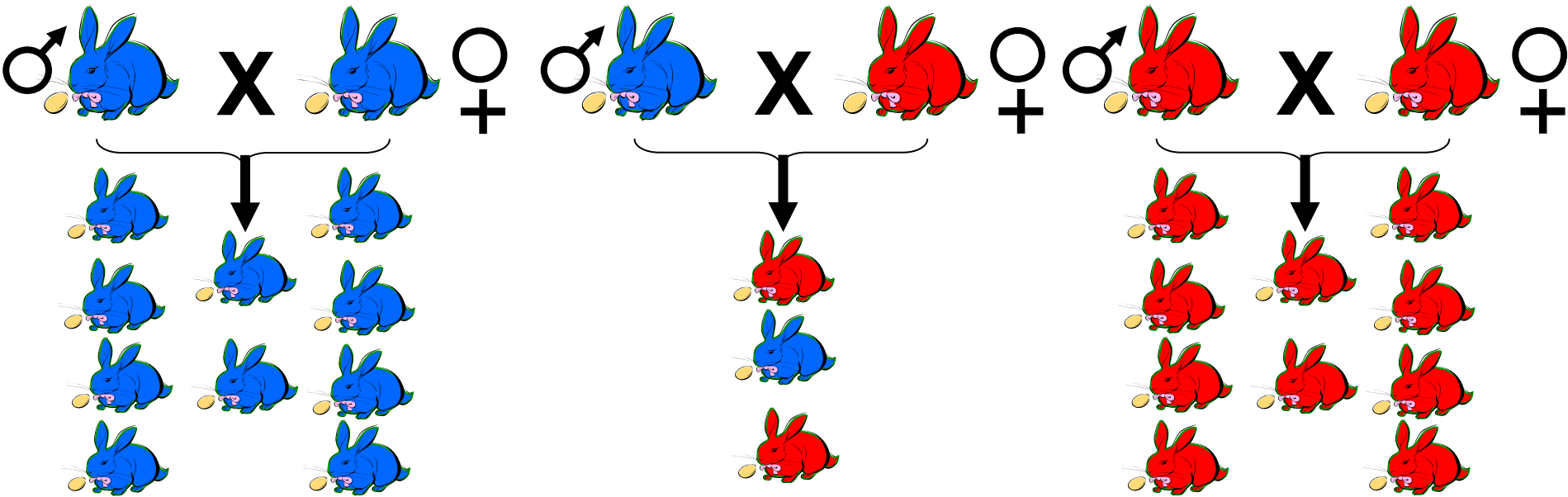
Kotlík P. et al. PNAS 2006



# Mother's curse

MtDNA mutations create variation in the efficiency of the oxidative phosphorylation pathway and therefore cellular energy production.

Deleterious mutations may reduce the performance of sperm cells due to their high energy requirements, with little or no effect on the viability of somatic cells or ova.

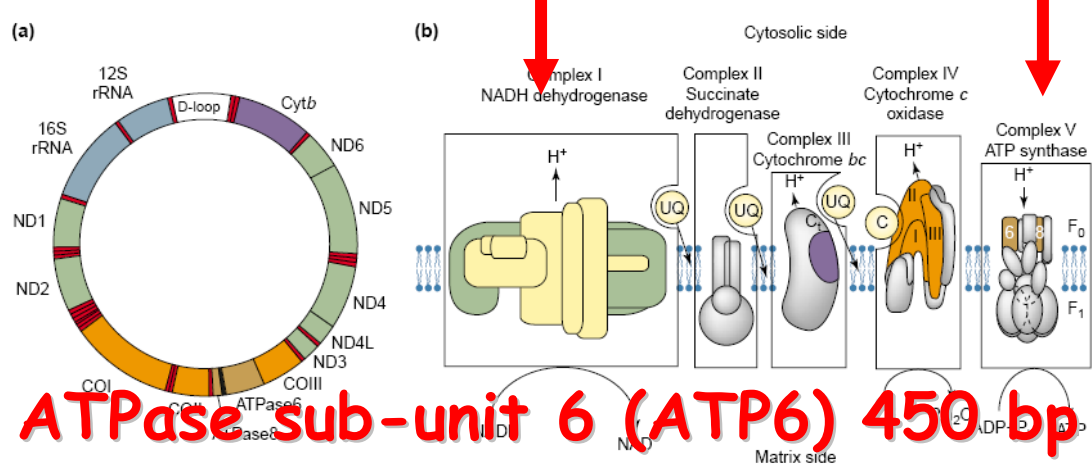


• These data have particular implications for phylogeography and the management of populations for conservation.

# Techniques: mtDNA PCR, SSCP, Sequencing

A clear divergence between the three phylogeographic regions

## OXPPOS complex



ATPase sub-unit 6 (ATP6) 450 bp  
NADH (ND2) 409 bp



LOW DIVERSITY

LOW DIVERSITY

REFUGE

REFUGE

REFUGE

GENE FLOW BARRIER

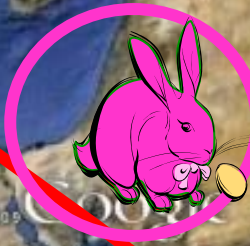
GENE FLOW BARRIER

REFUGE

REFUGE

One homogeneous species or two different subspecies

Even MC1R is different



# DNA Barcode:

Μικρές τυποποιημένες αλληλουχίες που ταυτοποιούν είδη σε ένα ευρύ φάσμα ζωντανών οργανισμών

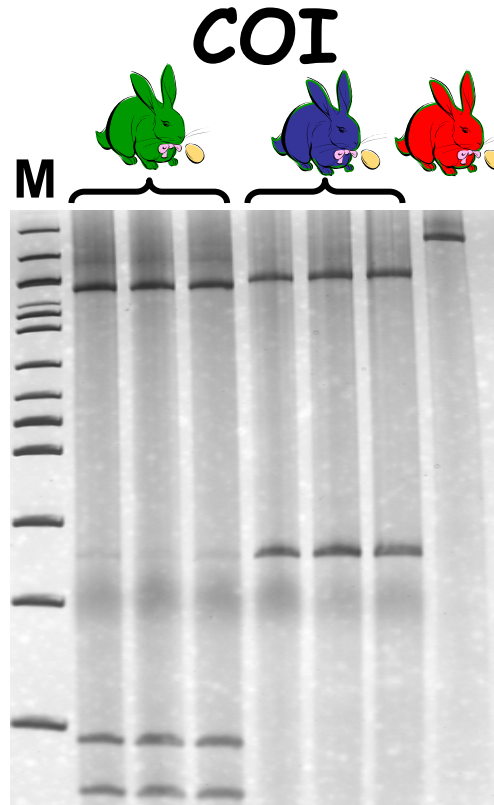
Δεν φιλοδοξεί να περιγράψει τα είδη, αλλά να προσδιορίσει τα όριά τους



Εξ ορισμού, είναι ένας μοναδικός δείκτης.

Μεταξύ ανθρώπων διαφέρει μόνο σε δύο το πολύ θέσεις. Ανάμεσα σε ανθρώπους και χιμπαντζήδες σε 60 και σε γορίλες σε 70. Περίπου το ίδιο συμβαίνει και με τα 65.000 είδη που έχουν ταυτοποιηθεί με barcode μέχρι σήμερα.

Ένα από τα σημαντικότερα μόρια στη διαδικασία αυτή είναι το μιτοχονδριακό γονίδιο της κυτοχρωμικής οξειδάσης (COI)



Έτσι, το barcode διαφέρει ελάχιστα μέσα στο είδος, αλλά έχει πολύ μικρή επικάλυψη ανάμεσα στα είδη.

# Research in progress

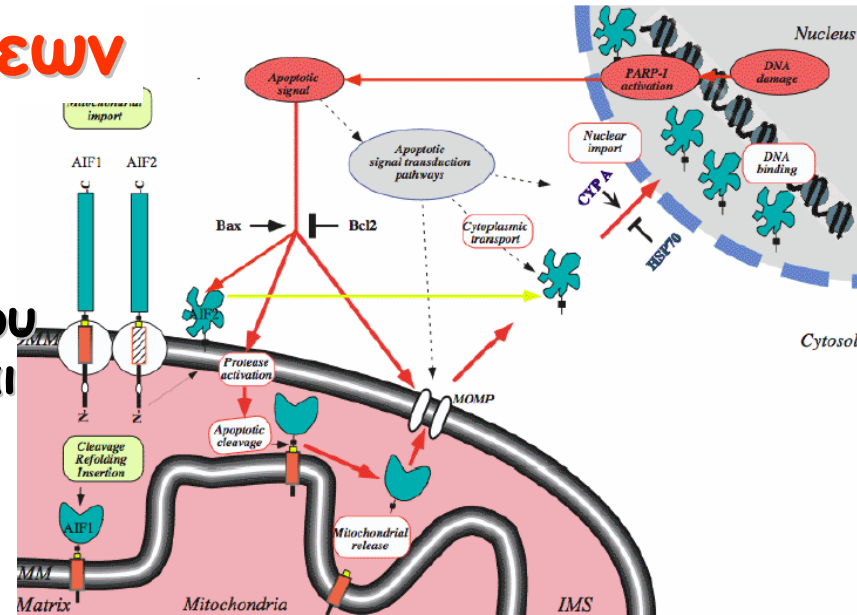
## Έλεγχος εξελικτικών υποθέσεων

Η επιβίωση του κυττάρου εξαρτάται από την αναπνοή, που συμβαίνει στα μιτοχόνδρια μέσω μεγάλων πρωτεϊνικών συμπλόκων, περίπου 43 υπομονάδων, που δημιουργούνται άλλες στα μιτοχόνδρια και άλλες από το γονιδίωμα του πυρήνα και πρέπει να αλληλεπιδράσουν στενά.

Ο υψηλός ρυθμός μεταλλάξεων του μιτοχονδριακού DNA ίσως βοηθάει τους οργανισμούς να προσαρμοστούν σε περιβάλλοντα που αλλάζουν.

Γονίδια του πυρήνα πρέπει να προσαρμοστούν γρήγορα στους συνεταιίρους τους στα μιτοχόνδρια.

Στα πρωτεύοντα πυρηνικά γονίδια που κωδικοποιούν για πρωτεΐνες μιτοχονδρίων είχαν ίδιους ρυθμούς μετάλλαξης με τα μιτοχονδριακά γονίδια και εξελίσσονταν 10 φορές γρηγορότερα από τα υπόλοιπα πυρηνικά. Δηλ. μιτοχονδριακά και πυρηνικά γονίδια, που συνεργάζονται, συνεξελίσσονται ταχύτητα γιατί οι ρυθμοί μεταβολής στα μιτοχόνδρια είναι πολύ γρήγοροι

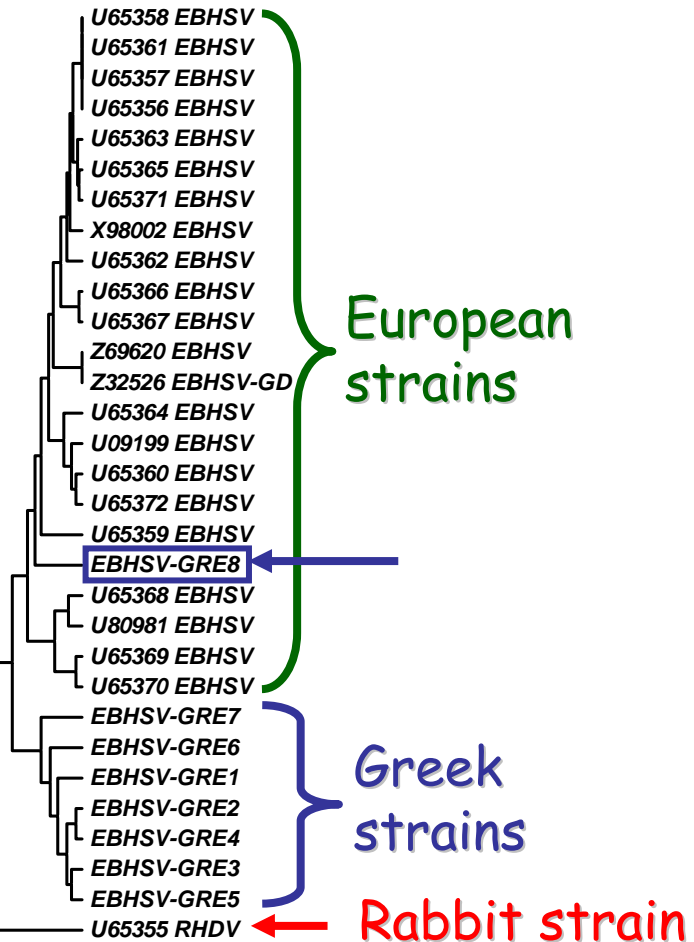
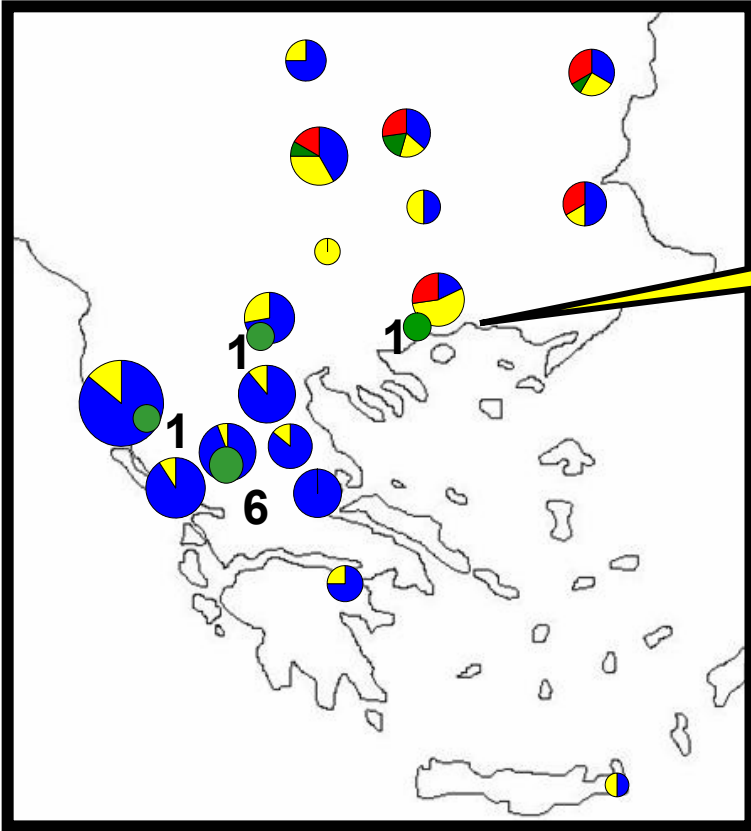


## Research in progress

- A study on the same brown hares for the DRB locus (more complicated due to four pseudogenes)
- A European epidemiological study on the European Brown Hare Syndrome (EBHS), including phylogenetic analysis of the virus' strains
- Multifactorial analysis, combining data of all studies both at individual and population level

# Combined genetics of hares and EBHS virus

A strain of EBHS virus phylogenetically different from the strains already found in Greece, probably developed in C. Europe and introduced in Greek wild by a reared-released hare



Probably with a serious impact on hares' health and survivorship

0.4 0.3 0.2 0.1 0.0

# Applied issues

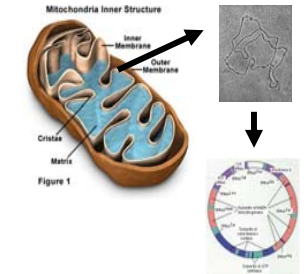
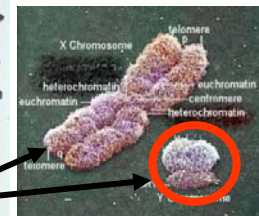
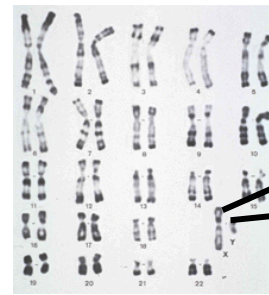
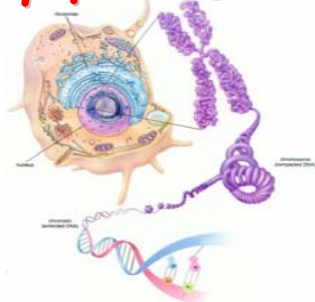
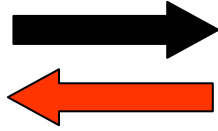


**PCR**

**Πυρηνικό DNA**

**Χρωμόσωμα Y**

**mtDNA**



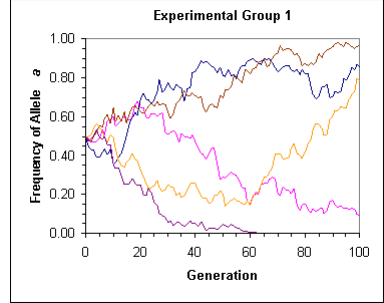
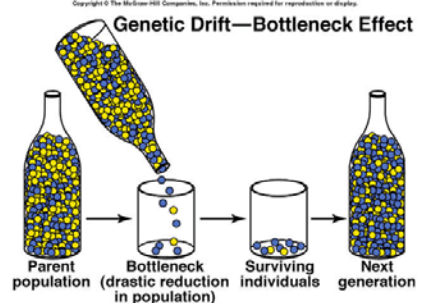
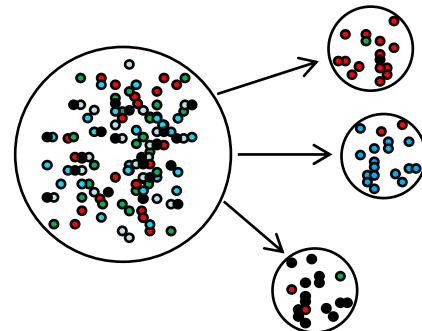
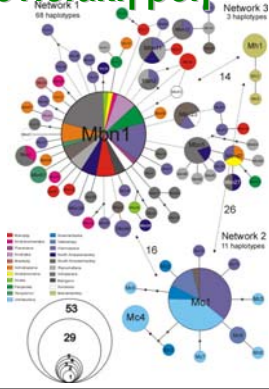
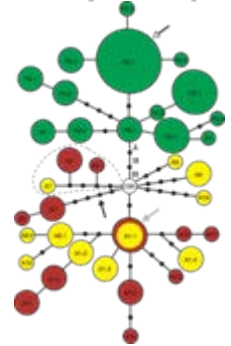
**Φυλογένεση**

**Μετανάστευση-Γονιδιακή ροή**

**Μετανάστευση-Αρχή Ιδρυτή**

**Φαινόμενα Στενωπού**

**Τυχαία Γενετική Παρέκκλιση**



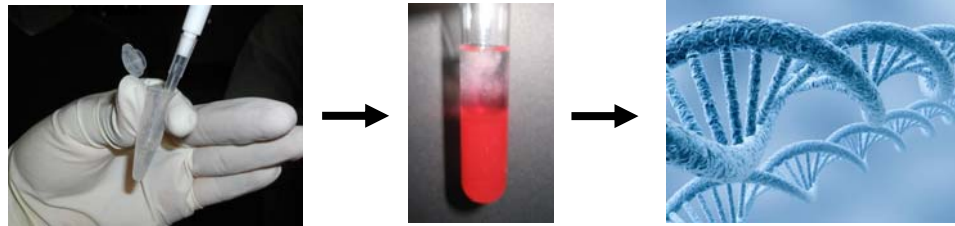
**Μικροεξέλιξη**

**Διατήρηση βιοποικιλότητας**

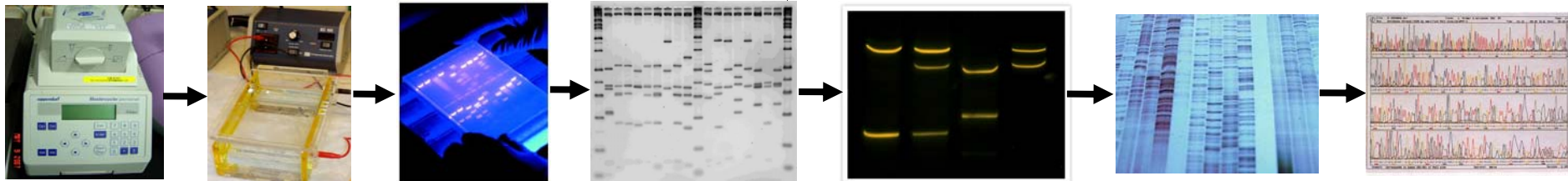
**Μηχανισμοί ειδογένεσης**



# Τροφή (κονσερβοποιημένη, επεξεργασμένη, μαγειρεμένη)



## Εξαγωγή και Απομόνωση DNA



## Επεξεργασία του DNA (PCR, ηλεκτροφόρηση, ανάλυση)



## Ταυτοποίηση των ειδών που περιέχονται στο αρχικό επεξεργασμένο προϊόν



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Thank you very much



Τμήμα  
Βιοχημείας &  
Βιοτεχνολογίας  
Πανεπιστημίου Θεσσαλίας

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Biotechnology  
University of Thessaly