Alma Mater Studiorum - Università di Bologna

Istituto Superiore per la Protezione e Ricerca Ambientale

DOTTORATO DI RICERCA

Biodiversità ed Evoluzione

Ciclo XXIII

Settore scientifico disciplinare di afferenza: BIO/11 BIOLOGIA MOLECOLARE

Phylogeny and genetic diversity of Italian species of hares (genus *Lepus*)

Presentata da: **Dott. CHIARA MENGONI**

Coordinatore Dottorato: **Relatore:**

Prof. MANTOVANI BARBARA **Prof. ETTORE RANDI**

Esame finale anno 2011

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CHAPTER FIRST: INTRODUCTION

1.1 INTRODUCTION TO THE SPECIES

Hares are placental mammals belonging to the family *Leporidae*, included in the order Lagomorphs. Lagomorphs retained many primitive characters and didn't develop special morphological adaptations and behavioural differences between the different species, despite their ancient origin (about 55 million years ago) and wide distribution, which originally included the Palaearctic and Ethiopian regions and the Americas. Currently they are also present in Australia and New Zealand as a result of recent introductions.

They're plantigrade terrestrial animals and they are of medium size and slender shape, with small head, big eyes and long ears, highly developed hind legs designed for running and jumping; front limbs are equipped with five toes, and back four.

The diet is essentially vegetarian; common features are the presence of four incisors with no roots in the upper jaw and the lack of canines.

In this study we consider different species belonging to the family *Leporidae*:

- *Lepus corsicanus* (Italian hare). The Italian hare, or Apennine hare, was described in 1898 by W.E. de Winton as a distinct species from *Lepus europaeus*, based on some morphological characters observed on specimens in museum collections. The Italian hare, which was probably widely distributed in the past in central-southern Italy and in Sicily, and which was introduced in the 16th century in Corsica (Vigne 1992), was later downgraded to a subspecies of *L. europaeus*. In the middle of last century, because of hunting pressure and restocking with the European hare also in central and southern Italy, the subspecies *corsicanus* was considered extinct (Toschi 1965). The description of diagnostic morphological characters (Palacios 1996), and the results of recent genetic studies (Pierpaoli *et al*. 1999), have confirmed the status of species and have shown the presence of residual populations of hares in different areas of central-southern Italy and Sicily.
- *Lepus europaeus* (European brown hare). The current Eurasian distribution of *Lepus europaeus* extends from the northern provinces of Spain, to introduced populations in the United Kingdom and southern regions of Scandinavia, south to northern portions of the Middle East, and has naturally expanded east to sections of Siberia (Flux and Angermann 1990). This species has been extensively introduced as a game species into several countries across the globe. These countries are: Argentina,

Australia, Barbados, Brazil, Canada, Chile, Falkland Islands, New Zealand (North and South Island), Rèunion, the United Kingdom, Ireland and the United States (Flux and Angermann 1990).

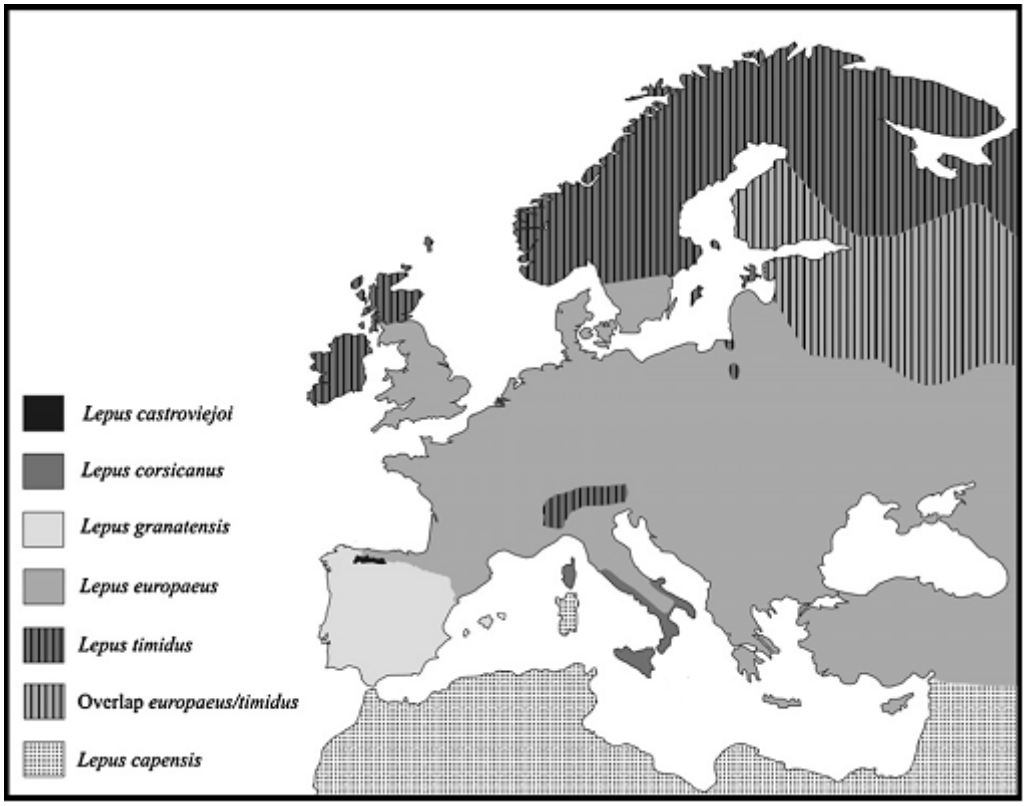


Fig. 1. Geographical distribution of the European and northern African hares (Alves *et al*. 2008)

In Italy the species has been subject to massive repopulation in the last century, that have led to the release of animals imported from abroad, or, in small part, raised in the peninsula. The populations of the subspecies *L. europaeus meridiei*, originally distributed throughout north-central Italy, have been replaced by introduced non-native hares and probably belonging to different subspecies.

- *Lepus timidus varronis* (Mountain hare). *Lepus timidus* has a widespread distribution and there are currently 15 recognized subspecies; we consider the subspecies *varronis*, distributed in the Alps. Historical hybridization events and genetic introgression with *L. europaeus*, recently documented in Scandinavia, in the Iberian Peninsula and in Russia (Thulin *et al*. 1997, Melo-Ferreira *et al*. 2005, Waltari and Cook 2005, Thulin *et al*. 2006, Melo-Ferreira *et al*. 2007), have made more complicated the identification of the genetic structure of populations.

- *Lepus capensis* (Cape hare). The geographic range of *Lepus capensis* (in Arabia) includes isolated populations scattered across the entire peninsula and extends east into India. It is also found on the

islands of Sardinia (ssp. *Lepus capensis mediterraneus*, but taxonomy is still uncertain (Suchentrunk *et al*., 1998) and Cyprus. Geographic range in Africa is extensive and separated into two distinct regions of non-forested areas (Boitani *et al.* 1999). The southern distribution includes the following countries: South Africa, Lesotho, Swaziland, Namibia, Botswana, Zimbabwe, southern portions of Angola, Mozambique, and Zambia (Boitani *et al*. 1999). The northern distribution includes: Tanzania, Kenya, Uganda, Eritrea, Sudan, Egypt, Libya, Chad, Niger, Tunisia, Algeria, Burkina Faso, Mali, Morocco, Western Sahara, Mauritania, and Senegal.

- *Lepus granatensis* (Iberian hare). The geographic range of *Lepus granatensis* includes Portugal and nearly the entire Spain (Alves *et al.* 2003). It is absent from northern regions of Spain where *L. castroviejoi* and *europaeus* exist (Alves *et al*. 2003). In most of the northern provinces (Navarra, Asturias, Cantabria, Aragon, Catalunya, and Basque Country), *L. europaeus* and *L. granatensis* exist in parapatry, the Iberian hare inhabits the southern region and the Brown hare can be found to the north (Fernandez *et al*. 2004). *L. granatensis* is also located on the island of Mallorca of the Balearic chain (Schneider 2001). It has been introduced in southern France and Corsica (Perpignan) (Alves *et al*. 2003).
- *Lepus castroviejoi* (Broom hare). The distribution of *L. castroviejoi* is limited to the Cantabrian Mountains in the northwest of Spain (Flux and Angermann 1990).

1.2 LEPUS CORSICANUS

1.2.1 Distribution

This research project wants to focus the attention especially on the Italian hare, as an important endemic threatened species.

In this century, the distribution area of the species has been subjected to a substantial contraction accompanied by a significant reduction in density of populations. The most important risk factors have been identified in the fragmentation of the distribution area, isolation and low population density, deterioration of the habitat, introduction of *L. europaeus* and over-hunting.

Lepus corsicanus may be considered a typical Italian endemism, because in Corsica the species was introduced by humans: it is important to adopt as soon as possible measures for the conservation and management.

Currently, the distribution area of the Italian hare (Fig. 3) recognizes as the northern limit Monte Amiata in the province of Grosseto, on the Tyrrhenian coast, and a small area near the National Park of Abruzzo, in the province of L'Aquila, on the Adriatic coast. South of these areas, the taxon is still present in all peninsular regions up to the province of Reggio Calabria, but with relict populations, often isolated in protected or inaccessible mountainous areas (Angelici, 2001).

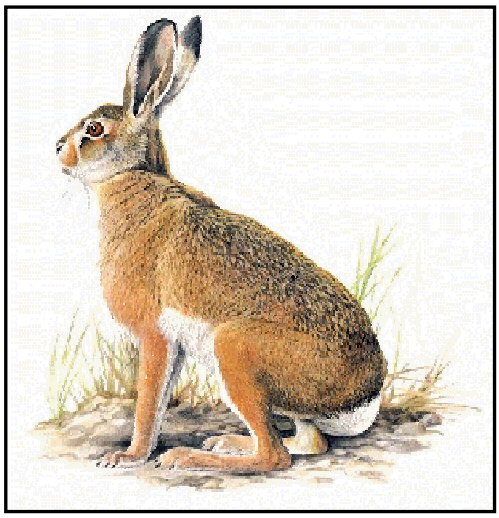


Fig.2. *Lepus corsicanus*

On the contrary, in Sicily the species is relatively widespread and is also observed in hunting areas far from protected parks (for example, in the province of Enna, where there aren't protected areas). Despite the identification of several tens of hares taken in recent years in the territory where hunting is practiced, it was not possible to confirm the presence of the Italian hare on the Island of Elba, but only the European Hare (introduced for hunting purposes).

Figure 4 shows, on a UTM map with 10 km mesh, the current distribution of *Lepus corsicanus* in Sicily, which is present in 70% of the 283 quadrants that divide the island; the absences in the south-eastern Sicily is due to the lack of information. The species remains absent from all small islands around Sicily (Lo Valvo, 2007).

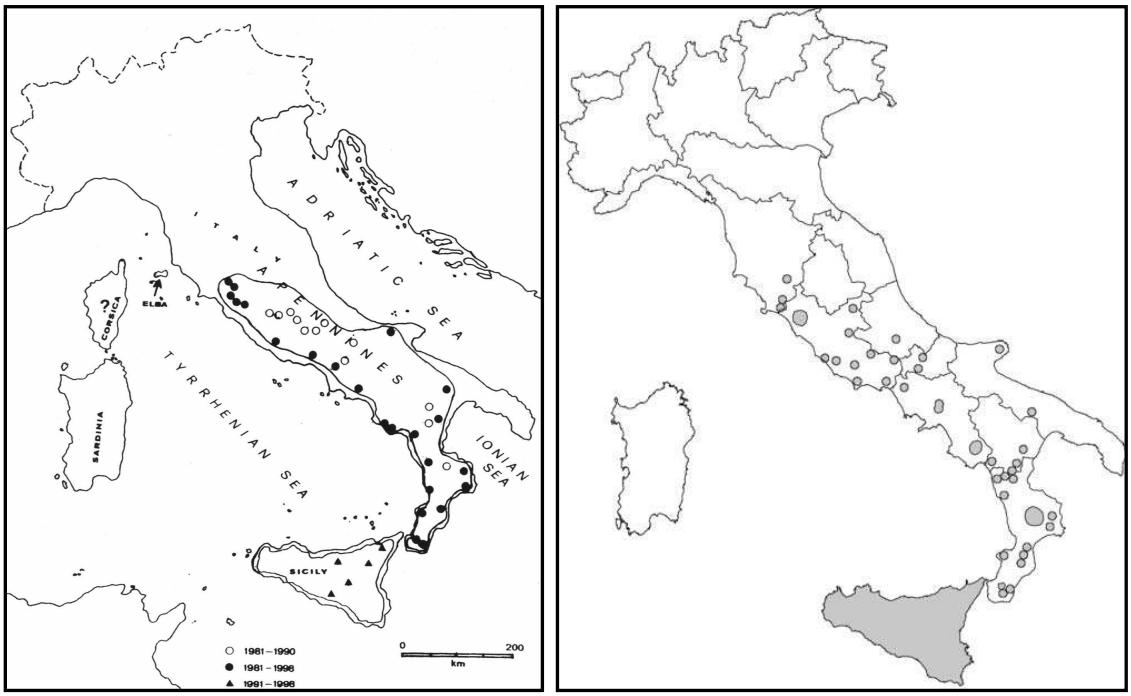


Fig. 3. Distribution of the Italian hare in Italy in the past (on the left) and in the present (on the right).

Lepus corsicanus was introduced also in Corsica for hunting purpose, such as other sedentary game species (Pietri, 2002), but currently there aren't data about his distribution.

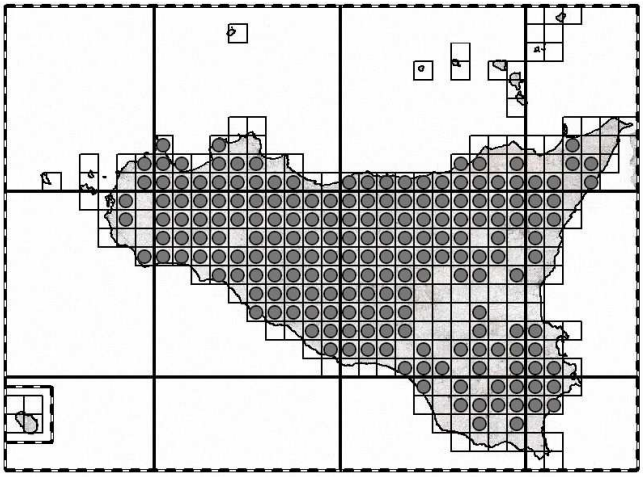


Fig. 4- Distribution of the Italian hare in Sicily.

1.2.2 Ecology

The Italian hare, as all *Leporidae*, shows a laterally compressed head, very long auricles, narrow and elongated body usually kept bent, hind legs much longer and stronger than the front legs and suitable for jumping, short tail. The fur is reddish-gray on the neck, shoulders, hips, grayish-black on the back, white on the belly; long ears are black-tipped, black is also the top of the queue, and eyes are big and brown. There isn't sexual dimorphism.

Although similar in general to the European hare, the Italian hare has a relatively more slender shape, in fact the head-body length, the back foot and the ears are proportionally longer, the average weight of adults is about 800 g lower. The morphological characteristics of *Lepus corsicanus* may imply a greater potential for thermal regulation and adaptation to the warm climate of the Mediterranean regions, whereas it is known that the European hare is well adapted to open environments with a continental climate.

The distinction between the two species in nature is not easy (Fig. 5), especially with the naked eye and with animals moving. The coat colour of the Italian hare differs from that of the European hare for tawny shades and for the clear transition between the reddish fur of the hip and the white belly.

Fig. 5. Differences in the coat colours between the European hare (on the left) and the Italian hare (on the right).

The ecological distribution of *L. corsicanus* confirms the adaptation to habitats characterized by a Mediterranean climate (Tomaselli *et al*. 1973, Blondel and Aronson, 1999), although it is present from sea level up to 1900 m above sea level in the Apennines and 2400 m above sea level on Mount Etna. Favourite habitats seems to be those with alternating clearings, also grown, bushy areas and broad-leaved woods; can also occupy areas with dense cover of Mediterranean vegetation, including dune environments.

The species seems to have a sedentary behaviour with relatively small living spaces, attending after sunset and for the entire night almost the same areas of pasture, in which close it sets up a day den.

In areas of sympatry with the European hare they were observed frequenting the same pastures.

The diet of *L. corsicanus*, studied in Sicily, varies seasonally as the available vegetation changes. Monocotyledones, Cyperaceae and Juncaceae, are ingested year round, while Gramineae and Labiatae are consumed during spring and summer, respectively (De Battisti *et al.* 2004). Dicotyledones ingested year round by *L. corsicanus* are Leguminosae and Compositae (De Battisti *et al*. 2004).

The sexual rest period is relatively short (about sixty-seventy days), between October and December and for the other months the species doesn't know practically sexual activity stops, although it is more intense in summer season.

The species is polygamous and doesn't form stable pairs, for the possession of the females, males often fight with aggression and violence, hitting with the front legs, and rarely, trying to bite.

Mating takes place mostly at dusk or at night and the act of copulation is often preceded by a sort of courtship; the female prepares a special haven where giving birth to leverets (the number of births varies from one to five), which born after a gestation of about 41-42 days. A female can reproduce an average of three or four times a year, but as the breeding season is more or less long in relation to latitude, in regions with a warmer climate also occur five births.
Hares have therefore a relatively high reproductive potential and this condition is well suited to a medium-sized herbivore that is subjected to a strong predation by several species of carnivores.

1.2.3 Threats

There are several conservation problems about the Italian hare that make this species threatened with extinction. Listed below are the main ones:

- Fragmentation and isolation of the distribution areas. The genetic differences observed between the haplotypes of specimens of *L. corsicanus* coming from central Italy, from south Italy and Sicily (Pierpaoli *et al*. 1999) reflect an evolutionary history with the presence of ancient subdivisions in the distribution area and consequently long periods of reproductive isolation. Current distribution data show an important fragmentation that must necessarily be attributed to anthropogenic causes, with very small populations isolated from each other, within an environmental matrix became increasingly unfavourable. The erosion and fragmentation of habitat due to human impacts are the major causes of isolation of the populations.
- Interspecific competition. The protracted restocking with *L. europaeus* for hunting purposes may have led to interspecific competition and the transmission of infectious diseases (Guberti *et al.* 2000). Competition may occur mainly through the use of the same food resources or breeding sites and shelters; this may affect the coexistence of the populations concerned, in terms of changes in their size, distribution and structure.
- Genetic pollution. In the genus *Lepus* hybridization between species has already been documented; in Sweden hybrids were observed between the native form *L. timidus* and introduced *L. europaeus* (Thulin *et al*., 1997), and in Spain the three Iberian species of hares (*L. granatensis*, *L. castroviejoi*, *L. europaeus*) harbour high frequencies of mitochondrial DNA (mtDNA) from *Lepus timidus*, now extinct in the region (Melo Ferreira *et al*. 2005). The absence of observation of intermediate phenotypes and the lack of introgression in mitochondrial haplotypes of a species in the other leads to the belief that hybridization

between the European and the Italian hare is an unlikely event. More concretely, however, is the risk of genetic pollution from translocated individuals (often from breeding station) in areas where genetically and morphologically different populations live (Pierpaoli *et al*., 1999; Riga *et al*., 2001).

- Hunting activity. Although the species is not included in the list of hunted species (L. n. 157/92) in the peninsula, the hunting exercise can be a real limiting factor: this is a complex issue because of the coexistence in the same areas of *L. corsicanus* and *L. europaeus*, of the difficulties in the recognition in nature, of the lack of a specific tradition in hares management and of the knowledge basis for sustainable management. These difficulties are reflected in a high impact on the residual populations of Italian hare and a practical impossibility in the implementation of conservation strategies, different between the two species.
- Poaching. In central and southern Italy and Sicily poaching on hares is traditional and widespread, encouraged by the lack of supervisory activities.
- Habitat degradation. Reforestation in general represents a threat to the habitat of the hare. Moreover, the intensification of cultivation occurred since the war has led to a series of very heavy impact on the agricultural environment and adjacent natural areas, as well as for wildlife directly. They are also various consequences about the use of chemicals products (fertilizers and pesticides): direct consequences for acute and chronic toxicity, and indirect consequences for trophic sources significant reduction.

1.2.4 Legal protection

In 2008 the species was classified as “vulnerable” according to the criteria of the IUCN Red List. In 2001 the National Action Plan for the Italian has been published, which contains guidelines for conservation actions for the species.

The DPCM 07.05.2003 (Official Gazette. July 3, 2003, No. 152) introduced this species among those hunted ("Only population living in Sicily" for the period October 15-November 30), of which art. 18, paragraph 1, letter e) of National Law 157/1992.

1.3 - INTRODUCTION TO CONSERVATION GENETICS

1.3.1 Conservation genetics

Conservation genetics is the application of genetic techniques and analysis methods to preserve species and dynamics entities capable of coping with environmental change. It deals with the genetic factors that affect extinction risk and genetic management regimes required to minimise these risks. It is a discipline that focuses on methods and techniques of population genetics, but also considers the ecology of the species, ethology, physiology, molecular biology, the evolution and demography. The role of population genetics is to investigate the origin, the maintenance, the organization and the causes of genetic variation between natural populations. Natural populations are treated as evolution units and their gene pools, resulting from the set of all alleles in various loci, constitute the raw material of evolutionary changes.

There are several genetic issues in conservation genetics (Frankham *et al.* 2002):

- The deleterious effects of inbreeding on reproduction and survival (inbreeding depression).
- Loss of genetic diversity and ability to evolve in response to environmental change.
- Fragmentation of population and reduction in gene flow.
- Random processes (genetic drift) overriding natural selection as the main evolutionary process.
- Accumulation and loss (purging) of deleterious mutations.
- Resolving taxonomic uncertainties.
- Defining management units within species.
- Use of molecular genetic analysis in forensics.
- Use of molecular genetic analysis to understand aspects of species biology (mating, dispersal and migration patterns, reproduction systems) important for conservation.

1.3.2 DNA structure and function

Each individual, with the exception of identical twins, is genetically unique because he possesses a unique patrimony of genetic information (DNA) organized in the chromosomes that are contained in cell nucleus (nuclear DNA), and in mitochondria, organelles present in cell cytoplasm (mitochondrial DNA or mtDNA).

Each DNA molecule takes the form of a double helix built by four nucleotides, the chemical building blocks (Adenine-A, Thymine-T; Guanine-G and Cytosine-C). The structure of the double helix consists of two ribbon-like entities that are entwined around each other and held together by crossbars composed of two bases that have strong affinities for each other. The bases within each chain are bound together by a pentose sugar and phosphate ion, while the opposing strands are held together by weak hydrogen bonds that are relatively easy to break by heating. The linear order in which these four nucleotides follow each other in the double helix of the DNA is called a nucleotide sequence. This very simple structure is extremely stable and allows the DNA to act as a template for protein synthesis and replication (Watson & Crick, 1953).

1.3.3 Mitochondrial DNA

Unlike most cells, whose functions are defined by the nuclear DNA, mitochondria have their own DNA and are believed to have evolved separately.

Vertebrate mitochondrial DNA is a circular double helix made up of 15.000-20.000 nucleotides, depending on the species (Hartl & Clark, 1993). It is replicated, independently from cell and DNA nuclear replication, each time the mitochondria divide. During the gametogenesis, the content of cytoplasm and, therefore, the number of mitochondria contained in the gametes significantly change. Mitochondria are provide entirely by cell eggs, therefore during fertilization is the egg cell of the mother that transmits all the mitochondria to the zygotes. Hence mtDNA is haploid and does not recombine. The different types of mtDNA that are originated from mutations and that are present in populations are called “mitochondrial haplotypes”**.**

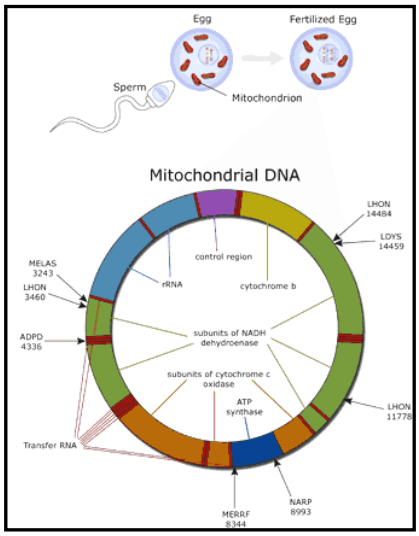


Fig. 6. Mitochondrial DNA structure.

1.3.4 Nuclear DNA

The genome of vertebrates and many other living organisms is largely made up of coding and non coding DNA sequences.

Coding regions are organized in functional domains and are necessary to regulate the protein

synthesis consisting of a first phase of transcription of DNA into messenger RNA (mRNA) followed by a phase of translation of the messenger RNA into protein.

Non coding, tandem repeated DNA exists in the genome of every species (repetitive DNA). Tandem repetitive sequences, commonly known as “satellite DNAs” are classified into three major groups:

- Satellite DNA: highly repetitive sequences with very long repeat lengths (up to 5.000.000 nucleotides), usually associated with centromeres.
- Minisatellite DNA: present in hundreds or thousands of *loci* in eukaryotic genomes. These tandem repeats often contain a repeat of more than 10 nucleotides and are present in multiple pairs that produce clusters of 500-30.000 nucleotides. Profiling of these minisatellite *loci* is done using *multi-locus* probes-MLP or *single-locus* probes-SLP to obtain DNA fingerprinting.
- Microsatellite DNA: present in many thousands of *loci* in eukaryotic genomes. They are made up of very short repeats, from 2 to 8 nucleotides, repeated only few times that produce clusters of a few dozen or few hundred nucleotides at every *locus*. Microsatellites are used extensively in forensic genetics and are profiled through PCR

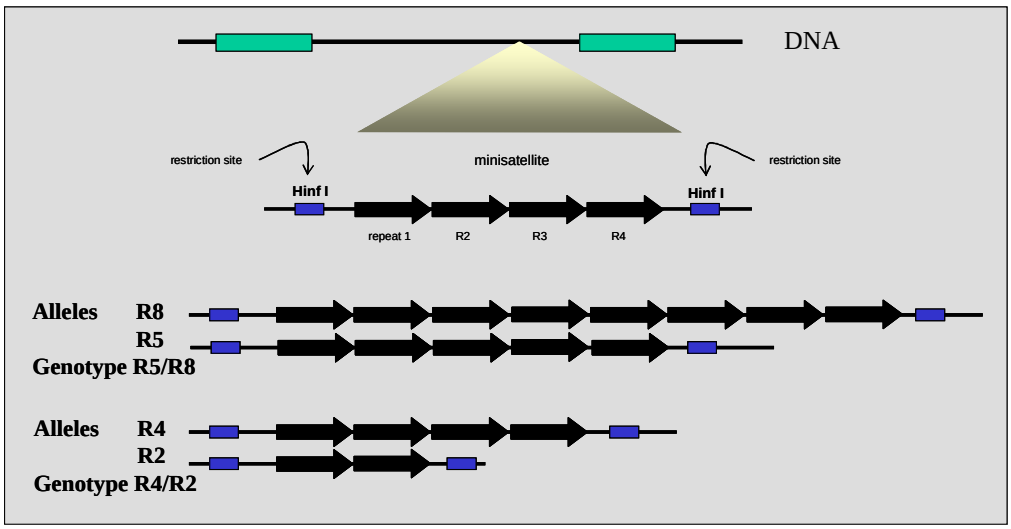


Fig. 7. Minisatellite’s scheme.

1.3.5 Genetic mutations and polymorphisms

A genetic mutation is any change in the nucleotide sequence of a genome or, more generally, of genetic material (DNA or RNA); mutations modify the genotype of an individual and can possibly change their phenotype depending on its characteristics and interactions with the environment.

Mutations generate variability in individuals and populations because they modify DNA sequences and produce the basis on which natural selection can act. Different mutational processes exist and they mainly depend on the structure and function of involved DNA:

- Nucleotide substitution: is the substitution of a nucleotide with another at a certain point in the DNA strand.
- Insertion or deletion of a single nucleotide or series of nucleotides.
- Crossing-over and recombination: crossing-over can be symmetrical, which produces exchanges of corresponding sequences and genetic recombination between two chromosomes, or asymmetrical, which occurs between tandemly repeat DNA that do not precisely align themselves and gives rise to the deletion of a DNA fragment from a chromatid and its insertion on another one.
- DNA slippage: can occur during tandemly repeated DNA replication when the single strand nascent DNA can pair in another point of the DNA template.
- Gene conversion: produces the transfer of a DNA sequence from one allele to another one.

The term polymorphism indicates the existence in a population of more than one allele for a given locus more frequently than 1% (a gene presenting two or more variations for the same nucleotide sequence). A polymorphism can be detected: from phenotypic frequencies, from the presence of different protein variants and from differences in gene sequences.

1.3.6 Genetic markers

Genetic markers are the main tools used to study the genetic variability within and among populations, in fact they allow to estimate which alleles are present inside them (Avise, 1994; Muller & Wolfenbarger, 1999; Parker *et al*., 1995; Sunnucks, 2000).

A genetic marker can be represented by any variable and in hereditable characteristics in populations, determined by genes and not by environment. The main characteristics of a molecular marker are: polymorphism, expression stability during environmental, ontogeny and morphologic changes, well identifiable and amplifiable, Mendelian heredity, expression codominance, many species application. Many kinds of markers exist:

- Visible polymorphisms: phenotype characters with few distinctive variants (*morfi*) not environmental influenced. They are not very common in the eukaryotic genome.
- Molecular markers: macromolecules (proteins, RNA, DNA) which can be separated through electrophoresis in agarose gel within an electric field with a migration speed depending on

their weigh and electric charge and visible under ultraviolet light. Alloenzymes belong to these markers (Murphy *et al*., 1996).

- DNA markers: they allow to isolate genetic variability in DNA fragments with different dimensions and weighs and to separate them within electrophoresis gel. Many kinds of markers belong to them:

  RFLP: restriction enzymes and restriction fragments length polymorphisms analysis (Jefferies *et al*., 1985).

  RAPD: random amplified polymorphic DNA (Williams, 1990).

  AFLP: amplified fragment length polymorphisms (Vos *et al.*, 1995).

  VNTRS: variable number of tandem repeats. They are non-coding regions characterized by tandemly repeated sequences. Each repeat can be made up from 10 to 64 nucleotides (minisatellites) or from 2 to 9 nucleotides (microsatellites).

  SNPs: Single Nucleotide Polymorphisms. They're widespread in all genomes (coding and non-coding regions), and they evolve in a manner well described by simple mutation models, such as the infinite sites model (Vignal *et al*., 2002). These polymorphisms are base substitutions, insertions, or deletions that occur at single positions in the genome (Budowle, 2004). They are hypothesized to become the marker of choice in evolutionary, ecological and conservation studies as genomic sequence information accumulates. As a biallelici marker, SNPs are innately less variable than microsatellites but they are the most prevalent form of genetic variation and hence there is a substantial increase in the number of loci available (Brumfield *et al*. 2003).

1.4 STATISTICAL METHODS

The aim of population genetics is to describe the genetic composition of population and to understand the causes of the evolutionary change. Genetic variability in population is described through allele frequencies. Allele frequencies at each locus can vary across the generations due to mutations, natural selection, migration or genetic drift.

The different combinations of alleles present at each locus determine individual genotypes, whose frequency in populations can be calculated. In an ideal population, in which population forces are not active, genotype frequencies remain constant from one generation to the next. Population genetics is based on an abstract, ideal population model, supported by a series of assumptions.

The Hardy-Weinberg law defines the relationship that exists between allele and genotypes frequencies at each locus in a population. In a locus with two alleles (a1 and a2), with frequencies p and q, with p+q=1, the genotype frequencies are obtained from the proportion:

a_1a_1: $2a_1a_2$:a_2a_2=p^2:2pq:q^2.

It is possible to estimate the genotype frequencies of a population in Hardy-Weinberg Equilibrium (HWE) using the observed allele frequencies. If a population is not in HWE an estimate of genotype frequencies, starting from the allele frequencies, may be wrong. Deviation from HWE may be caused from non-random mating, gene flow, founder effect, bottleneck and random drift.

Even though many reasonable statistic approaches are available to analyse the genetic structure of populations and to estimate the absolute and effective population sizes, most of them, used in this study are based on *F* and *Bayesian Statistics*.

In population genetics, *F*-statistics (also known as fixation indices) describe the level of heterozygosity in a population; more specifically the degree of a reduction in homozygosity when compared to Hardy-Weinberg expectation. Such changes can be caused by the Wahlund effect (the reduction of heterozygosity in a population caused by subpopulation structure), inbreeding, natural selection or any combination of these.

The concept of *F*-statistics was developed during the 1920s by the American geneticist Sewall Wright who was interested in inbreeding in cattle, but its applications deeply increased after the 1960s when the advent of molecular genetics allowed heterozygosity in populations to be reliably measured.

F-statistics measure the correlation between genes drawn at different levels of a (hierarchically) subdivided population. This correlation is influenced by several evolutionary forces, such as mutation and migration, but it was originally designed to measure how far populations had gone in the process of fixation owing to genetic drift.

The different F-statistic measures, FIS, FST, and FIT, are related to the amounts of heterozygosity at various levels of population structure. Together, they are called F-statistics, are derived from *F*, the inbreeding coefficient, and look at different levels of population structure: FIT is the inbreeding coefficient of an individual (I) relative to the total (T) population, as above; FIS is the inbreeding coefficient of an individual (I) relative to the subpopulation (S), using the above for subpopulations and averaging them; and FST is the effect of subpopulations (S) compared to the total population (T), and is calculated by solving the equation:

$$(1 - FIS)(1 - FST) = (1 - FIT).$$

In a simple two-allele system with inbreeding, the genotypic frequencies are:

$p2 + Fpq$ for AA; $2pq(1 - F)$ for Aa; and $q2 + Fpq$ for aa.

The value for F is found by solving the equation for F using heterozygotes in the above inbred population. This becomes one minus the observed number of heterozygotes in a population divided by its expected number of heterozygotes at Hardy–Weinberg equilibrium.

The expected value at Hardy–Weinberg equilibrium is given by

$$\mathrm{E}\,(\int(\mathrm{Aa})) = 2\,p\,q$$

where p and q are the allele frequencies of A and a, respectively. It is also the probability that at any locus, two alleles from the population are identical by descent.

A reformulation of the definition of F would be the ratio of the average number of differences between pairs of chromosomes sampled within diploid individuals with the average number obtained when sampling chromosomes randomly from the population (excluding the grouping per individual). One can modify this definition and consider a grouping per sub-population instead of per individual. Population geneticists have used that idea to measure the degree of structure in a population.

Unfortunately, there is a large number of definitions for FST, causing some confusion in the scientific literature. A common definition is the following:

$$F_{ST} = \frac{\mathrm{var}(p)}{p\,(1-p)}$$

where the variance of *p* is computed across sub-populations (Wright, 1951; 1965; 1969; 1978; Weir & Cockerham, 1984; Slatkin, 1991; Weir & Hill, 2002).

Bayesian Statistic is based on Bayes' theorem (also known as Bayes' rule or Bayes' law), set out by Thomas Bayes (1702-1761), an English clergyman in 1764. It is a result in probability theory relates the conditional and marginal probability distributions of random variables. In some interpretations of probability, Bayes' theorem tells how to update or revise beliefs in light of new evidence "*a posteriori*", according to which, the probability *a posteriori* of an event (given by evidence) can be obtained combining the observations (probability conditional or *likelihood*) with the subjective degree of belief (*a priori*) about the same event based on experiences or theories independent from data. Bayesian probability is an interpretation of the probability calculus where the concept of probability can be defined as the degree to which a person (or community) believes that a proposition is true. The probability of an event *A* conditional on another event *B* is generally different from the probability of *B* conditional on *A*. However, there is a definite relationship between the two, and Bayes' theorem is the statement of that relationship.

Some researchers consider the scientific method as an application of Bayesian probabilistic

inference because they claim Bayes' Theorem is explicitly or implicitly used to update the strength of prior scientific beliefs in the truth of hypotheses in the light of new information from observation or experiment. This is said to be done by the use of Bayes' Theorem to calculate a posterior probability using that evidence and is justified by the Principle of Conditionalisation that P'(h) = P(h/e), where P'(h) is the posterior probability of the hypothesis 'h' in the light of the evidence 'e', but which principle is denied by some. Adjusting original beliefs could mean (coming closer to) accepting or rejecting the original hypotheses.

Since the 1950s, Bayesian theory and Bayesian probability have been widely applied and it has recently been shown that Bayes' Rule and the Principle of Maximum Entropy (MaxEnt) are completely compatible and can be seen as special cases of the Method of Maximum (relative) Entropy (ME). This method reproduces every aspect of orthodox Bayesian inference methods. In addition this new method opens the door to tackling problems that could not be addressed by either the MaxEnt or orthodox Bayesian methods individually (Lindley, 1990; West & Harrison, 1989; O'Hagan, 1994; Sivia, 1996; Pritchard *et al*., 2000; Tijms, 2004).

The main differences between *F* (or *frequency*) and *Bayesian Statistics* lie in the definition, interpretations and in the effective calculus of probabilities (Press, 1972), in fact:

- F statistics assigns probabilities to random events according to their frequencies of occurrence or to subsets of populations as proportions of the whole and allows to compare the test hypothesis to a model/hypothesis (the "null" hypothesis). The probability *p* of an event *H* depends on the number of times (*n*) the event occurs on the total number of tests (*N*). The probability *p* of *H* corresponds therefore to its frequency:

$$pH = n(H)/N.$$

- Bayesian statistics assigns probabilities to propositions that are uncertain; conditions on the data actually observed, and is therefore able to assign posterior probabilities to any number of hypotheses directly. The requirement to assign probabilities to the parameters of models representing each hypothesis is the cost of this more direct approach. The probability *p* is an estimation of *likelihood* that that the event *H* occurs. We can have convictions (subjective) or information (objective, even though not exactly quantifiable) that an event may more or less occur frequently. Posterior probability of an event *H* corresponds on the probability that the event *H* occurs given the evidence *E*:

$$Pr\,(H) = Pr\,(H/E).$$

1.5 GENETIC STUDIES ON HARES

Evolution, phylogeny and population genetics of the hares are still poorly known and the taxonomic distinction is still unclear for some species (Petter 1961; Angermann 1983, Flux 1983, Chapman & Flux 1990, Hoffman 1993).

During the Pleistocene, the severe climatic changes induced major shifts in species distributions, forcing them to retract, expand, displace and/or fragment their ranges (Hewitt, 1996). In Europe, these continuous oscillations led to the production of greater subspecific and specific diversity in the southern peninsulas since they were the major ice age refugial areas (Hewitt, 1999). Hares (genus *Lepus*) seem to perfectly demonstrate these phenomena.

L. corsicanus, distributed in central and southern Italy and Sicily, and introduced in Corsica in the sixteenth century (Vigne 1992; Palacios 1996), was described by W.E. Winton in 1898. The proposal of a new species was immediately rejected by Miller (1912) and others (Ellerman & Morrison-Scott 1951; Toschi 1965), because they were considering *L. corsicanus* as a subspecies of *L. europaeus*, but no genetic information on intraspecific divergence and phylogenetic relationships was available about the hare.

The historical distribution suggests that natural populations of Italian hare and European hare were allopatric with apparent contact areas from central Tuscany to the Gargano promontory (Palacios 1996). However, no data was available to document the degree of reproductive isolation or possible gene flow between populations in contact. This lack of knowledge has encouraged an assessment of its current distribution range and genetic structure of its populations.

In 1999 Pierpaoli *et al*. assessed the genetic distinction of *L. corsicanus*, investigated the genetic variation among populations of the peninsula and Sicily, and reconstructed the phylogenetic relationships between the Italian hare and other species of hares from Europe and Africa. This research, based on mitochondrial DNA (mtDNA), has provided the first evidence that *L. corsicanus* is genetically distinct and deeply divergent from the other Eurasian and African hares (Fig. 8). In addition it was shown that Italian and European hares did not share any mitochondrial haplotype, suggesting the absence of interspecific flow past a long separate evolutionary history between the two species and reproductive isolation.

From the study of the Eurasian and African hares we can identify two main groups of haplotypes:

- Clade A: includes *L. granatensis, L. corsicanus, L. timidus.*
- Clade B: includes *L. c. mediterraneus, L. habessinicus, L. starcki, L. europaeus.*

These results suggest that the three species belonging to Clade A, with a common ancestor, would

have colonized Europe independently of *L. europaeus* and would have originated for isolation during the Pleistocene glaciations in the southern or northern areas of refuge.

A surprising result is the close relationship between the Italian hare and the Mountains hare: times of divergence and biogeographical structure of the evolution of the genus *Lepus* in Europe indicates that *L. corsicanus* and *L. timidus* are relict species that originated before the dispersal of *L. europaeus* in western Europe.

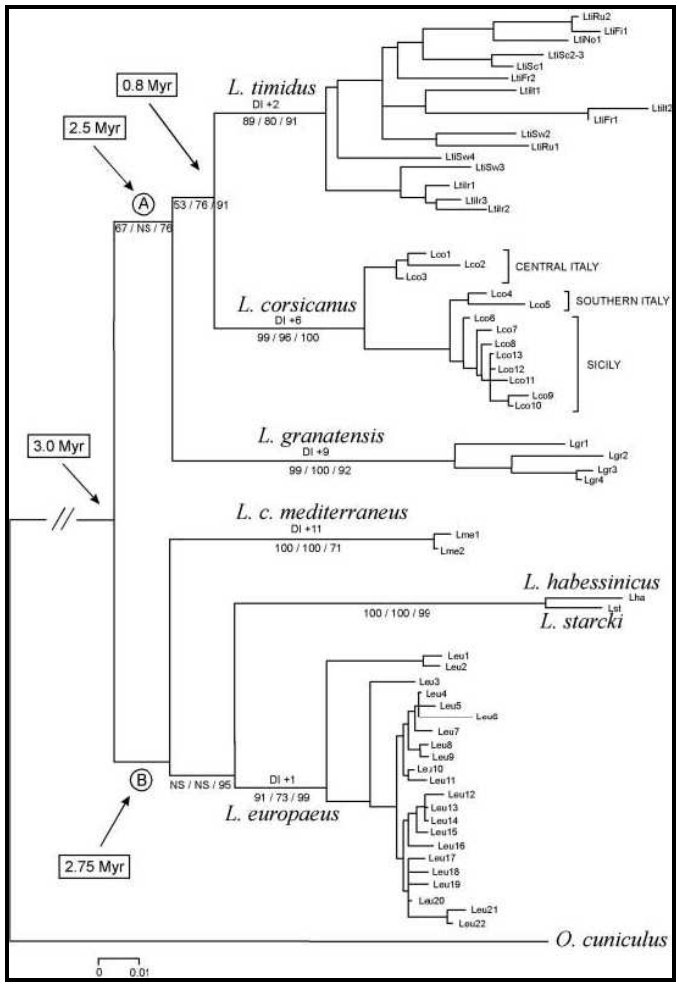


Fig. 8. Phylogenetic relationships between *L. corsicanus* and other hare species (Pierpaoli *et al*. 1999).

L. europaeus probably originated from an African ancestor and then spread to Europe, perhaps recently and by two different settlements: evidence for the first settlement would be the oldest haplotypes found in three altitude zones in the Apennines.

In historical times and in particular during the last century, there has been a massive spread of individuals with different haplotypes from Europe and South America, due to the translocation of hares for hunting purposes.

For the *L. corsicanus* haplotype divergence time is estimated between 45,000 and 121,000 years ago, suggesting the hypothesis of an ancient isolation in glacial refuge areas in central and southern Italy; during this period it was possible the colonization of Sicily due to sea level drop (about 110 meters from the current as a consequence of the glacial period).

To confirm these results we can see that hares sampled in central Italy have unique haplotypes, not found in hares sampled in southern Italy (Campania and Calabria) and Sicily. The separation of Sicily, from the end of the last ice age, may explain the divergence between Sicilian hares and peninsula's hares (Pierpaoli *et al*., 1999).

L. castroviejoi and *L. corsicanus* have allopatric and restricted ranges: the first one lives in the Cantabrian Mountains of the Iberian Peninsula and the second one in the Apennines from central and southern Italy and in Sicily.

Analysis of partial sequences of mtDNA cytochrome b showed that *L. corsicanus* and *L. castroviejoi* are closely related to *L. timidus* (2.2–2.7% of divergence) and, further, that the level of differentiation between them is very low when compared with the levels among typical hare species (circa 1.4% vs. 9% average between *Lepus* species; Alves *et al*., 2003).

Moreover the results based on three independent nuclear loci suggest that *L. corsicanus* and *L. castroviejoi* might be conspecific and distinct from *L. timidus*. These findings emphasize once again the fundamental role of the southern European peninsulas as deposits of biodiversity and natural laboratories for the study of evolution and speciation (Alves *et al*. 2008). These two southern european endemisms occupy highly specialized patches of scarce habitat and thus the establishment of suitable conservation mechanisms is a major concern (e.g., Temple and Terry, 2007).

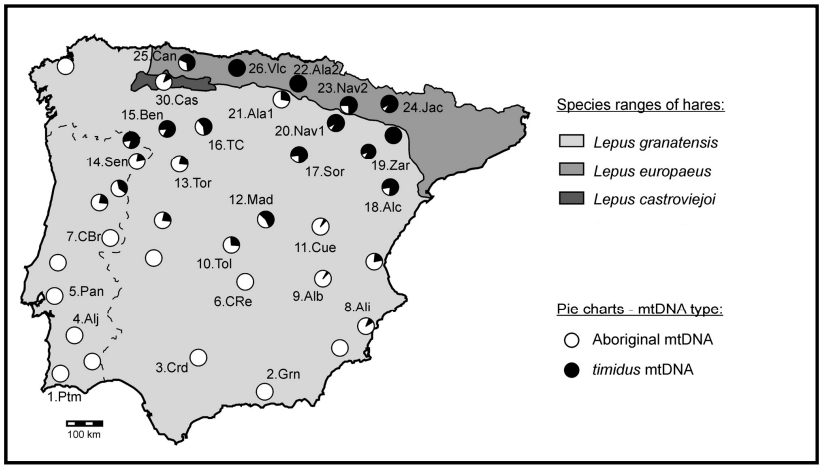


Fig. 9. Geographical distribution of *Lepus granatensis*, *L. europaeus*, *L. castroviejoi* in the Iberian Peninsula. The pie charts show the frequencies of mtDNA of *L. timidus* origin in Iberia (Melo-Ferreira *et al*. 2009).

In some areas the alternation of species due to climatic fluctuations during glaciations set the conditions for competition and eventually hybridization. Hares in the Iberian Peninsula appear to illustrate this phenomenon: populations of the three species of hares present in the Iberian Peninsula harbour high frequencies of mitochondrial DNA (mtDNA) from *Lepus timidus*, an arctic/boreal species now extinct in the region (Fig. 9).

The hypothesis is that this massive introgression of mtDNA occurred during the competitive replacement of the arctic species by the temperate ones as climate became warmer at the end of the last glaciation (Melo-Ferreira *et al*. 2009).

1.6 AIMS

Present-day distribution of the Italian hare is extremely fragmented in central and southern Italy. Populations survive at low density, mainly in protected areas and National Parks, where the species has managed to escape overhunting and competition with introduced Brown hares.

Extensive human disturbance (overhunting and restocking) could have threatened, severely restricted and eventually eradicated the Italian hare from most of its former historical range.

The knowledge of the genetic status of Italian hare populations and in particular the certainty of its reproductive isolation from the European brown hare are indispensable for the design of adequate management and conservation plans of this species in the country.

The main purposes of this conservation genetic study are:

- to investigate the extent of genetic variability among Italian hares collected in peninsular Italy and Sicily;
- to detect any signs of hybridization (and thus of possible gene flow) between the species *L. corsicanus* and *L. europaeus* in sympatric areas of Italy;
- to confirm the phylogenetic relationships between the Italian and the other European species;
- to evaluate the use of new genetic markers (SNPs) which allow to identify with precision the species of individual samples (e.g. in cases of genotyping of faecal samples collected in non-invasive genetic programs), and of geographic populations.
- to investigate Major Histocompatibility Complex (MHC) variability at class II DQA locus between the brown hare and the Italian hare.

For the development of this work various molecular genetic techniques have been used such as DNA extraction from biological samples (using different extraction methods), DNA amplification by PCR, genotyping and sequencing by special laboratory equipment.

CHAPTER SECOND: MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

We analyzed nearly 700 samples belonging to six different species; sampling details are shown in Table 1. Most of the samples were collected in Italian regions, but sampling also covered other european and non-european countries between 1992 and 2009.

The distribution map in Fig. 10 shows sampling areas in the Italian peninsula, in Sicily, in Sardinia and in Corsica.

SPECIES	ORIGIN	SAMPLES
L. corsicanus	Italy-Corsica	154
L. capensis?	Africa	12
L. cap. mediterraneus	Sardinia	92
L. castroviejoi	Spain	5
L. europeaus	Italy-Hungary-Romania-Austria-Bulgaria-Greece-Uruguay	343
L. granatensis	Spain	29
L. timidus	Italy-Finland-Sweden-Ireland-Scotland	75

Tab. 1. List of species analysed, collecting areas, and number of samples for every species.

Fig. 10. Map of sampling areas in Italy and Corsica: red points represents samples belonging to *L. corsicanus*, green points to *L. europaeus*, yellow points to *L. c. mediterraneus* and blue points to *L. timidus*.

The sample collection phase is fundamental to ensure a good success of the following genetic analysis based on PCR techniques because analysis procedures and the quality of the results are dependent on the quality of samples and possible contaminations. For these reasons it necessary to collect and preserve biological samples in the best possible way.

We analysed invasive biological samples (tissues or blood) coming, for the most part, from individuals killed during the hunting seasons. Tissue samples should be kept in sterile plastic tubes airtight containing 90-100% ethanol (EtOH 100%) according to a report alcohol-sample 1 to 10, this is because ethanol dehydrates the tissues by blocking the biochemical reactions subsequent to cell death, which would lead to degradation of DNA. Blood samples are placed in a preservative solution like Longmire Buffer respecting the proportions of 1 to 1 (for example 1 ml of solution must be added to 1 ml of blood).

Samples can then be frozen at -20 ° C to -80 ° C in liquid nitrogen or, alternatively may be kept at room temperature or refrigerated at all temperatures below room temperature (ethanol and buffer make DNA stable).

2.2 MOLECULAR ANALYSES

2.2.1 DNA extraction

The extraction process is a crucial step because it must isolate DNA molecules which are present in a sample producing available solutions of DNA without contaminants and must impede further degradations during laboratory procedures. In this study both manual and automated extraction methods to isolate available DNA from tissues and blood were used (for details see Box 1). Negative controls (no biological material added to the extractions) were always used to check possible contaminations during both extraction processes.

Manual extraction uses a guanidinium thiocyanate and diatomaceus earth (guanidinium-silica) protocol (Gerloff *et al.*, 1995). The used solutions are characterized by the presence of:

TRIS: it maintains a constant pH value that inhibits the activity of enzymes that degrade DNA;

EDTA: it acts as chelants of bivalent calcium and magnesium ions inhibiting the activity of DNase that requires the presence of these ions;

GUS (Guanidinium Thiocyanate): it produces the chemical disintegration of protein structures.

Guanidinium-silica protocol (summary)

Preparation of the samples:

- a piece of tissue (50 mg) is cut and transferred into an "eppendorf" test tube of 1.5 ml containing 500 µl of *GUS Lysis Buffer*; flamed sterilized scalpels and forceps are used.
- a small amount of blood is added to 800 µl of water into an "eppendorf" test tube of 1.5 ml to produce cell lysis and extract hemoglobin, which would otherwise interfere with the extraction process. We centrifuge for 1 minute, eliminate the supernatant and add 500 µl of *GUS Lysis Buffer*.

Digestion of the samples:

- in rotation at 56°C overnight.

Collecting DNA:

- centrifuge at room temperature for 10 minutes and collect the supernatant;
- add 500 µl of *GUS Binding Solution* and in rotation for 1 hour;
- centrifuge at room temperature for 1 minute and eliminate the supernatant.

DNA is now bound to micro-granules of pelleted silica at the bottom of the test tube. Each pellet is washed twice, each time with 500 µl of *GUS Washing Solution* and then centrifuged at room temperature for 1 minute. The supernatant is eliminated, each pellet is washed again twice, each time with 500 µl of EtOH 70% and centrifuged at room temperature for 3 minutes. The pellet is dried in open "eppendorf" in a thermostatic multiblock at 56 °C for 10 minute. The pellet is re-suspended in 300 µl of TE for 15 minutes at 56°C, transferred in a new "eppendorf" and preserved at -20°C.

QUIAGEN Stool and tissue extraction kit protocol (summary)

Manual phase:

Preparation of the samples:

- Preparation is the same written above in the Guanidinium-silica protocol; in this case we add to the sample 20 µl of *Proteinase K* and 180 µl of *ATL Lysis Buffer* (previously warmed up at 57°C for 5 minutes); flamed sterilized scalpels and forceps are used.

Digestion of the samples:

- in rotation at 56°C for 30 minutes.

Collecting DNA:

- centrifuge at room temperature for 10 minutes and collect the supernatant;
- transfer the supernatant in a new "eppendorf" and centrifuge at room temperature for other 10 minutes;
- transfer the supernatant in a new appropriate QUIAGEN tube.

Automated phase:

- link the multiblock with QUIAGEN tubes to the robot's platform containing a vacuum pump system to aspirate liquid solutions and a serious of silica-gel filters to trap the DNAs.
- the mechanical hands add 410 µl of *AL/E Lysis Buffer* (previously warmed up at 57°C for 5 minutes) to each QUIAGEN tube containing digested sample solutions and the software activates the pup system to isolate the DNA;
- the mechanical hands add 500 µl of *AW1 Washing Solution* and the software activates the vacuum for 10 minutes;
- the mechanical hands add 500 µl of *AW2 Washing Solution* and the software activates the vacuum for 10 minutes;
- the mechanical hands add 300 µl of *AE Solution* (elution solution) to each sample re-suspending the DNA linked to silica filters at room temperature for 1 hour.

The solution with the DNA is transferred in a new "eppendorf" and preserved in freezer at - 20°C.

Box.1. DNA extraction protocols.

Automated extraction in an automated manner by the *MULTIPROBE IIEX* robot (Perkin Elmer) and using the QUIAGEN Stool and tissue extraction kit (QUIAGEN). The robot consists of 2 mechanical hands controlled by an appropriate software which can be set up each time according to the number of samples and to the extraction kind and conditions. This procedure consists of a first manual phase and of a second automated one.

2.2.2 DNA amplification

DNA amplification is a necessary procedure to obtain sufficient DNA quantity for molecular analysis. DNA sequences made up of a few dozen or thousands nucleotides and present in a single copy in DNA samples can be amplified effectively up to 10 million times in a few hours using Polymerase chain reaction (PCR) (Mullis *et al.*, 1986).

PCR occurs by reconstructing the chemical conditions necessary to obtain DNA synthesis in vitro.

First, it is necessary to identify the gene or DNA sequence that one wishes to amplify. The sequence to be amplified is flanked on both side by sequences that must be at least partially known, in fact to start off PCR it is necessary to chemical synthesise a pair of oligonucleotides (20-30 bp) “primers” that are at least partially complementary to the flanking sequences and can bind to flanking regions starting the duplication process of the target sequence. PCR occurs in a test tube that contains: the DNA sample, the two primers, the DNA polymerase enzyme, a certain quantity of free nucleotides, all this in a buffer solution that optimises DNA synthesis. Every test tube for PCR is placed in a thermal cycler that carries out a prefixed thermal cycle made up of the following steps and repeats it many times:

- denaturation of the DNA sample at temperatures up to 90-95°C;
- annealing of the primers to the flanking sequences: it occurs at temperatures which vary from 40°C and 55°C, depending on the length of the primers and their base sequence;
- extension of the primers through the enzymatic action of a thermoresitant DNA polymerase (Taq Polymerase) which catalyses the extension of the primers: it occurs at 72°C end ends in the complete replication of both strands of the target sequence.

By the end of the first cycle, every form of the target sequence present in the sample is replicated once, and the thermal cycle of the PCR is repeated a second time and then many other times (20-40) producing an exponential replication of the target sequence because with every successive cycle the synthesised DNA is doubled.

The advantage of using PCR is that the DNA does not have to be in large amounts or even purified

to be amplified. It has also been successfully used to amplify ancient DNA (Hofreiter *et al*., 2001). PCR efficiency depends on the capacity to faithfully amplify the target DNA. If the primers anneal to the target sequence and also to other sequences present in the DNA samples, then the PCR would amplify "aspecific" sequences which would make the analysis and interpretations of the results problematic and even impossible.

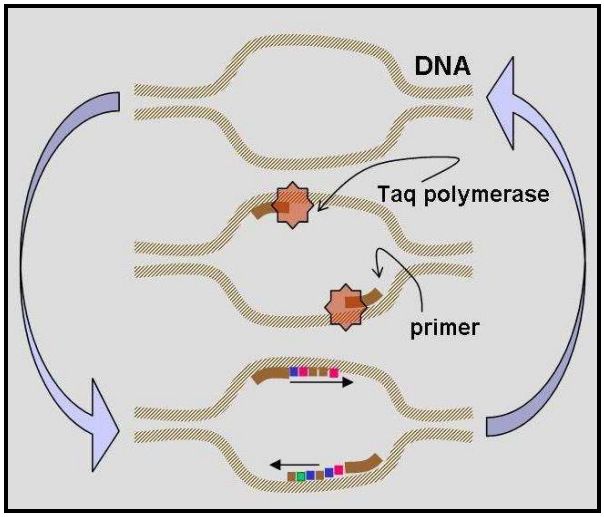


Fig. 11. Different phases of the Polymerase Chain Reaction.

2.2.3 DNA markers used for the analysis

In this study we used three different molecular markers:

- **Mitochondrial DNA (mtDNA)**. The mtDNA has only maternal inheritance, infact during gametogenesis cytoplasmic content changes significantly, and with it the number of mitochondria, to the benefit of female gametes that, being rich in cytoplasm and mitochondria, are the only ones able to transmit the mtDNA zygote. Almost the whole mtDNA has a coding function with the exception of a large region that controls the replication of the genome itself (D-loop or control region): genes coding for proteins involved in the breathing processes or for ribosomal and transfer RNA molecules are been identified inside this region). In mammals the length of the D-loop varies 880 to 1400 bp (Sbisà *et al.*, 1997) and its rate of nucleotide substitution, especially in mammals, appears to be five to ten times higher than that of single-copy nuclear genes (Hartl & Clark, 1993); mutational events are rare within the genes coding for proteins, but they increase in frequency in non-coding regions. Control region is the most variable part of the

mitochondrial genome and therefore the most interesting from the evolutionary point of view: this allows to successfully use it as molecular marker in the genetic studies, at both interspecific and intraspecific level. Most of the studies in which control region sequences have been used have focused on intraspecific patterns of variability and phylogenetic relationships of closely related species.

- **Nuclear DNA: Microsatellites**. They have quickly become of standard usage as genetic markers in DNA fingerprinting. They are nuclear DNA sequences made up of a simple motif of 2-8 nucleotides, that is repeated in tandem for a certain number of times with or without interruptions due to the insertion of other nucleotides or other sequences. Microsatellites have been identified in the genome of all organisms analysed up to now and are distributed in a more or less random way in chromosomes (Mellersh & Ostrander, 1997). They are not frequent in coding sequences of genes (exons), while they may be present in introns. The composition of microsatellite sequences is variable. In fact the short DNA segments can be made up of mono, di, tri or tetranucleotides (Mellersh & Ostrander, 1997; Stallings *et al.*, 1991; Tautz & Renz, 1984). Microsatellites present very high estimated mutation rates (in vertebrates 10-4-10-5 mutations per *locus* for every generation) which determine high levels of polymorphisms, in fact in a single *locus* more than 10 alleles can be present which differ for the number of repeats and therefore for their molecular weight. Moreover they find many applications in population genetics, in fact they represent particularly useful tools to study population story and structure, their genetic variability and allow to correctly assign the belonging species and to detect potential hybrids.
- **Nuclear DNA: Single Nucleotidic Polymorphisms (SNPs).** This marker consists just in a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. For such a base position with sequence alternatives in genomic DNA to be considered as an SNP, it is considered that the least frequent allele should have a frequency of 1% or greater. Although in principle, at each position of a sequence stretch, any of the four possible nucleotide bases can be present, SNPs are usually biallelic in practice. One of the reasons for this, is the low frequency of single nucleotide substitutions at the origin of SNPs, estimated to be between 1 x 10-9 and 5 x 10-9 per nucleotide and per year at neutral positions in mammals (Li *et al.*, 1981, Martinez-Arias *et al.*, 2001). Therefore, the probability of two independent base changes occurring at a single position is very low. Another reason is due to a bias in mutations, leading to the prevalence of two SNP types. Mutation mechanisms result either in

transitions: purine-purine (A_G) or pyrimidine-pyrimidine (C_T) exchanges, or transversions: purine-pyrimidine or pyrimidine-purine (A_C, A_T, G_C, G_T) exchanges. Some authors consider one base pair indels (insertions or deletions) as SNPs, although they certainly occur by a different mechanism. The very high density of SNPs in genomes usually allows to analyse several of them at a single *locus* of a few hundred base pairs, so that SNPs could represent a more reliable and faster genotyping method.

- **Nuclear DNA**: **Major Histocompatibility Complex (MHC genes).** In all vertebrates studied to date, the MHC is a multigene family encoding receptors that act at the interface between the immune system and infectious diseases (Koutsogannouli *et al.* 2009). The primary role of the MHC is to bind fragments of antigenic proteins within cells and then transport them to the surface of the cell membrane. There, the complex is recognized by T-cell receptors (TCRs), which can initiate the cascade of immune responses (Janeway *et al.* 2005). The peptide-binding region (PBR) is responsible for antigen recognition, binding and presentation, and a match between the PBR, antigenic peptide and TCR is required to initiate an immune cascade (Brown *et al.* 1993; McFarland & Beeson 2002). Although PBRs show a degree of specificity, a single MHC molecule can bind multiple peptides that share common amino acids at specific anchor positions (Rammensee *et al.* 1995). Many of the MHC genes that have been studied are highly polymorphic across a wide taxonomic range in vertebrates. Polymorphism occurs mainly within the PBR, and the majority of studies have revealed that the pattern of nucleotide substitutions in the PBR deviates from neutral evolution expectation (Klein & Takahata 1990; Hill *et al.* 1991; Abbott *et al.* 2006). It has been suggested that the pattern observed can be maintained by overdominance and/or frequency dependence, reinforced by maternal–foetal incompatibility and mating preference (Penn & Potts 1999; Piertney & Oliver 2006). Nevertheless, generally it is accepted that this variability in the PBR is the key factor that enables the MHC proteins to bind a variety of pathogens. In addition, different MHC alleles have been associated with other important biological characteristics, such as susceptibility to infectious or autoimmune diseases, individual odours, mating preferences, kin recognition, cooperation and outcome of pregnancy (Hedrick 1994; Bernatchez & Landry 2003; Sommer 2005). Due to these functions and characteristics, the MHC has been the focus of studies of population genetics and evolutionary ecology that are concerned with the mechanisms and significance of molecular adaptation in vertebrates (Potts & Wakeland 1993; Hedrick 1994; Bernatchez & Landry 2003). Although different selective models have been proposed with regard to the

mechanisms that maintain MHC polymorphism in natural populations, this field still remains an open question and a central goal in evolutionary biology (Potts & Slev 1995; Edwards & Hedrick 1998).

2.3 MITOCHONDRIAL DNA (MTDNA)

2.3.1 MtDNA amplification

We sequenced nearly 450 nucleotides of the mtDNA D-loop using the forward primer Lepcyb2L (5'-GAAACTGGCTCCAATAACCC-3') and the reverse primer LepD2H (5'-ATTTAAGAGGAACGTGTGGG-3'), (Pierpaoli *et al*. 1999).

Amplification was performed in 10 µl of volume, using 2 µl of DNA solution, 1 µl of PCR Buffer 10X (1,5 mM of MgCl2), 1 µl of BSA (Bovine Serum Albumin), 0,4 µl of deossinucleotides (dATP, dCTP, dTTP, dGTP) 2,5mM, 0,15 µl of each primer 10 µM, 0,25 units of Taq and 5,25 µl of PCR water, in a 9700 ABI Thermocycler (Applied Biosystems) using the following protocol:

94°C x 2'→(94°C x 30''→ 50°C x 30''→ 72°C x 30'') for 40 cycles →

72°C x 10' → 4°C x 10' → 15°C

Positive amplifications were detected on a 2% agarose gel and binding DNA with an UV fluorescent reagent (Gel Red); PCR products were purified using 1 µl of a mixture of Exonuclease I and Shrimp Alkaline Phosphatase that remove respectively unincorporated primers and dNTPs using the following thermocycling program:

37°C x 30'→ 80°C x 15' →4°C x 10' →15°C

Sequencing PCR was carried out in 10 µl of volume, using 1 µl of PCR product, 0,7 µl of Big Dye terminator Mix, 0,2 µl of the extension primer 10 µM, 8,1 µl of PCR water using the following thermocycling program:

(96°C x 10'' → 55°C x 5'' → 60°C x 4') for 25 cycles → 4°C x 10' → 15°C

The main difference from the first PCR is that involves the use of one primer only to start DNA replication. The Big Dye terminator Mix contains a reaction buffer, Taq polymerase, deossinucleotides (dATP, dGTP, dCTP, dTTP) and dideossinucleotides (ddATP, ddGTP, ddCTP, ddTTP); dideossinucleotides (ddNTPs) are modified bases which posses an OH in 3'-position and avoid the formation of a phosphodiesteric link with another deossinucleotide, so that when a ddNTP is randomly incorporated in the chain, it stops the extension of the same and thus generate

fragments terminating with one of the four ddNTPs.

PCR products are purified by precipitation using 3M Sodium acetate (Na acetate) and Etoh 70-100%. One µl of each sequencing PCR product was resuspended in a denaturation solution (Formamide) and analysed by electrophoresis on an AB Prism 3130 Genetic Analyser with a 36 cm capillary array, POP4 polymer.

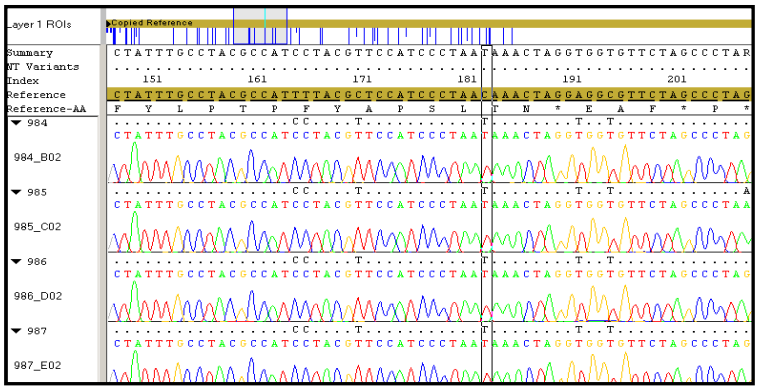
2.3.2 Sequence analysis

The sequencing process allows to read sequences of DNA strands amplified by the use of special equipment (automated sequencers). The methods currently used for DNA sequencing were developed by Maxam and Gilbert, and Sanger and colleagues in 1977: currently DNA sequences are read almost exclusively by using automated sequencers based on the Sanger method.

The automated sequencer modified the usual electrophoretic technique because they monitor the run of the nucleotide fragments with a laser instead of a gel, which is capable in detecting the fluorescence emitted by specific chemical molecules bound to DNA.

Sequencers own a series of multiple capillaries (usually 16 or 96), containing a polymer; inside capillaries occurs the electrophoretic run supported by an electrolyte.

Fluorescent marking systems use molecules called fluorophores; they are incorporated into DNA during sequencing PCR or amplification, by using primers that were previously labelled with a fluorophore (this is the case of microsatellite analysis), or by incorporating a labelled nucleotide into DNA synthesized (this nucleotides are dideossinucleotides (ddATP, ddGTP, ddCTP, ddTTP) labelled with different colours (A=green; C= black; G=blue; T=red)). During electrophoresis within each capillary, labelled DNA fragment passes through a viewing window, the fluorophore is excited by a laser beam and emits fluorescence that is detected and measured: there are different fluorophores emitting different wavelengths that are read as different colours.



We can label the DNA fragments with different colours that are recognized and analysed at the same time: we can label the four nucleotides with four different colours and analyse the results

Fig. 12. Electropherogram obtained during a sequencing process.

of a sequencing reaction in a single capillary. During electrophoresis the computer connected to the sequencer builds one or more image files, to track the performance of real-time analysis; results of sequence analysis are saved in shape of electropherograms.

When a fluorophore is excited by the laser produces a light emission recorded as peak: the peak height indicates the intensity of the emission and the colour indicates the colour of the fluorophore. Because each colour is uniquely associated with a specific termination reaction (i.e. one of the four nucleotides), the coloured peaks sequence of the electropherogram corresponds exactly to the DNA sequence (Fig. 12).

Softwares mainly used to process sequencing data are:

- *SeqScape v. 2.5* (Applied Biosystems 2001), an application especially designed for the processing of genetic which makes it possible to automatically align sequences with a sequence reference (appropriately chosen from those obtained from the sequencer or contained in the database) and it also allows to view electropherograms to correct any ambiguous nucleotides.
- *BioEdit* (Hall, 1999) allows to align sequences when they present gaps, sequences can also be edited and may be cut in order to take all the same length.
- *Dnasp v. 5* (Giulio Rosaz *et al*. 2003) is an interactive computer program for the analysis of DNA polymorphism from nucleotide sequence data.
- *Mega 5* (Tamura *et al.* 2011) allows to calculate a distance matrix between different sequences on the basis of a comparison in pairs (i.e. counting the number of mutations existing between them and comparing it, every time, with the number of total nucleotides) and allows to build phylogenetic trees based on different statistical methods; it is also particularly useful for identifying various types of mutations found by differentiating transitions, transversions or indel (deletions and insertions).
- *Network 4.5.1.6.* (Fluxus Technology, 2004-2010) is used to reconstruct phylogenetic networks and trees, infer ancestral types and potential types, evolutionary branchings and variants, and to estimate datings.

2.4 MICROSATELLITES

2.4.1 Microsatellites amplification

In this work we analysed 13 microsatellites loci (Tab. 2); 12 of them were amplified using QIAGEN Multiplex PCR Kit in four multiplex PCR in 7 µl of volume, using 2 µl of DNA solution, 3,5 µl of Qiagen Master Mix, 0,7 µl of Q-solution, 0,14 µl of each primer used in the multiplex PCR, 0,38 µl of RNase-free water.

Cycling conditions were optimized for each multiplex, starting from the following general PCR program:

95°C x 15'→ (94°C x 30''→Ta°C (57/60°C) x 90''→72°C x 60'') for 40 cycles →

60°C x 30' → 4°C x 10' → 15°C

Only locus SOL30, because of his large allele range, was amplified separately with a simplex PCR in 10 µl of volume, using 2 µl of DNA solution, 1 µl of PCR Buffer 10X (1,5 mM of MgCl2), 1 µl of BSA (Bovine Serum Albumin), 0,4 µl of deossinucleotides (dATP, dCTP, dTTP, dGTP) 2,5mM, 0,15 µl of each primer 10 µM, 0,25 units of Taq, 5,25 µl of PCR water, with the following general PCR program:

94°C x 2'→(94°C x 30''→ 60°C x 30''→ 72°C x 30'') for 40 cycles →

72°C x 10' → 4°C x 10' → 15°C

Locus	Multiplex	Size	Dye	Reference
SAT12	1	102-134	6-FAM	Mougel *et al.*, Animal Genetics, 1997.
LSA1		161-175	HEX	Kryger *et al.*, Molecular Ecology, 2002.
SOL33		199-226	6-FAM	Surridge *et al.*, Animal Genetics, 1997.
OCLS1B	2	142-180	HEX	Hamill *et al.*, Heredity, 2006.
LSA2		234-251	6-FAM	Kryger *et al.*, Molecular Ecology, 2002.
D7UTRI		110-168	6-FAM	Hamill *et al.*, Heredity, 2006.
LSA8	3	179-193	6-FAM	Kryger *et al.*, Molecular Ecology, 2002.
OCELAMB		106-130	HEX	Hamill *et al.*, Heredity, 2006.
LSA3		197-213	HEX	Kryger *et al.*, Molecular Ecology, 2002.
LSA4	4	100-110	6-FAM	Kryger *et al.*, Molecular Ecology, 2002.
LSA5		222-273	HEX	Kryger *et al.*, Molecular Ecology, 2002.
LSA6		164-173	6-FAM	Kryger *et al.*, Molecular Ecology, 2002.
SOL30	simplex	149-236	HEX	Hamill *et al.*, Heredity, 2006.

Tab.2. List of microsatellites loci used for the analysis**.**

One µl of each PCR product was diluted in 100 µl of water (10 µl for locus SOL30), then resuspended in a denaturation solution (Formamide) and analysed by electrophoresis on an AB Prism 3130 Genetic Analyser with a 36 cm capillary array, POP4 polymer. GeneScan-350 Rox Size Standard (Applied Biosystems), labelled with red colour that was not used to mark the nucleotides, was used as internal size standard.

2.4.2 Microsatellites analysis

Microsatellite analysis consists in separating different alleles (the alleles differ for the number of repetitions of the repeat) by electrophoresis in a denaturing gel which clearly separates the 2 alleles present at the heterozygous *loci*. In automated capillary sequencers the electrophoresis does not require the gel preparation because they can automatically inject it in a serious of capillaries through which fragment migration takes place (mechanism of operation of an automated sequencer was described in the previous paragraph). When the labelled DNA fragment passes a pre-set location the fluorescent dye is picked up by a laser and the emission of fluorescence is detected and measured by the software that analyses the results of electrophoresis and convert the weights of the different alleles (the alleles differ for the number of repeats) in an image file and in an electropherogram in which the molecular weights of the alleles is precisely determined by the use of internal standards.

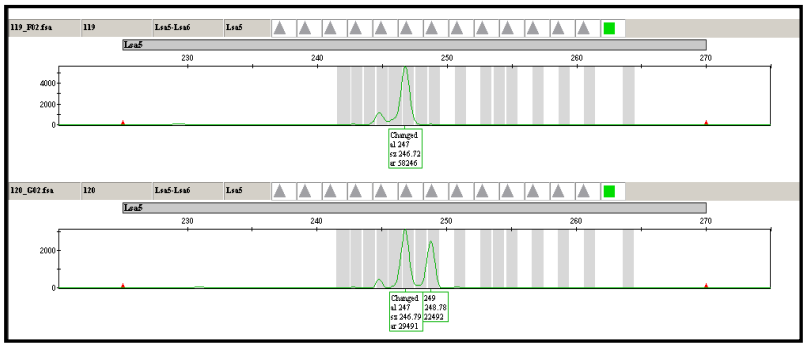


Fig. 13. Example of a microsatellite's electropherogram, the single peak stands for a homozygous sample at a given *locus*, the double peaks indicate a heterozygous sample at a given *locus*.

Homozygous sample at a given *locus* present a single band (that appears as a single peak in an electropherogram) while heterozygous samples present 2 bands (that appear as 2 different peaks in an electropherogram) (Fig. 13). In automated sequencers it is possible to analyse several microsatellite *loci* in the same capillary column simultaneously. The analysis of multiple *loci* can be done via multiplex PCR or via electrophoresis of mixtures of single PCR (electrophoresis multiplex). In multiplex systems (both PCR and electrophoresis systems) it is necessary to choose microsatellite *loci* that produce clean and clear signals (electropherograms). As in the automatic

analysis of microsatellite one of the two PCR primers is labelled with a fluorescent dye, in multiplex systems it is necessary to label primers at different *loci* with different colours. Three colours (yellow, green and blue) are currently used to label the primers while a fourth colour (red) is used to label the standard molecular weight.

Microsatellite whose alleles have different molecular weights can be combined in multiplex systems and PCR products are separated in different areas of the gel or capillary and the identification of alleles is facilitated by reading the coloured signals that do not overlap.

Softwares mainly used to process microsatellites data are:

- *GeneMapper v 3.0* (Applied Biosystems ABI), a software used to manually or automatically correct the results of the automated analysis. When the electrophoresis ends every allele may be made up of a single band (that appear as a single peak in an electropherogram) or of a main band plus a serious of secondary bands that represent aspecific amplification products. After defining the variation range of molecular weight and of the main peak of the electropherogram as well as the colour of the *locus*, the software allows to identify the signal produced by the main band and assign the respective molecular weight. The program uses an algorithm to filter that information which ignores the secondary signals and assigns the correct molecular weight to the principle signal of the allele. The final result can be visualized as a correct electropherogram, and the data, that contains the values of the molecular weight assigned to each allele, can be exported to database Microsoft Excel-type format, or to input formats of various data elaboration software.
- *GenAlex v. 6.0* (Peakall & Smouse, 2005; 2006) is a software provided as an Excel add-in, with a compiled module and an associated menu, particularly useful to study population genetics and produce output files which can be directly used in other elaboration software. It can be used to estimate allele frequency by *locus* and population, observed (*H*O) and expected unbiased (*H*E) heterozygosities, mean number of alleles per *locus* (*N*A), number of private alleles (NP) per population (i.e. the number of alleles unique to a single population in the data set), to compute the HWE and Chi-square testing procedures, to perform genetic distance and assignment tests (through Principal Coordinate Analysis PCA) and several other parameters.
- *Structure v. 2.1* (Pritchard *et al*. 2000); it is an important software that perform population assignment and hybrids detection using a Bayesian clustering procedure. The program *Structure* implements a model-based clustering method which uses *multilocus* genotype data, consisting of unlinked markers, to infer population structure and to assign individuals

to populations.

- *Genetix v. 4.2* (Belkhir *et al.* 2001) allows to simplify the graphic visualization of *Structure* and *GenAlex* results; it can describe in three dimensions all the variability analysed in *GenAlex* by Principal Coordinate Analysis and the different *Structure* clusterings.

2.5 SINGLE NUCLEOTIDE POLYMORPHISM (SNP)

2.5.1 SNPs amplification

We checked 13 SNPs loci found in four nuclear coding genes, but only 9 of these were variable (see Tab. 3, Melo-Ferreira *et al*. 2009) and were used for the analysis of 150 samples belonging to *Lepus corsicanus* (88 samples collected in central-southern Italy, 62 from Sicily) and 15 samples belonging to *Lepus europaeus*.

GENE	SNPs	SIZE	REFERENCE
SPTBN1 (Spectrin Beta non-erythrocytic 1)	SPTBN1-7Bf	40	Melo-Ferreira *et al.*, Molecular Ecology 2009.
	SPTBN1-7Cr	48	Melo-Ferreira *et al.*, Molecular Ecology 2009.
UCP2 (Uncoupling Protein 2)	UCP2-10Cf	44	Melo-Ferreira *et al.*, Molecular Ecology 2009.
	UCP2-10Df	60	Melo-Ferreira *et al.*, Molecular Ecology 2009.
	UCP2-10Br	76	Melo-Ferreira *et al.*, Molecular Ecology 2009.
CA2 (Carbonic Anhydrase 2)	CA2-2Ar	52	Melo-Ferreira *et al.*, Molecular Ecology 2009.
	CA2-2Cr	80	Melo-Ferreira *et al.*, Molecular Ecology 2009.
HPX (Hemopexin)	HPX-4Br	58	Melo-Ferreira *et al.*, Molecular Ecology 2009.
	HPX-4Af	84	Melo-Ferreira *et al.*, Molecular Ecology 2009.

Tab.3. List of SNPs belonging to four nuclear genes used for the analysis.

Sequence containing SNPs were PCR-amplified separately for every gene in 8 µl of volume, using 2 µl of DNA solution, 0,8 µl of PCR Buffer 10X (1,5 mM of MgCl2), 0,8 µl of BSA (Bovine Serum Albumin), 0,32 µl of deossinucleotides (dATP, dCTP, dTTP, dGTP) 2,5 mM, 0,12 µl of each primer 10 µM, 0,25 units of Taq and 3,8 µl of PCR water.

In this case we use a Touchdown PCR method which allows to avoid amplification of nonspecific sequences; the earliest steps of a touchdown PCR cycle have high annealing temperatures, then the annealing temperature is decreased in increments for every subsequent set of cycles (in this case temperature (starting from 60°C or 55°C depending on primer) decreases every cycle of 0,5°C for a total of 10 cycles).

Cycling conditions were optimized for each primer pair (annealing temperature changes from 60°C to 55°C depending on primer), and this is the general PCR program:

94°C x 2'→(94°C x 30''→ Ta°C (Touchdown PCR) x 30''→ 72°C x 30'') for 10 cycles →
(94°C x 30''→ Ta°C x 30''→ 72°C x 30'') for 40 cycles→
72°C x 10' → 4°C x 10' → 15°C

PCR products were purified using 1 µl of a mixture of Exonuclease I (EXO) and Shrimp Alkaline Phosphatase (SAP) that remove respectively unincorporated primers and dNTPs using the following thermocycling program:

37°C x 30'→ 80°C x 15' →4°C x 10' →15°C

The SNaPshot extension reaction (Minisequencing Multiplex PCR) were carried out in 10 µl of volume, using 1 µl of PCR product for every SNPs locus analysed, 1 µl of SNaPshot Reaction Mix, 0,2 µl of the extension primer 10 µM for every SNPs locus, and a variable quantity of PCR water using the following thermocycling program:

(96°C x 10''→ 55°C/50°C x 5''→60°C x 30'') for 25 cycles →4°C x 10' →15°C

SNaPshot Reaction Mix contains a reaction buffer, the enzyme Taq polymerase and the four dideossinucleotides (ddATP, ddGTP, ddCTP, ddTTP) labelled with different colours: A=green; C= black; G=blue; T=red. Dideossinucleotides are modified bases which posses an OH in 3'-position and avoid the formation of a phosphodiesteric link with another deossinucleotide so that the incorporation of one of them stop the extension generating fragments consisting of the primer and the SNP at that *locus*.

PCR products were purified using 1 µl of Shrimp Alkaline Phosphatase (SAP) that removes unincorporated dNTPs, using the thermocycling program written above for EXO/SAP.

One µl of each purified minisequencing PCR product was resuspended in a denaturation solution (Formamide) and analysed by electrophoresis on an AB Prism 3130 Genetic Analyser with a 36 cm capillary array, POP4 polymer. GeneScan-120 Liz Size Standard (Applied Biosystems), labelled with orange colour that was not used to mark the nucleotides, was used as internal size standard.

2.5.2 SNaPshot analysis

SNaPshotTM (Applied Biosystems) is a solution-based assay that uses the single nucleotide primer extension assay (Syvanen *et al.*, 1990; Syvanen, 1999; Budowle *et al.*, 2004). The method is based on the use of three primers for the analysis of each SNP: an external forward, an external reverse and an internal primer consisting of a few nucleotides until the one which precedes the mutation.

The first two primers are necessary for a first amplification of the fragment containing the SNP, while the SNP extension primer is used during a second amplification to detect the polymorphism. During this minisequencing PCR the SNP extension primer is annealed to the denatured template amplicon and is extended at the SNP site by the incorporation of one of the four fluorescently labelled terminator ddNTPs. The primer cannot be extended further, because only ddNTPs are in the extension reaction. The extended SNP primer is subjected to capillary or slab-gel electrophoresis.

The particular incorporated nucleotide is identified by the different labelled fluorescent tag as in Sanger sequencing. The specific SNP *locus* (or in actuality the extended SNP primer) in a multiplex assay is identified by its mobility during electrophoresis. The mobility can be modified by incorporating varying-length polynucleotide tails or by incorporating mobility modifiers at the 5' end of the SNP primer.

Sequencing results are saved in the form of electropherograms (Fig. 14) and visualized in the form of peaks because during the electrophoresis, when a fluorescent dye is picked up by a laser the data produce a luminous emission that is registered as a peak. The height of the peak indicates the intensity of the emission and the colour indicates the colour of the fluorescent dye.

Softwares used to correct the electropherograms and to process data from SNaPshot analysis are the same seen for microsatellites analysis.

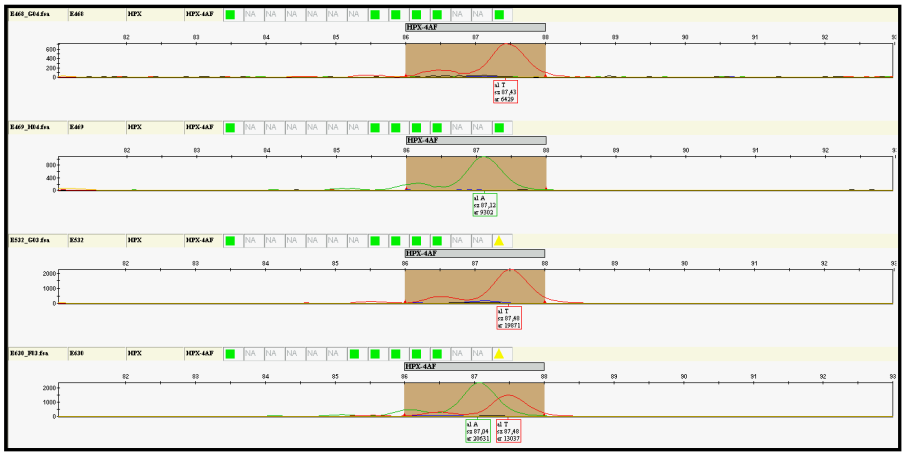


Fig. 14. Example of a SNP's electropherogram, the single peak stands for a homozygous sample at a given *locus*, the double peaks indicate a heterozygous sample at a given *locus*. In this case the green peak stands for the nucleotide A, the red peak stands for the nucleotide T.

2.6 SEX IDENTIFICATION

Sex of individuals belonging to the species *L. corsicanus* was determined by selective PCR amplification of a fragment of the Sry sex-determining gene on the Y-chromosome in duplex with a segment of the Transferrin gene, and PCRs were subjected to electrophoresis on 2% agarose gels and binding DNA with an UV fluorescent reagent (Gel Red).

PRIMER	SEQUENCE	SIZE	REFERENCE
SRYf	CTGTGGCAGCATGCTTTGAG	1700 bp	Melo-Ferreira *et al.*, 2009.
SRYr	GATTTGACGAATGCCAAGTGTTTC		
TFf	GCCTTTGTCAAGCAAGAGACC	500 bp	Alves *et al.*, 2003.
TFr	CACAGCAGCTCATACTGATCC		

Tab.4. Primers used for sexing analysis.

Amplification was performed in 10 µl of volume, using 2 µl of DNA solution, 1 µl of PCR Buffer 10X (1,5 mM of MgCl2), 1 µl of BSA (Bovine Serum Albumin), 0,4 µl of deossinucleotides (dATP, dCTP, dTTP, dGTP) 2,5mM, 0,15 µl of each primer 10 µM, 0,25 units of Taq and 4,95 µl of PCR water, in a 9700 ABI Thermocycler (Applied Biosystems) using the following protocol:

94°C x 2'→(94°C x 30''→ 55°C x 30''→ 72°C x 90'') for 40 cycles →

72°C x 10' → 4°C x 10' → 15°C

Gel photographs were examined by eye to detect the number of amplicons for each individual (two fragments amplified for a male, only one for a female). With every set of reactions carried out, 2 positive controls (1 male and 1 female) were included in the PCR and gel run. If these bands did not show up as expected, PCR was repeated. If an individual failed to produce any band or the result was not conclusive, PCR for that individual was repeated.

2.7 MAJOR HISTOCOMPATIBILITY COMPLEX

2.7.1 MHC loci amplification

A total of 84 samples (43 belonging to *L. corsicanus*, 41 to *L. europaeus*) collected in different regions of Italy were used in this analysis.

Exon 2 of DQA MHC class II locus was amplified using the primers Lepus-DQA-F2 (5'-

CTTTCACTCATCAGCTGACC-3') and Lepus-DQA-R1 (5'-ACAGCAGCAGTAGAGTTGGA-3'), (de Bellocq *et al.* 2009): forward primer was labelled with 6'FAM dye and reverse primer was labelled with NED dye.

PCR was carried out in 12,5 µl of volume, using 1 µl of DNA solution, 2,5 µl of Phusion HF Buffer 5X, 1,25 µl of deossinucleotides (dATP, dCTP, dTTP, dGTP), 0,2 µl of each primer, 0,1 µl of Phusion DNA polymerase (Fynnzymes), and 7,25 µl of RNase-free water using the following protocol:

98°C x 5'→ (98°C x 10''→58°C x 20''→72°C x 15'') for 34 cycles →72°C x 5'

One µl of each PCR product was diluted in 10/30 µl of water, then resuspended in a denaturation solution (Formamide) and analysed by electrophoresis on an AB Prism 3130 Genetic Analyser with a 36 cm capillary array, POP4 polymer. GeneScan-350 Rox Size Standard (Applied Biosystems), labelled with red colour that was not used to mark the nucleotides, was used as internal size standard. The solution was denaturated at 96°C for 3 min and immediately placed on ice.

2.7.2 SSCP analysis

Often population genetic studies need sequencing large number of samples. A widely used technique to achieve this goal is single-stranded conformation polymorphism (SSCP) analysis, which can also be used to characterize genotypes also in paternity testing.

The SSCP analysis detects point mutations and other electrophoretic mobility differences that can result from small changes in nucleotide sequences. A single base change can cause a conformational change in the DNA molecule. Under non-denaturing conditions and reduced temperature, single-stranded DNA molecules assume unique conformations that vary, depending on their nucleotide sequences. These conformational changes result in detectable differences in mobility.

SSCP analysis, which is widely used for mutation detection because it is simple and fast, includes the following processes: PCR amplification of the DNA region containing the potential mutation of interest, using primers that flank the desired region; denaturation of the resulting double-stranded PCR product with Formamide and heat (to separate the component single DNA strands), followed by rapid chilling to prevent re-annealing of the complementary strands; separation of the single-stranded DNA by capillary electrophoresis, using a non-denaturing sieving medium, such as a flowable polymer; comparison of the mobility of an unknown sample to that of wild-type DNA or

DNA with known mutations; confirmation of mutations by automated fluorescent DNA sequencing.

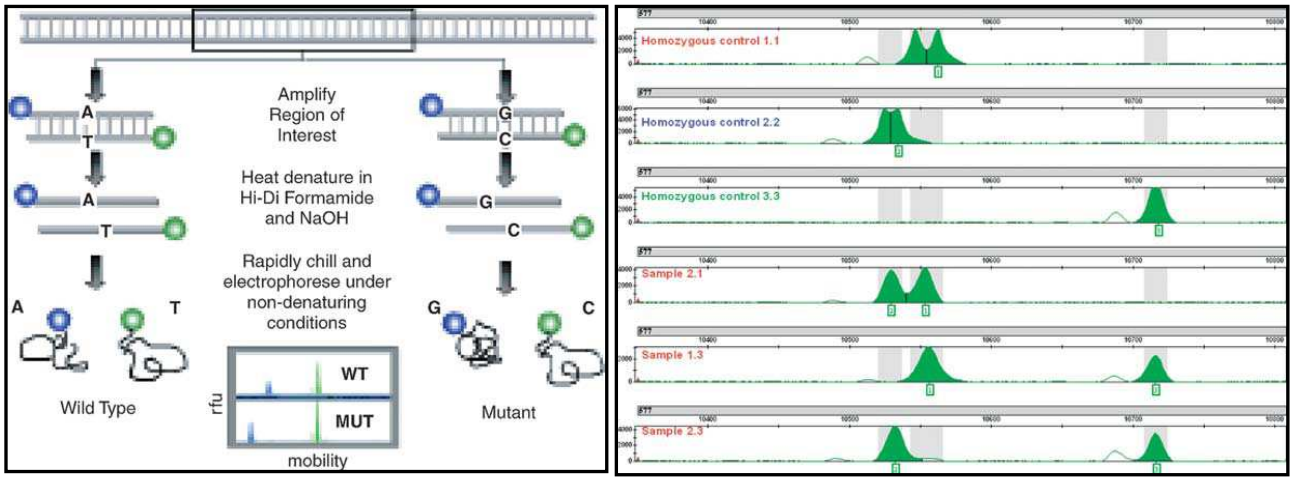


Fig. 15. SSCP process and example of mutation screening in *GeneMapper* software.

Data collected during electrophoresis were analyzed with *GeneMapper v 3*.0. Results were validated by sequencing the region of interest and comparing sample sequences using *SeqScape v* 2.5 (Applied Biosystems 2001).

Softwares used to process sequencing data are: *BioEdit* (Hall, 1999) to align sequences and to translate DNA or RNA to protein; *Dnasp v. 5* (Giulio Rosaz *et al*. 2003) for the analysis of DNA polymorphism from nucleotide sequence data; *Mega 5* (Tamura *et al*. 2011) to calculate a distance matrix between different sequences on the basis of a comparison in pairs (i.e. counting the number of mutations existing between them and comparing it, every time, with the number of total nucleotides) and to build phylogenetic trees based on different statistical methods.

CHAPTER THIRD: RESULTS

3.1 MITOCHONDRIAL DNA

We sequenced 459 nucleotides of the mtDNA D-Loop, which included 204 (44,4%) polymorphic sites and 146 (31,8%) parsimony-informative sites in nearly 700 samples of six species of hare. Haplotype diversity (*Hd*), nucleotide diversity statistics, genetic distances and other parameters were computed using DNASP 5 (Rozas *et al*. 2003).

The alignment identified 248 different haplotypes (40 in *L. corsicanus*, 104 in *L. europaeus*, 44 in *L. timidus*, 21 in *L. granatensis*, 5 in *L. castroviejoi*, 27 in *L. c. mediterraneus*, 7 in hares collected in different areas of Africa); Italian and brown hares had different haplotypes that diverged by an average TN93 genetic distance of 0.138 (Table 6).

Mountain hares and Iberian hares showed approximately one different haplotype per individual, while brown hares and Italian hares showed one haplotype per three individuals (Table 5); also the number of polymorphic sites was high for *L. timidus* and *L. granatensis* when compared to the number of samples.

Species	**Samples**	**Haplotypes**	**Polymorphic sites (*s*)**	**Haplotype diversity (*h*)**	**Nucleotide diversity (π)**	**Theta (Watterson)**
L. corsicanus	136	40	48/459	0.935 (0.016)	0.016 (0.0008)	0.020 (0.005)
L. europaeus	314	104	113/459	0.942 (0.006)	0.018 (0.001)	0.042 (0.009)
L. timidus	63	44	73/459	0.982 (0.007)	0.038 (0.001)	0.036 (0.010)
L. granatensis	26	21	83/459	0.978 (0.022)	0.070 (0.004)	0.051 (0.016)
L. castroviejoi	5	5	67/459	1.000 (0.126)	0.081 (0.018)	0.075 (0.038)
L. cap. med.	86	27	38/459	0.830 (0.032)	0.012 (0.001)	0.017 (0.005)
L. capensis?	8	7	82/459	0.964 (0.077)	0.084 (0.009)	0.074 (0.032)

Tab. 5:. Values of interpopulation genetic diversity between the six species (standard deviations are in parenthesis).

With the software *Mega* we calculated a distance matrix: the software compares the various sequences in pairs, counting the number of mutations existing between them, reporting, from time to time, the total number of nucleotides and allows us to identify the type of mutations by differentiating transversions, transitions and indel (deletions and insertions).

The genetic distances thus obtained were then displayed by a phylogenetic tree, constructed with the Neighbor Joining (NJ) algorithm (Saitou & Nei, 1987) and Tamura and Nei TN93 genetic

	L. europaeus	*L. castroviejoi*	*L. cap. med*	*L. cap.?*	*L. corsicanus*	*L. granatensis*	*L. timidus*
L. europaeus	-						
L. castroviejoi	0.137	-					
L. c. med.	0.106	0.138	-				
L. capensis?	0.105	0.145	0.076	-			
L. corsicanus	0.138	0.076	0.144	0.142	-		
L. granatensis	0.142	0.100	0.136	0.145	0.119	-	
L. timidus	0.135	0.078	0.133	0.141	0.088	0.094	-

Tab. 6:. Interspecific genetic distances (Tamura & Nei 1993) computed using control region sequences in six species of hares.

distance model (Tamura & Nei 1993) widely used for displaying the phylogenetic relationships among taxa. In addition to hare samples analysed was added a sequence belonging to *Oryctolagus cuniculus* (wild rabbit) with the function of outgroup to emphasize the time of divergence between different species.

The phylogenetic tree shows two main distinct groups of haplotypes:

- Clade A: including *Lepus europaeus*, *Lepus capensis* e *Lepus capensis mediterraneus*;
- Clade B: including *Lepus timidus*, *Lepus granatensis*, *Lepus castroviejoi* e *Lepus corsicanus*.

The tree structure (Fig. 16) shows a clear genetic distinction among the mtDNA sequences of *L. corsicanus* and the other taxa included in this study. D-Loop haplotypes of Italian hares group in a strongly supported (bootstrap value 98) monophyletic clade, and DNA distinction is concordant with both qualitative and quantitative morphological classification (Riga *et al.* 1998). Italian and brown hares seem to have a long-lasting history of independent evolution and seem to be reproductively isolated.

The tree confirms that Eurasian and African hares belong to two different phylogenetic clades; brown hares originated from an African ancestor and dispersed in Europe, and Italian and Mountain hares are relictual species that originated before the dispersal of *L. europaeus* in western Europe, and which became adapted to the different Mediterranean and Alpine habitats.

A high frequency of *Lepus timidus* mitochondrial haplotypes was found in Iberian species (*L. castroviejoi* and *L. granatensis*), although the Mountain hare is extinct in these regions; this could result from the invasive replacement of *L. timidus* by the temperate species during deglaciation.

The sequence analysis of the control region also confirms the close relationship between *L. corsicanus* and *L. castroviejoi:* two haplotypes belonging to individuals of *L. catroviejoi* (Fig.17) in

Fig. 16. Neighbour-joining (NJ) phylogenetic tree computed by *Mega* using TN93 genetic distances among the 248 aligned haplotypes of mitochondrial DNA (mtDNA) control region I (CR-I) of six species of hares, and using the correspondent sequence of rabbit as an outgroup.

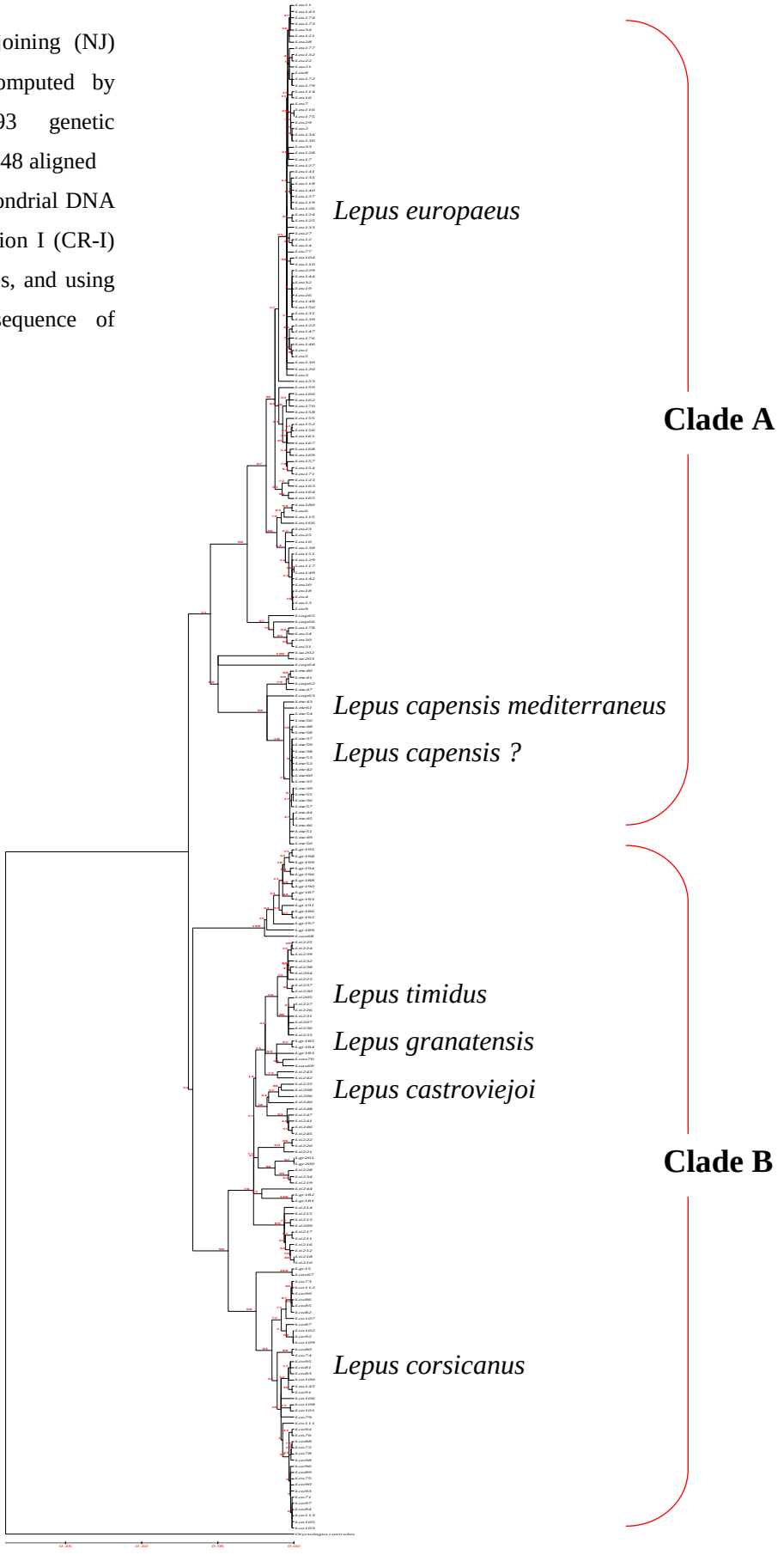
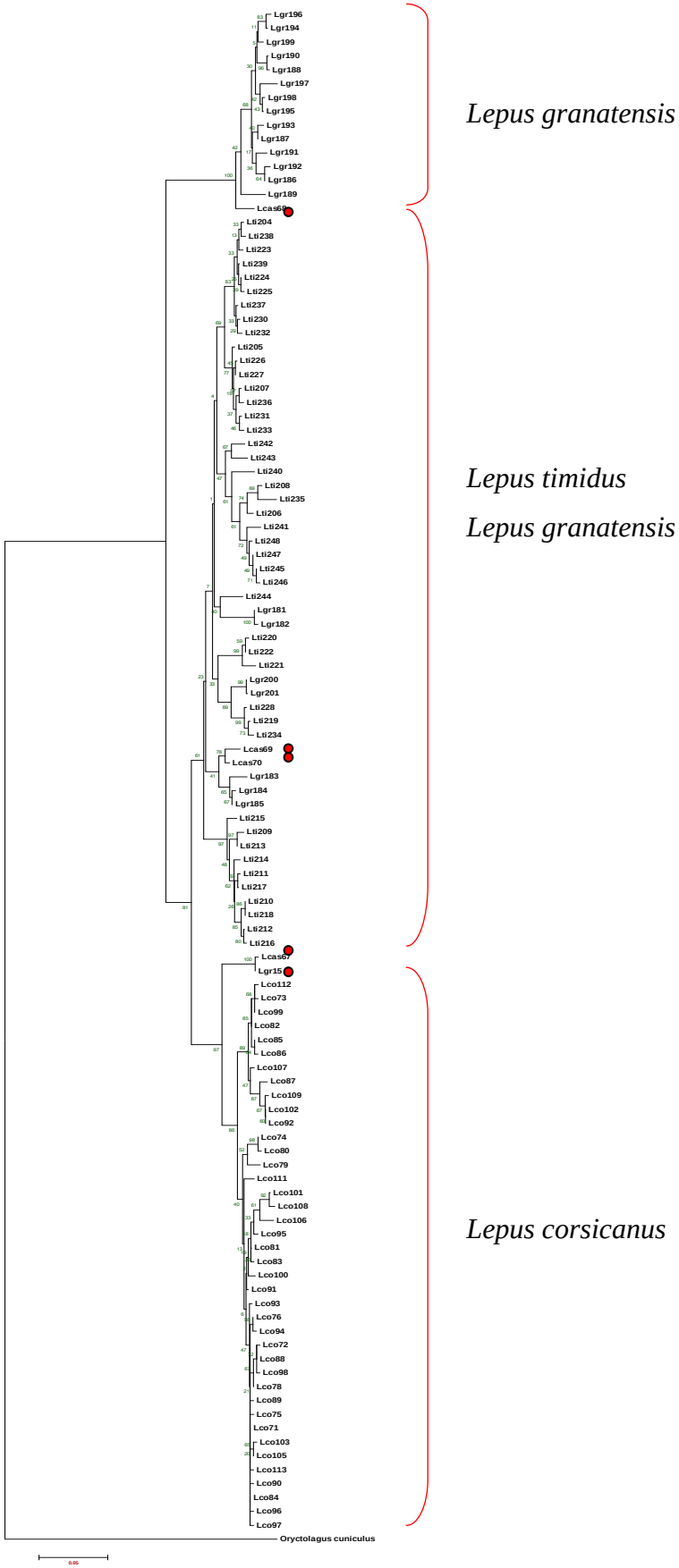


Fig. 17. Neighbour-joining (NJ) phylogenetic tree computed by *Mega* using TN93 genetic distances among the 110 aligned haplotypes of mitochondrial DNA (mtDNA) control region I (CR-I) of species belonging to clade B (*L. corsicanus*, *L. timidus*, *L. granatensis*, *L. castroviejoi*) and using the correspondent sequence of rabbit as an outgroup. The red points stand for *L. castroviejoi* samples.



fact are included in the clade of the Italian hare.

The phylogenetic tree in Figure 19 shows that *L. corsicanus*'s populations are geographically differentiated and confirms the existence of a genetic diversity of mitochondrial haplotypes between the samples collected in central-southern Italy and Sicily; the Sicilian samples in fact are grouped together and show a slight differentiation compared to samples of the peninsula.

An important result concerns the Leu41 haplotype, found in two samples collected in 1999 in Calabria identified morphologically and genetically (through microsatellites analysis) as belonging to *L. europaeus*; analysing mitochondrial DNA we verified with surprise that this sample owns an Italic haplotype. This finding can be interpreted as an introgression of *L. corsicanus* mitochondrial genome in *L. europaeus* genome occurred in historical times as a result of an hybridization (eventually anthropogenic) event.

Networks are better suited than phylogenetic methods to infer haplotype genealogies at the population level because they explicitly allow for extant ancestral sequences and alternative connections (Bandelt *et al*. 1999). We used 41 haplotypes identified in 138 *L. corsicanus* samples with the median-joining network procedure (Bandelt *et al*. 1999), implemented in *Network* 4.5.1.6. (http://www.fluxustechnology. com/) to draw the network (Fig. 18).

Haplotype diversity was high (Hd= 0,935) and the haplotype H1 (Lco1), found in 28 samples from Sicily, has the highest frequency; in the network sicilian haplotypes are identify with pink colour and the haplotype Leu41 with blue colour. In the network we can identify several haplotypes groups, and this variability indicates an ancient origin of these populations; it was not possible to observe any spatial structure considering sampling regions.

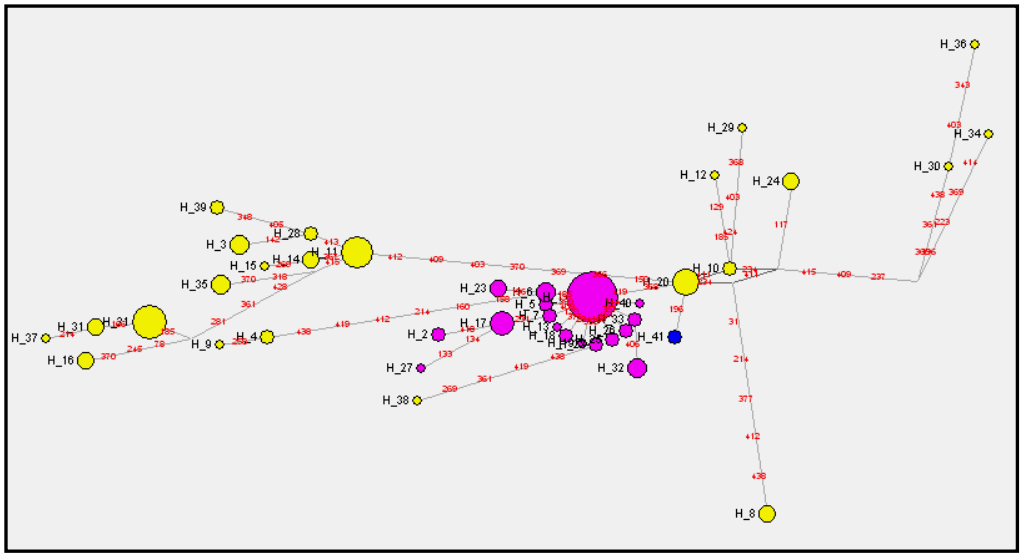
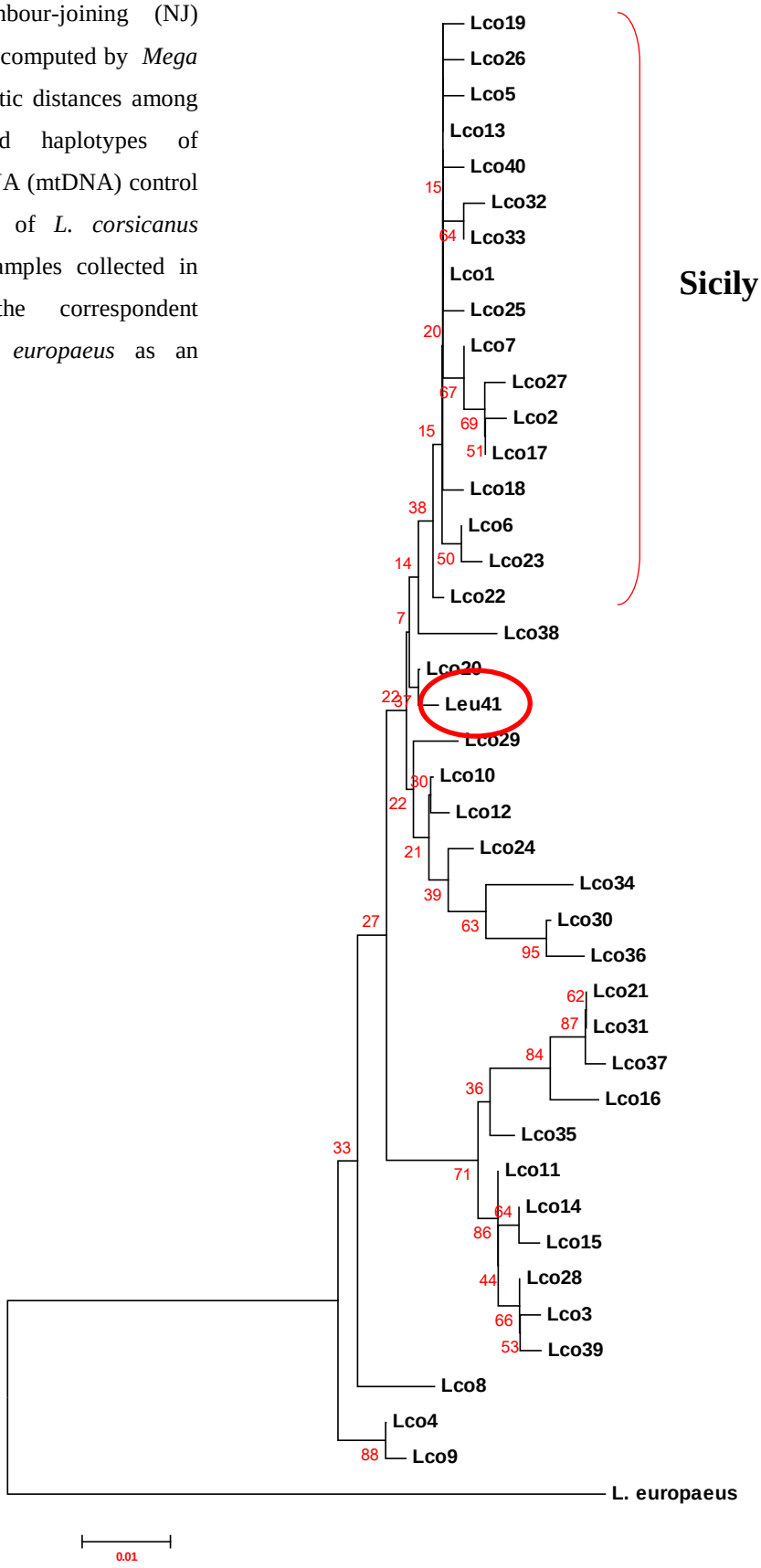


Fig. 18: Mitochondrial DNA haplotypes network with *L. corsicanus* samples: pink colour stands for samples collected in Sicily, yellow colour for samples from the peninsula, the blue dot identify haplotype Leu41.

Fig. 19. Neighbour-joining (NJ) phylogenetic tree computed by *Mega* using TN93 genetic distances among the 41 aligned haplotypes of mitochondrial DNA (mtDNA) control region I (CR-I) of *L. corsicanus* obtained from samples collected in Italy, using the correspondent sequence of *L. europaeus* as an outgroup.



3.2 MICROSATELLITES

In this study *GenAlex v. 6* (Peakall & Smouse, 2005; 2006) was used to estimate allele frequency by *locus* and population, observed (HO) and expected unbiased (HE) heterozygosities, mean number of alleles per *locus* (NA), number of private alleles (NP) per population (i.e. the number of alleles unique to a single population in the data set) and to compute the HWE and Chi-square testing procedures. *GenAlex* was also used to perform the AMOVA (analysis of molecular variance), which was used to assess the level of global and pairwise population differentiation based on PhiPT, an analogue of *F*ST , which estimates the proportion of the genotypic variance among populations, relative to the total variance.

The thirteen microsatellite loci showed 97.44% polymorphic loci and an average number of alleles of 6.8 for each locus. Higher alleles numbers were retrieved in *L. europaeus* populations (with an average of 13 alleles per locus); *L. corsicanus* shows lower average values between 6.23 alleles per locus (for samples collected in the peninsula) and 3.84 alleles per locus (for samples collected in Sicily). We found 71 private alleles in a total of 240 alleles (29.5% private alleles) over all the populations.

	n	Hexp.	Hobs.	P (0.99)	A
L. timidus	75	0.66 (0.23)	0.48 (0.20)	0.92	9
L. europaeus	343	0.69 (0.19)	0.57 (0.21)	1.00	13
L. corsicanus	88	0.46 (0.27)	0.33 (0.19)	1.00	6.23
L. corsicanus (Sicily)	66	0.29 (0.24)	0.23 (0.21)	0.84	3.84
L. capensis mediterraneus	92	0.59 (0.25)	0.52 (0.24)	1.00	8
L. granatensis	29	0.65 (0.21)	0.60 (0.24)	1.00	6.92
L. castroviejoi	5	0.62 (0.12)	0.33 (0.20)	1.00	3.69
L. capensis	12	0.77 (0.10)	0.58 (0.18)	1.00	7.92

Tab. 7: Sample size (n), expected heterozygosity, observed heterozygosity and allele number for each population.

Heterozygosity didn't show high values (Ho= 0,23-0,60; He= 0,29-0,77), and in particular *L. corsicanus* populations showed the lowest values (observed Heterozygosity in the Sicilian population is 0,23).

Deviation from HWE was significant in all populations, probably due to sampling method; in fact we have a low sample number for some populations, often collected in an heterogeneous way

considering the different distribution areas.

Differentiation between all populations was assessed also by Analysis of MOlecular VAriance (AMOVA). A significant average *multilocus* FST = 0.37 (P = 0,01; computed from AMOVA) indicated that genetic diversity was significant.

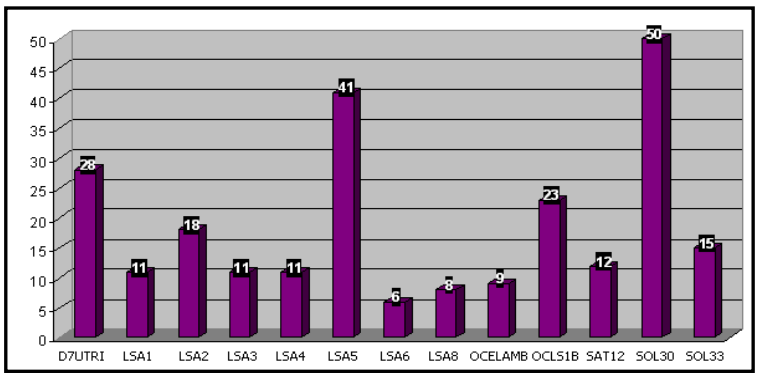


Fig. 20: Number of alleles found in each locus for all populations.

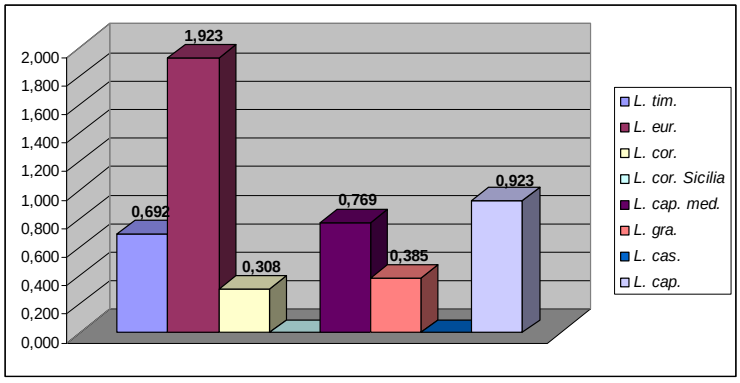


Fig.21: Number of private alleles for each population.

GenAlex was also utilized for assignment tests and Principal Coordinates Analysis (PCA): this software calculate for each sample the expected genotype frequency at each *locus* and logtransform to give a log likelihood value which is calculated even for each population, using the allele frequencies of the respective population. A sample is assigned to the population with the highest log likelihood. Genetic distance and assignment tests allow, through Principal Coordinate Analysis (PCA), to detect the different considered populations despite a Cartesian axe system not linked to a geographic reference system. The software, in fact, synthesizes all variability of the populations, expressed by many variables, in 2 or 3 variability axes around which the analysis and the further assignments occur.

To simplify the graphic visualization of *GenAlex* results the program *Genetix v.4.2* (Belkhir *et al.*, 2001; http://www.University-montp2.fr/-genetix/genetix.htm) was used.

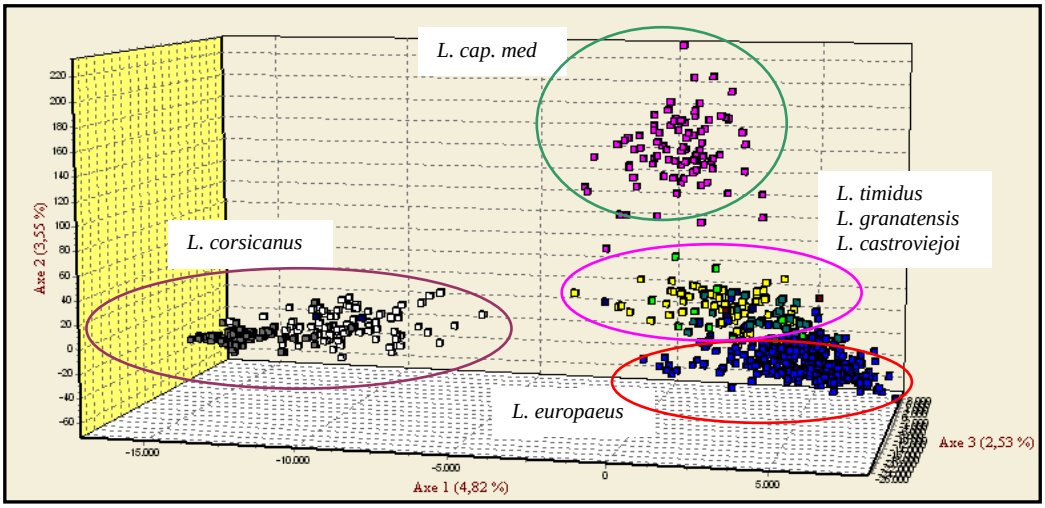


Fig. 22: Principal Coordinates Analysis of samples genotyped at 13 microsatellite loci. Gray dots stand for *L. corsicanus* samples from Sicily, white dots for *L. corsicanus* samples from the peninsula, blue dots for *L. europaeus* samples, pink dots for *L. cap. mediterraneus* samples, yellow dots for *L. timidus*, green dots for *L. capensis*, dark green dots for *L. granatensis*, dark blue dots for *L. castroviejoi*.

Variability is described with three principal components: the first axe describe the 49,13% of the variability, the second 19,05%, and the third 9,26%.

PCA results (Fig. 22 and Fig. 24) show a deep genetic differentiation between *L. corsicanus* and *L. europaeus*, confirming that they are distinct and distantly related biological species. The absence of intermediate genotypes suggests a lack of hybrid individuals.

All specimens identified morphologically and genetically with the *L. corsicanus* mitochondrial haplotype were assigned to Italian hare population, no individual has been assigned to different species. The two hare samples collected in Calabria, morphologically identified as belonging to *L. europaeus* but identified with a *L. corsicanus* haplotype in mitochondrial DNA analysis, showed in microsatellites analysis an allelic pattern typical for the European brown hare.

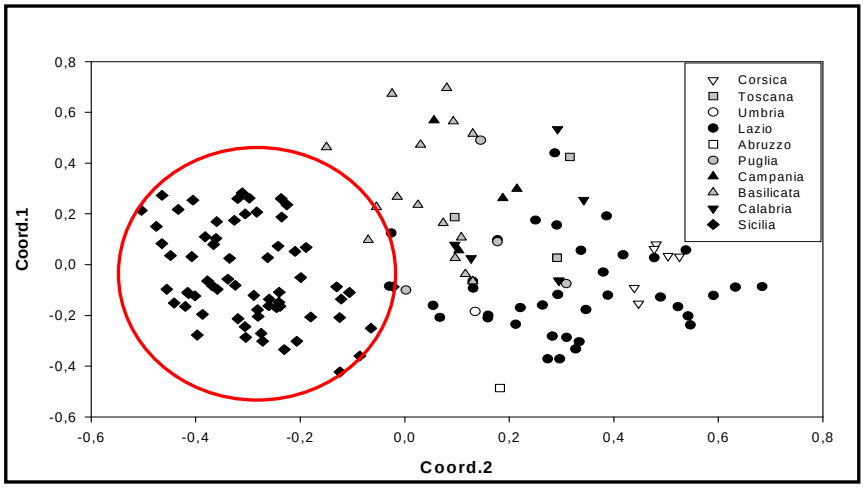


Fig. 23: Principal Coordinates Analysis of Italian hare samples genotyped at 13 microsatellite loci. The red circle show samples collected in Sicily.

As we showed in the analysis of the mitochondrial DNA, also in nuclear DNA we found a level of genetic differentiation between hare populations in Sicily and in the peninsula (Fig.23 shows a PCA carried out only with Italian hare samples).

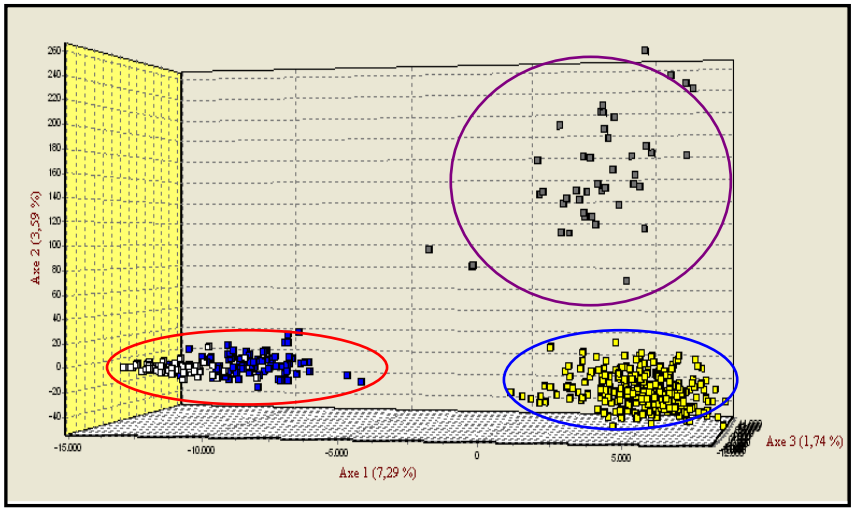


Fig. 24: Principal Coordinates Analysis of all samples collected in Italy belonging to *L. corsicanus*, *L. europaeus*, *L. timidus* genotyped at 13 microsatellite loci. White dots stand for *L. corsicanus* samples from Sicily, blue dots for *L. corsicanus* samples from the peninsula, yellow dots for *L. europaeus* samples, grey dots for *L. timidus* samples.

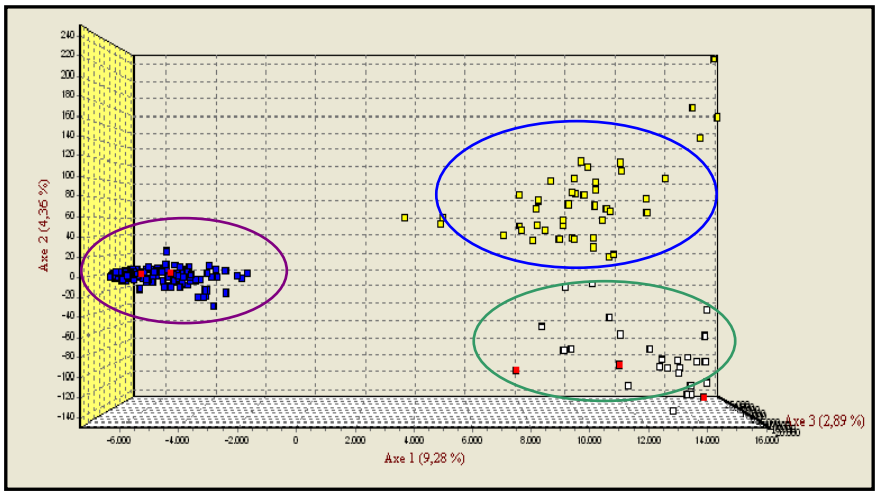


Fig. 25: Principal Coordinates Analysis of all samples belonging to *L. corsicanus*, *L. granatensis*, *L. castroviejoi*, *L. timidus* genotyped at 13 microsatellite loci. Blue dots stand for *L. corsicanus* samples, yellow dots for *L. timidus* samples, white dots for *L. granatensis* samples, red dots for *L. castroviejoi* samples.

Fig. 25 shows results obtained from Principal Coordinates Analysis regarding the species *L. corsicanus*, *L. granatensis*, *L. castroviejoi* and *L. timidus*. As for mitochondrial DNA analysis, we can confirm the close genetic relationship between the Italian hare and the Broom hare; in fact the two individuals from Spain (morphologically identified as belonging to *L. castroviejoi*) presenting *L. corsicanus* haplotype, show an allelic pattern typical of the Italian hare also with microsatellites analysis.

All genotypes were analyzed also with the software *Structure v. 2.1* (Pritchard *et al*., 2000; Falush *et al.*, 2003) to establish their belonging population considering their microsatellite allele frequencies. The program uses *multilocus* genotype data to infer population structure and to assign individuals to populations.

The model assumes Hardy-Weinberg (HWE) and linkage (LE) equilibrium among the unlinked *loci*, and that there are *K* populations (where *K* may be unknown), each of which is characterized by a set of allele frequencies at each *locus*. Individuals in the sample are assigned (probabilistically) to populations, or jointly to two or more populations if their genotypes indicate that they are admixed (or hybrids). Departures from HWE and LE lead the population to be split into subpopulations, to which individuals are assigned.

The program starts with a series of simulations to randomly assign the individuals, computing each time the reliability of these clusterings through a *likelihood* value estimation. Clustering occurs through *Markov Chain* and *Monte Carlo* algorithms that are able to maximize results, collecting only the permutations with high *likelihood* values. As the first simulations are usually not reliable and they are considered as *burnings*, they are deleted from the results interpretation that are based only on the following permutations. In this study 130000 simulations were used (30000 as *burnings*) to carried out the assignment, with the admixture model and we set *K*=1-15. Each run was 5 time independently replicated.

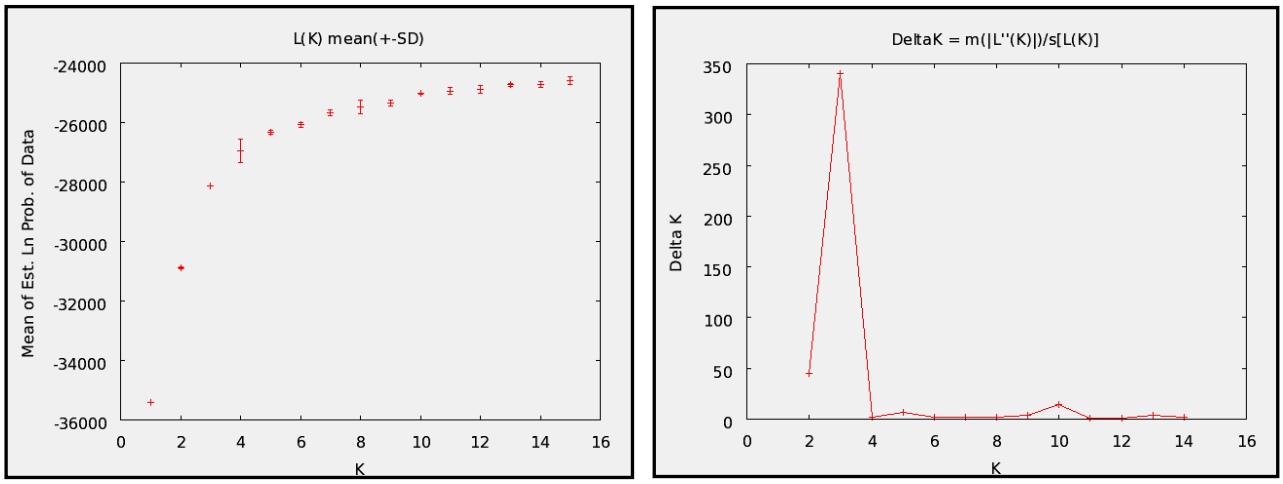


Fig. 26: Likelihood values and Delta K graphs obtained with the software *Structure* analyzing all samples belonging to the six species.

The clustering produced by *Structure* is shown with a graphs (Fig. 27). Using a cartesian reference system, individuals belonging to the different populations have been reported on the horizontal axis and the likelihood of belonging to one of the cluster considered in this assignment test for every single genotype on the vertical axis; assignment groups more distinguishable were marked with different colours to have a clearer view.

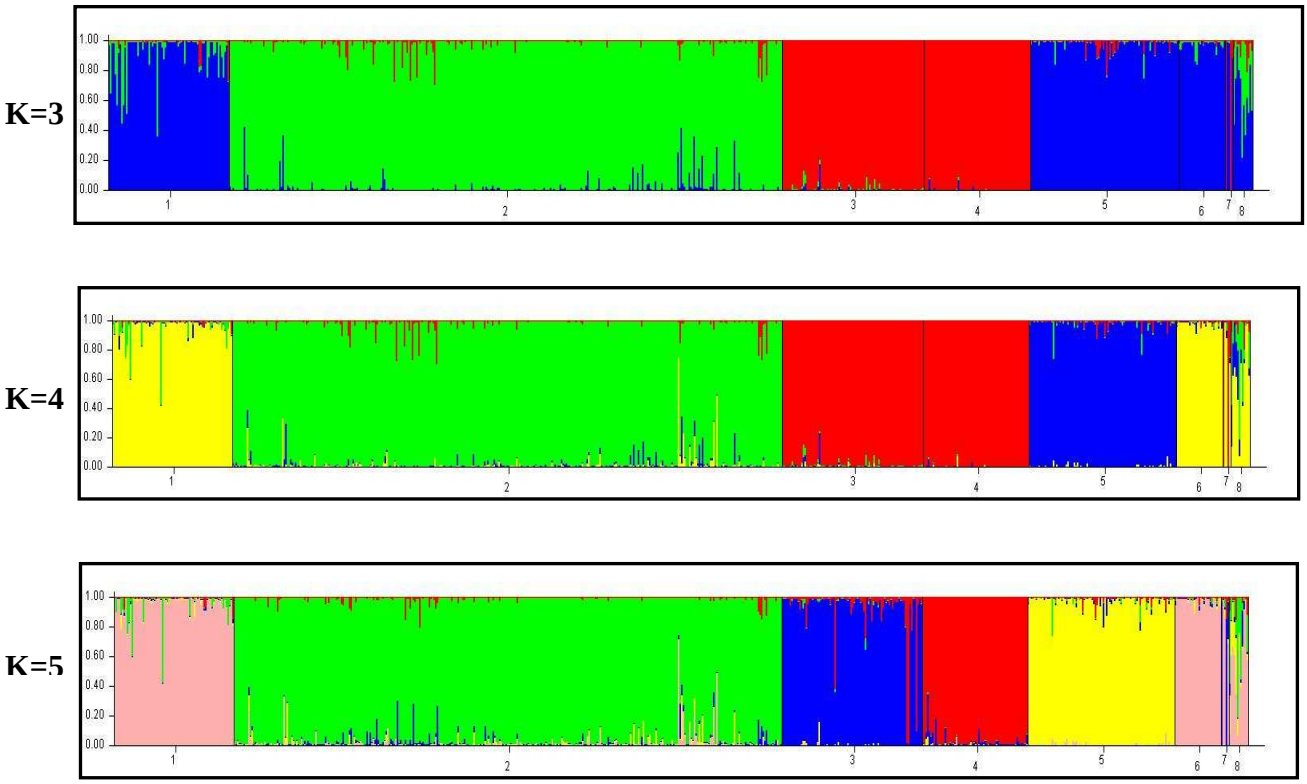


Fig. 27: Bayesian clustering of the populations obtained with the software *Structure*: population N°1 is *L. timidus*, N°2 *L. europeus*, N°3 *L. corsicanus*, N°4 *L. corsicanus* from Sicily, N°5 *L. c. mediterraneus*, N°6 *L. granatensis*, N°7 *L. castroviejoi*, N°8 *L. capensis.*

Genetic variability was described with K between 4 and 5 (Fig. 26), it has not the best likelihood, but permit to identify different populations.

Considering K=3 *L. corsicanus* and *L. europaeus* populations are clearly separated, but the other species seem not to be differentiated.

Considering K=4 *L. corsicanus*, *L. europaeus*, *L. c. mediterraneus* populations are distinct, while *L. timidus* and Iberian species are assigned to the same cluster; as already seen for microsatellites, two individuals belonging to *L. castroviejoi* are assigned to the *L. corsicanus* cluster.

Considering K=5 we get the same clustering, but there is a further division between sicilian populations and peninsular populations of *L. corsicanus*.

We repeated the same analysis considering only samples belonging to *L. corsicanus*, *L. timidus*, *L. granatensis*, *L. castroviejoi*; 130000 simulations were used (30000 as *burnings*) to carried out the assignment, with the admixture model and we set *K*=1-10. Each run was 5 time independently replicated.

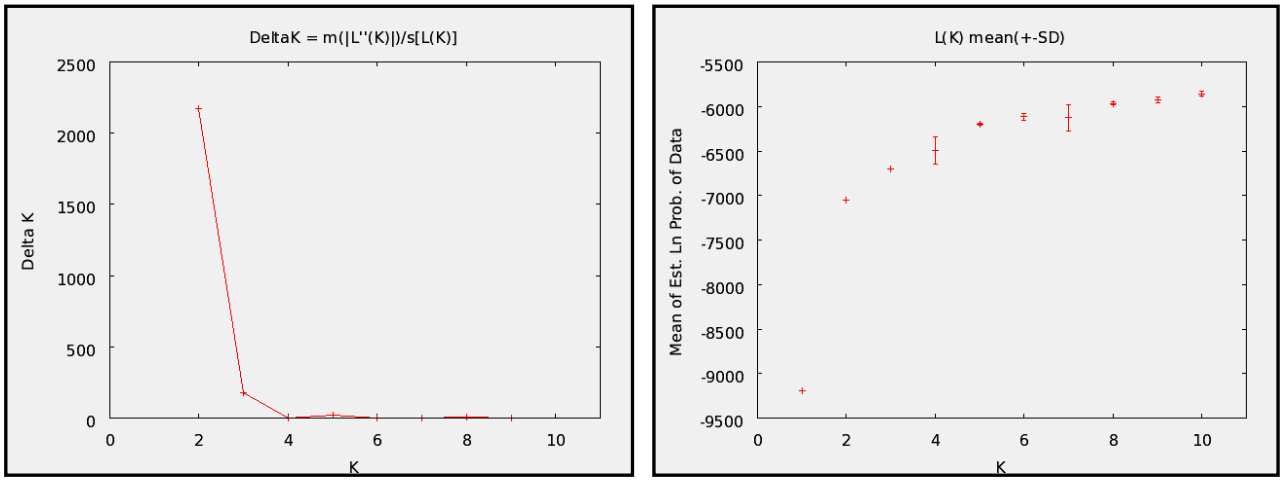


Fig. 28: Likelihood values and Delta K graphs obtained with the software *Structure* analyzing samples belonging to *L. corsicanus*, *L. timidus*, *L. granatensis*, *L. castroviejoi*.

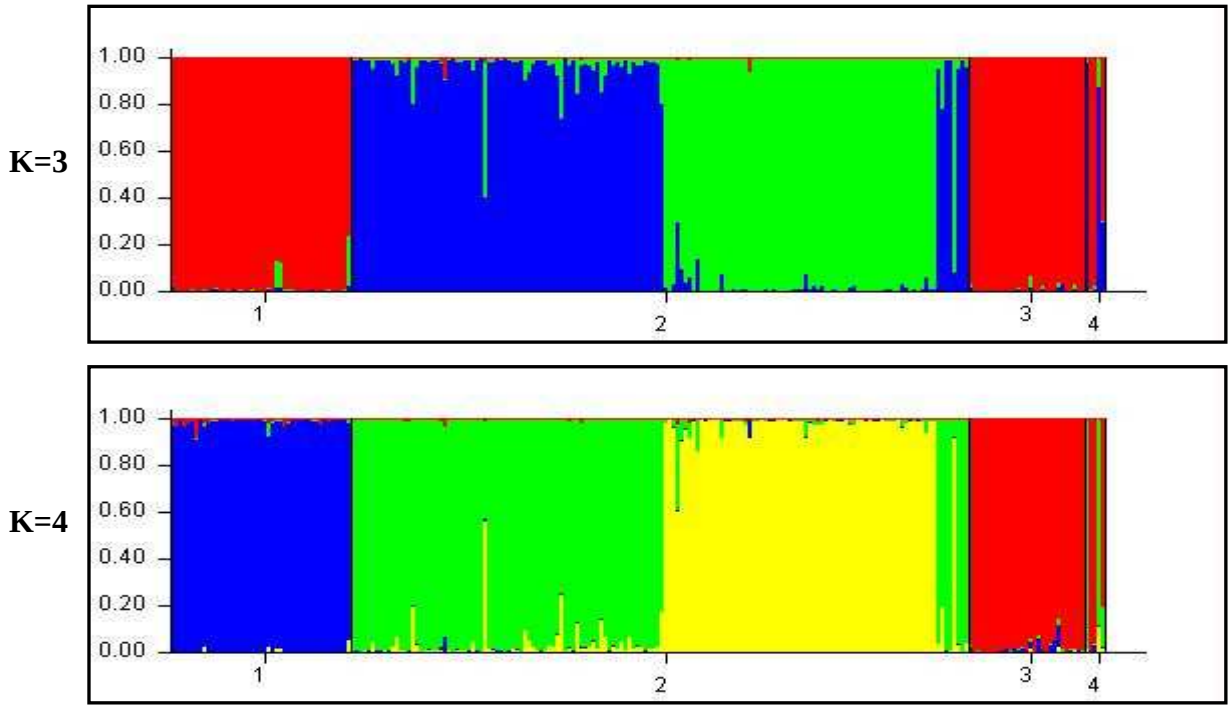


Fig. 29: Bayesian clustering of the populations obtained with the software *Structure*: population N°1 is *L. timidus*, N°2 *L. corsicanus*, N°3 *L. granatensis*, N°4 *L. castroviejoi*.

Considering K=3 *L. timidus* population is assigned to the same cluster with Iberian populations, considering K=4 we have two distinct clusters.

In both cases *L. corsicanus* populations show genetic differentiation between Sicily and the peninsula, and we get the same results obtained with previous analysis also on *L. castroviejoi*.

3.3 SINGLE NUCLEOTIDES POLYMORPHISM (SNP)

Genetic distance and Principal Coordinate Analysis (PCA) were calculated using *GenAlex* and *Genetix* (the same softwares used for microsatellites analysis) on 150 samples belonging to *L. corsicanus* and 15 samples belonging to *L. europaeus* collected in Italy analysed with nine single nucleotides polymorphism loci found in four nuclear coding genes (see Table 3).

All the nine SNP loci were polymorphic and often *L. europaeus* population showed diagnostic and private alleles (NP=0,556).

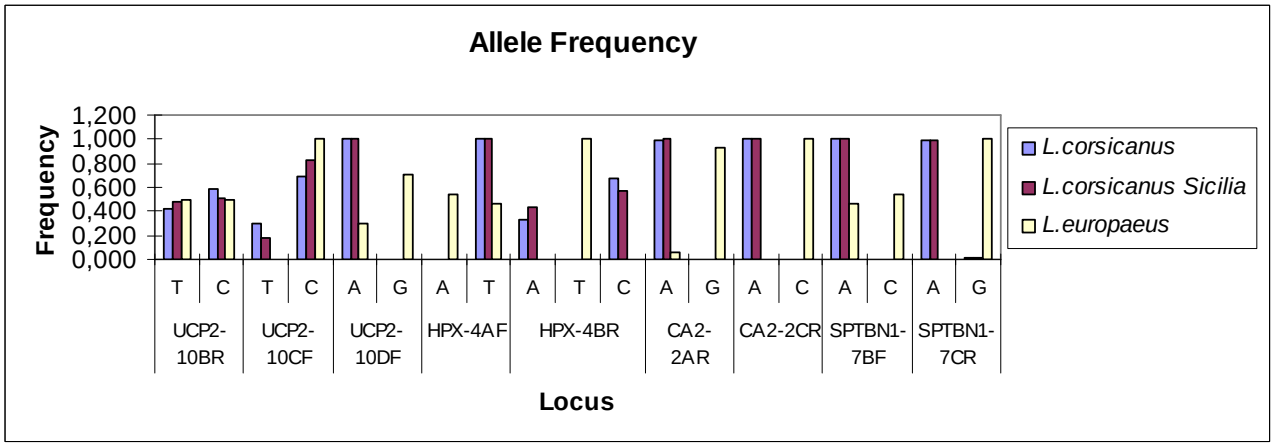


Fig. 30: Allele frequency for each SNP locus for the two considered species.

Variability is described with three principal components: the first axe describe the 64,49% of the variability, the second 20,87%, and the third 6,93%.

PCA results (Fig. 31) show a clear and deep distinction between the two species *L. corsicanus* and *L. europaeus*, and the absence of shared genotypes. We repeated the analysis (PCA) also combining genotypes obtained from microsatellites and SNPs and the result is shown in Figure 32. All specimens identified morphologically and genetically with the *L. corsicanus* mitochondrial haplotype were assigned to Italian hare population, no individual has been assigned to different species. The two hare samples collected in Calabria, morphologically identified as belonging to *L. europaeus* but identified with a *L. corsicanus* haplotype in mitochondrial DNA analysis, also with SNPs analysis showed an allelic pattern typical for the European brown hare.

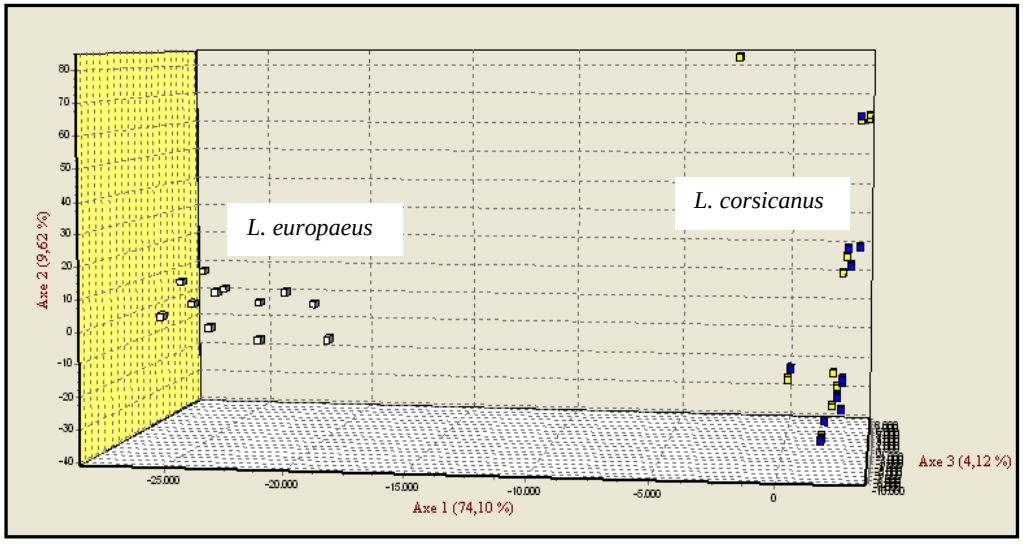


Fig. 31: Principal Coordinates Analysis of samples genotyped at 9 SNP loci. Blue dots stand for *L. corsicanus* samples collected in Sicily, yellow dots for *L. corsicanus* samples collected , white dots for *L. europaeus* samples.

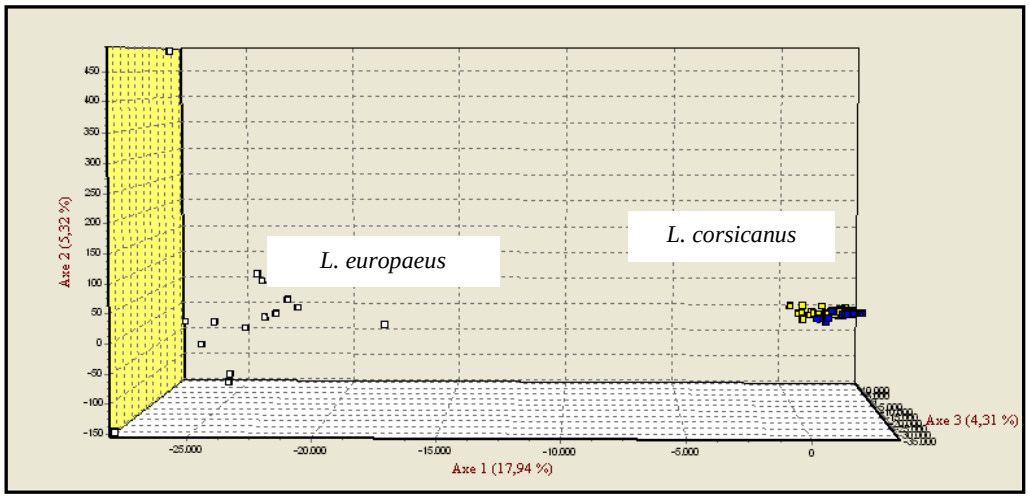


Fig. 32: Principal Coordinates Analysis of samples genotyped at 9 SNP loci and 13 microsatellite loci. Blue dots stand for *L. corsicanus* samples collected in Sicily, yellow dots for *L. corsicanus* samples collected , white dots for *L. europaeus* samples.

Combining microsatellites and SNPs results we found 78,79% polymorphic loci. Heterozygosity didn't show high values (Ho= 0,23-0,48; He= 0,23-0,49); *L. corsicanus* populations showed the lowest values (observed Heterozygosity in Sicilian population is 0,23), *L. europaeus* Ho=0,48.

3.4 SEX-BIASED DISPERSAL

For this analysis we used only samples collected in Sicily, as it is the only region that has a good number of samples collected fairly homogeneous throughout the territory (Fig. 33). Samples analyzed allowed to detect 24 males and 42 females with a sex ratio among detected individuals greater than one (1,00M:1,75F).

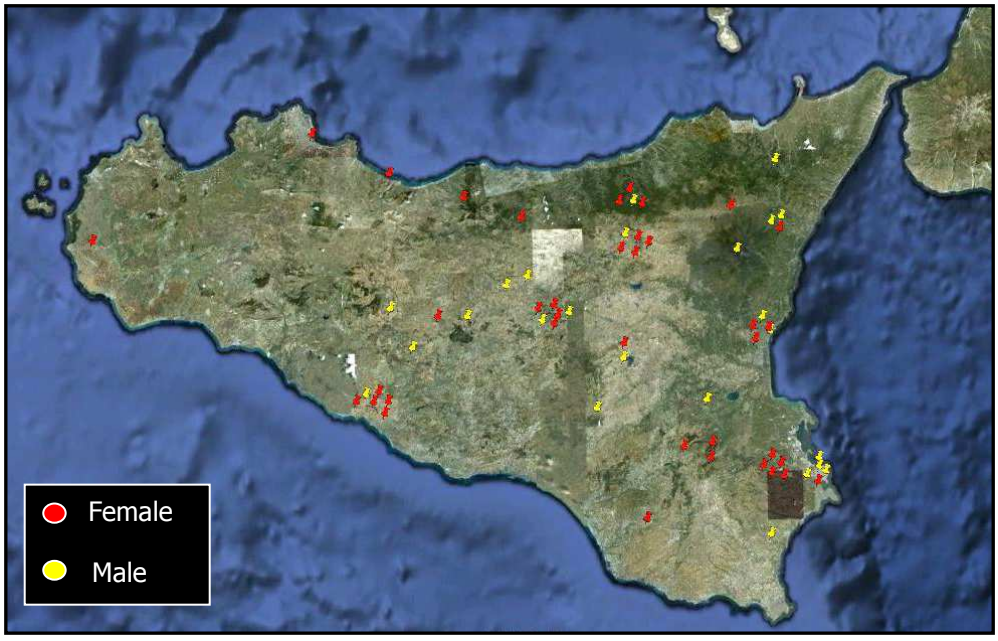


Fig. 33: Sicily sampling map and sex of individuals collected.

Sex-biased dispersal is a common reproductive strategy adopted by many species (Prugnolle and de Meeus 2002). In polygynous species of mammals, dispersal is predicted to be male-biased because of the greater benefits to females of remaining in familiar territory (Greenwood 1980), increased competition among related males for access to breeding partners (Dobson 1982; Hamilton 1972), inbreeding avoidance (Monard and Duncan 1996; Wolff 1993), or a combination of these factors (Favre et al. 1997).

Sex-biased dispersal among populations of hares has been investigated in few species and the results are equivocal. Both brown and snowshoe hares (*Lepus americanus* Erxleben) are polygynandrous (Burton and Krebs 2003; Hewson 1990) and have complex mating behaviors and both are predicted to be male-biased in dispersal under each of the above models (Dobson 1982). Mitochondrial and microsatellite evidence identified male-biased dispersal in a population of brown hares (Fickel et al. 1999, 2005). By contrast, assignment indices of microsatellite data indicate that

dispersal is equal among the sexes in snowshoe hares (Burton et al. 2002).

Spatial autocorrelation is a well established tool in biology for exploring the relationships between ecological or genetic variables and geographic location; we used the software *GenAlex v.6* to assess spatial autocorrelation in our samples.

GenAlex works with the multivariate spatial autocorrelation methods developed by Smouse and Peakall (1999) and extended by Peakall et al. (2003), Double et al. (2005) and Smouse et al. (2008); these methods employ a multivariate approach to simultaneously assess the spatial signal generated by multiple genetic loci. Unlike classical spatial autocorrelation analysis, usually executed one allele at a time, the procedure is intrinsically multivariate, avoiding the need for allele-by-allele, locus-bylocus analysis (although such analyses can always be conducted, if desired). The autocorrelation coefficient generated (*r*) is a proper correlation coefficient, bounded by [–1, +1] and is closely related to Moran's-I. The autocorrelation coefficient *r* provides a measure of the genetic similarity between pairs of individuals whose geographic separation falls within the specified distance class. A key feature of the autocorrelation method of Smouse and Peakall (1999) is that the starting point for analysis is a pair of genetic and geographic distance matrices.

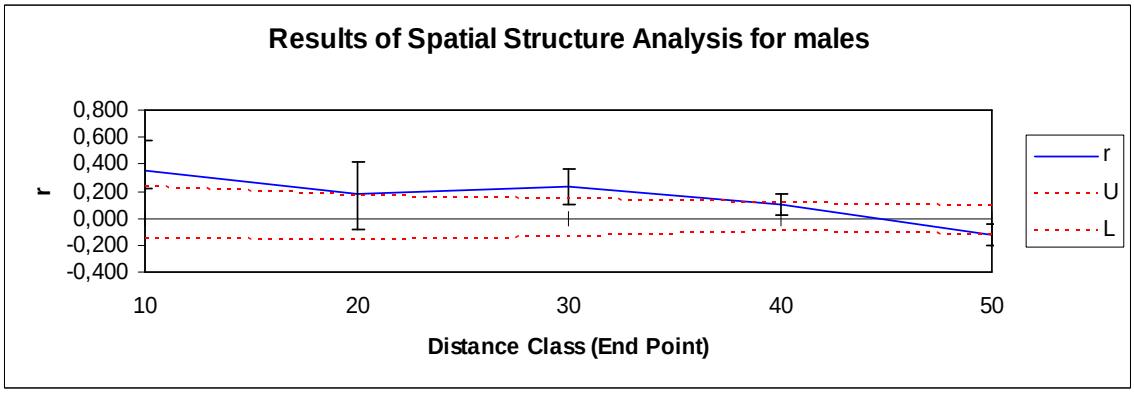


Fig. 34: Spatial autocorrelation analysis on males individuals collected.

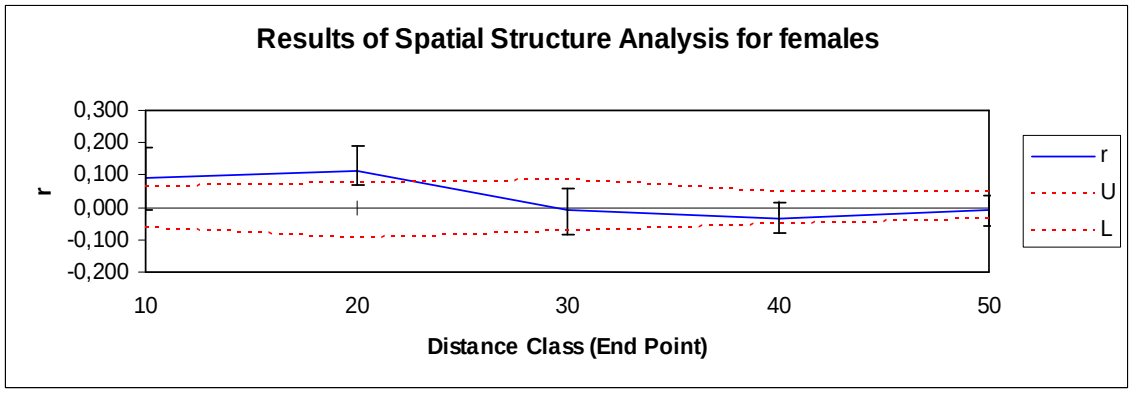


Fig. 35: Spatial autocorrelation analysis on females individuals collected.

We investigated on spatial autocorrelation between males and females collected in Sicily, testing for biased dispersal: graphs in Fig. 34 and Fig. 35 show an autocorrelation coefficient *r* bigger for males (tending to zero between 40-50 Km) and probably reveal a tendency toward higher dispersal in males.

These results have a clear biological meaning: because females take care for offspring, a male may disperse to avoid mating with his mother (Kerth et al. 2002; Wolff 1993) or to avoid competition with close relatives for mates. A female may require a good knowledge of the resources of her territory in order to provide optimal care for her offspring, hence remaining within natal territories more frequently than males.

3.5 MHC DQA LOCUS

3.5.1 Variability analysis

Sequence variation in exon 2 of DQA locus was investigated by PCR-SSCP analysis followed by sequencing of representative samples.

Nucleotide and amino acid sequences were aligned using *Bioedit* (Hall 1999); allelic frequencies, expected (*He*) and observed (*Ho*) heterozygosities were estimated using *Genetix* (Belkhir 2001). Haplotype diversity (*Hd*), nucleotide diversity statistics and other parameters were computed using *DNASP 5* (Rozas *et al*. 2003). The neighbour-joining tree and the rate of nonsynonymous and synonymous substitutions were calculated according to Nei & Gojobori (1986), applying the correction of Jukes and Cantor for multiple hits, using the *Mega 5* software (Tamura *et al*. 2011).

We sequenced 219 nucleotides of the exon 2 of the DQA locus, which included 49 (22,3%) polymorphic sites and 46 (41%) parsimony informative polymorphic sites: 19 haplotypes were identified in the two species.

Ten new alleles (Tab. 8 in red colour) were detected among the 84 *L. corsicanus* and *L. europaeus* samples (LeDQA51, LeDQA52, LeDQA53, LeDQA54, LcDQA01, LcDQA02, LcDQA03, LcDQA04, LcDQA05, LcDQA06), while nine alleles had been previously described by Koutsogiannouli *et al.* (2009) in different european populations of the brown hare; alleles named LcDQA were found only in *L. corsicanus* samples and only two alleles (LeDQA10-11) were shared between the two species.

Alleles	*L. corsicanus*	*L. europaeus*
LeDQA01	-	0.0244
LeDQA04	-	0.1707
LeDQA06	-	0.4024
LeDQA08	-	0.0976
LeDQA09	-	0.0366
LeDQA10	0.0581	0.1220
LeDQA11	0.0349	0.0244
LeDQA12	-	0.0488
LeDQA13	0.7674	-
LeDQA51	-	0.0122
LeDQA52	-	0.0122
LeDQA53	-	0.0122
LeDQA54	-	0.0366
LcDQA01	0.0465	-
LcDQA02	0.0116	-
LcDQA03	0.0233	-
LcDQA04	0.0116	-
LcDQA05	0.0116	-
LcDQA06	0.0349	-
N	43	42
A	9	12
H exp.	0.4021	0.7778
H obs.	0.1860	0.6341

Tab. 8: Alleles frequency, sample size (N), expected heterozygosity, observed heterozygosity and allele number (A) for each species; red colour indicates new alleles found.

Species	Samples	Haplotypes	Polymorphic sites (*s*)	Haplotype diversity (*h*)	Nucleotide diversity (π)	Theta (Watterson)
L. corsicanus	43	9	37/219	0.407 (0.067)	0.023 (0.005)	0.033 (0.009)
L. europaeus	41	12	46/219	0.787 (0.035)	0.049 (0.006)	0.042 (0.012)

Tab. 9: Values of interpopulation genetic diversity between the two species based on MHC sequences (standard deviations are in parenthesis).

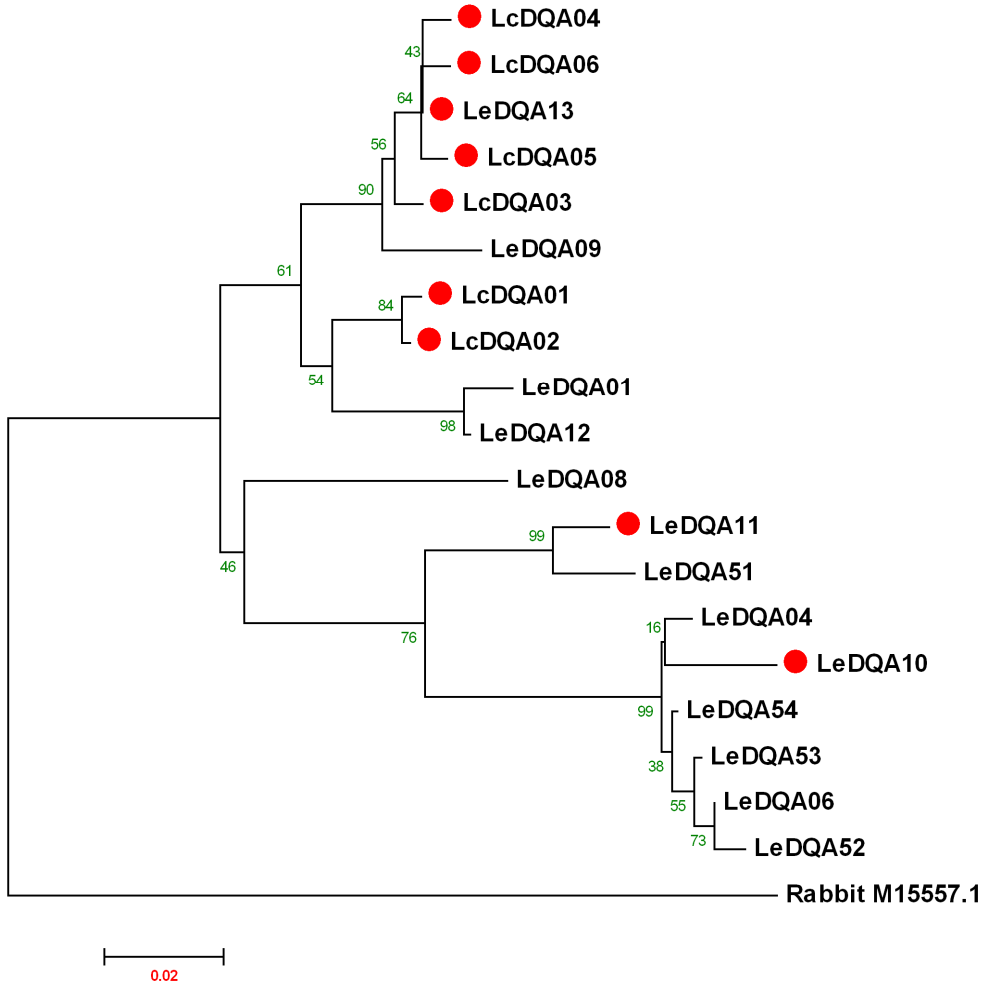


Fig. 36: Neighbour-joining phylogenetic tree for MHC class II DQA exon 2 sequences using a rabbit (*Oryctolagus cuniculus*) sequence as an outgroup; sequences marked with a red circles are alleles found in *L. corsicanus* samples.

Allele LeQDA13, previously described by de Bellocq *et al.* (2009) in Belgian populations of the brown hare, was found with a high frequency in the Italian hare population. The estimation of heterozygosity values showed that all populations exhibited lower levels of heterozygosity than expected, especially *L. corsicanus*.

The relationships among the 19 alleles identified in the present study were assessed by the construction of a neighbour-joining tree (Fig. 36), using a rabbit sequence as an outgroup (GenBank AN: M15557.1); alleles named "*Lc*" were found only in *L. corsicanus* samples, alleles named "*Le*" were found in *L. europaeus* samples. Alleles LeDQA10, LeDQA11, LeDQA13 were shared between the two species.

Although some of the branches did not have very strong bootstrap support, many internal allele lineages were supported strongly. However, the phylogenetic analysis showed clearly that

population-specific alleles did not cluster together, and no separation of alleles on the basis of geographical distances was observed.

3.5.2 Testing for selection

Examined the level of MHC genetic diversity, we tried to search for signal of selection in the evolution of the DQA gene.

Forty-nine out of 219 (22,37%) nucleotide and 27 out of 73 (36,98%) amino acids positions were variable (Fig. 37).

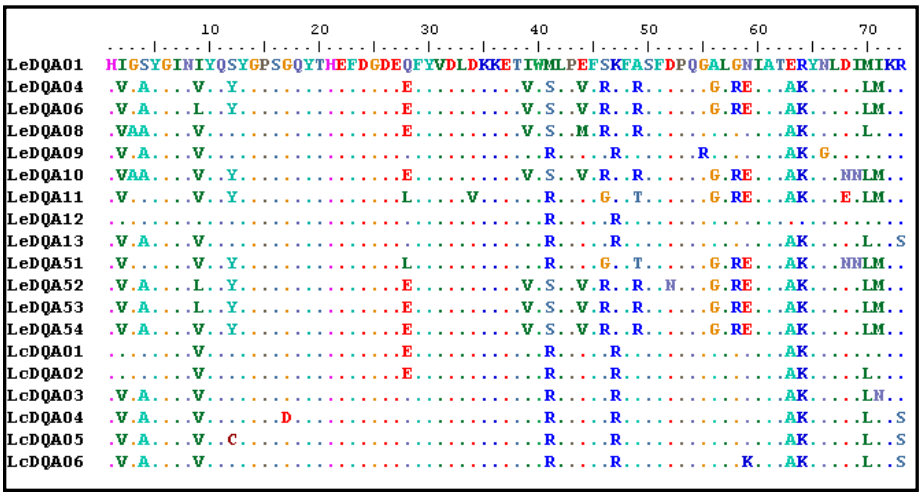


Fig. 37: Amino acid sequence alignment of 19 exon 2 DQA alleles of the brown hare and the Italian hare.

Examination of synonymous vs. nonsynonymous substitutions and Selection Test were computed with both softwares *Mega 5* (Tamura *et al*. 2011) and *DNASP 5* (Rozas *et al*. 2003), and they gave the same results. We computed the analysis first on alleles sequences found in *L. corsicanus*, then on alleles found in *L. europaeus* (Tab. 10).

The Tajima's D (Neutrality Test) test is a widely used test of neutrality in population genetics. The purpose of the test is to distinguish between a DNA sequence evolving randomly ("neutrally") and one evolving under a non-random process, including directional selection or balancing selection, demographic expansion or contraction, genetic hitchhiking, or introgression. A randomly evolving DNA sequence contains mutations with no effect on the fitness and survival of an organism. The randomly evolving mutations are called "neutral", while mutations under selection are "non-neutral".

This statistic illustrates the allele frequency distribution of nucleotide sequence data and is based on the difference between two estimators of **Θ** (the population mutation rate 4Neμ): (1) Tajima's estimator, which is based on the average number of pairwise differences between sequences, and (2) Watterson's estimator, which is based on the number of segregating sites in the sample.

To standardize the pairwise differences, the mean or 'average' number of pairwise differences is used. This is simply the sum of the pairwise differences divided by the number of pairs, and is signified by π.

Tajima's statistic computes a standardized measure of the total number of segregating sites (these are DNA sites that are polymorphic) in the sampled DNA and the average number of mutations between pairs in the sample. The two quantities whose values are compared are both method of moments estimates of the population genetic parameter theta, and so are expected to equal the same value. If these two numbers only differ by as much as one could reasonably expect by chance, then the null hypothesis of neutrality cannot be rejected. Otherwise, the null hypothesis of neutrality is rejected.

High positive values of Tajima's D suggest an excess of common variation in a region, which can be consistent with balancing selection, population contraction. High negative values of Tajima's D, on the other hand, indicate an excess of rare variation, consistent with population growth, or positive selection. The null hypothesis of the Tajima's D test is neutral evolution in an equilibrium population. This implies that no selection is acting at the locus and that the population has not experienced any recent growth or contraction (Tajima 1989).

Tajima's Neutrality Test: *L. corsicanus*

m	***S***	p_s	Θ	π	***D***	***P***
9	37	0.168950	0.062163	0.057839	-0.351579	Not significant (P>0.10)

Tajima's Neutrality Test: *L. europaeus*

m	***S***	p_s	Θ	π	***D***	***P***
13	47	0.214612	0.069158	0.081782	0.815934	Not significant (P>0.10)

Tab. 10: Results from Tajima's Neutrality Test. *Abbreviations*: *m* = number of sequences, *S* = Number of segregating sites, $p_s = S/m$, $\Theta = p_s/a_1$, π = nucleotide diversity, and *D* is the Tajima test statistic.

Codon#	Triplet	Syn sites (S)	Nonsyn sites (N)	dS	dN	dN-dS	P-value
1	CAC	0,381104	2,6189	2,624	0	-2,624	1
2	ATT	0,980462	2,01954	0	0,4952	0,49516	0,6732
3	GGC	1	1,99257	2	0,5019	-1,4981	0,9627
4	TCC	1	2	0	1	1	0,4444
5	TAT	0,391507	2	0	0	0	0
6	GGC	1	2	0	0	0	0
7	ATA	0,449654	2,55035	0	0	0	0
8	AAC	0,270933	2,72907	3,691	0	-3,691	1
9	GTC	1	2	0	0	0	0
10	TAC	0,263584	2	0	0	0	0
11	CAG	0,538753	2,19766	1,8561	0	-1,8561	1
12	TCT	0,641134	2,00262	0	0,9987	0,99869	0,5738
13	TAT	0,391507	2	0	0	0	0
14	GGT	1	2	0	0	0	0
15	CCC	1	2	0	0	0	0
16	TCT	1	2	0	0	0	0
17	GGC	0,991982	2,00802	0	0,498	0,498	0,6693
18	CAG	0,522816	2,2136	0	0	0	0
19	TAC	0,263584	2	0	0	0	0
20	ACC	1	2	1	0	-1	1
21	CAT	0,391507	2,60849	0	0	0	0
22	GAA	0,550346	2,31405	0	0	0	0
23	TTT	0,391507	2,60849	0	0	0	0
24	GAT	0,391507	2,60849	0	0	0	0
25	GGA	1	1,8644	0	0	0	0
26	GAT	0,391507	2,60849	0	0	0	0
27	GAA	0,525055	2,33934	1,9046	0	-1,9046	1
28	GAG	0,565374	2,21762	0	1,3528	1,3528	0,506
29	TTC	0,263584	2,73642	0	0	0	0
30	TAT	0,391507	2	0	0	0	0
31	GTG	1	2	2	0	-2	1
32	GAC	0,263584	2,73642	0	0	0	0
33	CTG	1,26358	1,73642	0	0	0	0
34	GAT	0,426466	2,57353	0	0,3886	0,38857	0,8578
35	AAG	0,522816	2,25841	0	0	0	0
36	AAG	0,522816	2,25841	0	0	0	0
37	GAG	0,522816	2,34158	0	0	0	0
38	ACT	1	2	0	0	0	0
39	ATC	0,659495	2,34051	0	0,4273	0,42726	0,7802
40	TGG	0	1,95437	0	0	0	0
41	AGG	0,694177	2,30582	0	0,4337	0,43369	0,7686
42	CTT	1,04331	1,95669	0,9585	0	-0,9585	1
43	CCT	1	2	0	0	0	0
44	GAG	0,601228	2,28545	0	0,4376	0,43755	0,7917
45	TTT	0,391507	2,60849	0	0	0	0
46	AGC	0,689877	2,31012	0	0,8658	0,86576	0,593
47	AGA	0,647574	2,13365	0	0,4687	0,46868	0,7672
48	TTT	0,391507	2,60849	0	0	0	0
49	GCA	0,961034	2,02698	1,0406	0,9867	-0,0539	0,7562
50	AGT	0,391507	2,60849	0	0	0	0

51	TTT	0,391507	2,60849	0	0	0	0
52	GAT	0,391507	2,60849	0	0	0	0
53	CCA	1	2	0	0	0	0
54	CAG	0,538753	2,19766	1,8561	0	-1,8561	1
55	GGT	1	2	0	0	0	0
56	GCA	1	1,9215	0	0,5204	0,52043	0,6577
57	CTG	1,26358	1,73642	0	0	0	0
58	GGA	0,873355	1,94289	0	0,5147	0,5147	0,6899
59	AAC	0,416774	2,49859	0	1,2007	1,20068	0,6295
60	ATA	0,449654	2,55035	0	0	0	0
61	GCC	1	2	1	0	-1	1
62	ACA	1	2	0	0	0	0
63	GCA	1	2	0	0	0	0
64	AAA	0,550346	2,23088	0	0	0	0
65	TAC	0,263584	2	0	0	0	0
66	AAC	0,263584	2,73642	0	0	0	0
67	TTG	0,914323	1,71744	0	0	0	0
68	GAC	0,280058	2,71215	0	0,7374	0,73742	0,8216
69	ATC	0,538485	2,46151	0	0,4063	0,40625	0,8205
70	ATG	1,24982	1,75018	0	0,5714	0,57137	0,5834
71	ATT	0,315932	2,68407	0	0,7451	0,74514	0,8005
72	AAA	0,550346	2,23088	0	0	0	0
73	CGC	0,943829	2,05617	0	0,4863	0,48634	0,6854

Tab. 11: Examination of synonymous vs. nonsynonymous substitutions for *L. corsicanus* alleles obtained with *Mega 5* software using the HyPhy software package.

We did not find any evidence of selection, in fact Tajima's D values are low and close to zero, indicating the hypothesis that no selection is acting at the locus analysed.

With the software Mega we computed also, for each codon and for each species (Tab. 11-12), the numbers of sites that are estimated to be synonymous (S) and nonsyonymous (N). These estimates are produced using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model (Muse *et al.* 1994) of codon substitution and Tamura-Nei model (Tamura *et al*. 1993) of nucleotide substitution. The test statistic dN - dS is used for detecting codons that have undergone positive selection, where dS is the number of synonymous substitutions per site (s/S) and dN is the number of nonsynonymous substitutions per site (n/N). A positive value for the test statistic indicates an overabundance of nonsynonymous substitutions. In this case, the probability of rejecting the null hypothesis of neutral evolution (P-value) is calculated (Kosakovsky *et al*. 2005; Suzuki *et al*. 1999). Values of P less than 0.05 are considered significant at a 5% level and are highlighted. Also in this case, codons with significant P values show test statistic values compatible with the null hypothesis of strict neutrality (dN = dS) for both species.

Codon#	Triplet	Syn sites (S)	Nonsyn sites (N)	dS	dN	dN-dS	P-value
1	CAC	0,375835	2,62416	2,6607	0	-2,6607	1
2	ATT	0,977995	2,02201	0	0,4946	0,49456	0,674
3	GGC	1	1,99532	4	1,0024	-2,9977	0,982
4	TCC	1	2	0	1	1	0,4444
5	TAT	0,384468	2	0	0	0	0
6	GGC	1	2	0	0	0	0
7	ATA	0,327929	2,67207	0	0	0	0
8	AAC	0,297281	2,70272	3,3638	0	-3,3638	1
9	ATC	0,956139	2,04386	0	0,9785	0,97854	0,4642
10	TAC	0,285813	2	0	0	0	0
11	CAG	0,615863	2,09832	1,6237	0	-1,6237	1
12	TCT	0,699938	2	0	0,5	0,5	0,7408
13	TAT	0,384468	2	0	0	0	0
14	GGT	1	2	0	0	0	0
15	CCC	1	2	0	0	0	0
16	TCT	1	2	0	0	0	0
17	GGC	1	2	0	0	0	0
18	CAA	0,571998	2,14219	1,7483	0	-1,7483	1
19	TAC	0,285813	2	0	0	0	0
20	ACC	1	2	1	0	-1	1
21	CAT	0,384468	2,61553	0	0	0	0
22	GAA	0,672071	2,20906	0	0	0	0
23	TTT	0,384468	2,61553	0	0	0	0
24	GAT	0,384468	2,61553	0	0	0	0
25	GGA	1	1,88113	0	0	0	0
26	GAT	0,384468	2,61553	0	0	0	0
27	GAG	0,57176	2,30937	1,749	0	-1,749	1
28	CAG	0,646495	2,14829	0	1,3965	1,39646	0,4542
29	TTC	0,285813	2,71419	0	0	0	0
30	TAT	0,384468	2	0	0	0	0
31	GTA	1	2	4	0	-4	1
32	GAC	0,285813	2,71419	0	0	0	0
33	CTG	1,28581	1,71419	0	0	0	0
34	GAT	0,393602	2,6064	0	0,3837	0,38367	0,8688
35	AAG	0,57176	2,22257	1,749	0	-1,749	1
36	AAG	0,562401	2,23193	0	0	0	0
37	GAG	0,562401	2,31873	0	0	0	0
38	ACT	1	2	0	0	0	0
39	ATC	0,641014	2,35899	0	0,8478	0,84782	0,6183
40	TGG	0	1,8752	0	0	0	0
41	ATG	0,580984	2,41902	0	1,2402	1,24017	0,5243
42	CTT	1,05671	1,94329	1,8927	0	-1,8927	1
43	CCT	1	2	1	0	-1	1
44	GAG	0,629683	2,28271	0	1,3142	1,31423	0,4815
45	TTT	0,384468	2,61553	0	0	0	0
46	AGC	0,833415	2,16658	0	0,9231	0,92311	0,5216
47	AAA	0,698789	2,09554	0	0,9544	0,95441	0,5624
48	TTT	0,384468	2,61553	0	0	0	0
49	GCA	0,922084	2,05623	1,0845	1,459	0,37448	0,6347
50	AGT	0,384468	2,61553	0	0	0	0

51	TTT	0,384468	2,61553	0	0	0	0
52	GAT	0,384468	2,61553	0	0,3823	0,38233	0,8718
53	CCA	1	2	0	0	0	0
54	CAG	0,615863	2,09832	1,6237	0	-1,6237	1
55	GGT	1	2	0	0,5	0,5	0,6667
56	GCA	1	1,94205	0	0,5149	0,51492	0,6601
57	CTG	1,28581	1,71419	0	0	0	0
58	GGA	0,899738	1,93908	0	0,5157	0,51571	0,6831
59	AAC	0,421816	2,51821	2,3707	0,7942	-1,5765	0,9442
60	ATA	0,327929	2,67207	0	0	0	0
61	GCC	1	2	1	0	-1	1
62	ACA	1	2	0	0	0	0
63	GAA	0,971303	2,01829	0	0,4955	0,49547	0,6751
64	AGA	0,682769	2,11156	0	0,4736	0,47358	0,7557
65	TAC	0,285813	2	0	0	0	0
66	AAC	0,297504	2,7025	0	0,7401	0,74006	0,8115
67	TTG	0,946869	1,68906	0	0	0	0
68	GAC	0,291544	2,70669	0	1,1084	1,10836	0,7357
69	ATC	0,487108	2,51289	0	0,7959	0,7959	0,7016
70	ATG	0,985896	2,0141	0	0,4965	0,4965	0,6714
71	ATT	0,38364	2,61636	0	0,3822	0,38221	0,8721
72	AAA	0,672071	2,12226	0	0	0	0
73	CGC	1	2	0	0	0	0

Tab. 12: Examination of synonymous vs. nonsynonymous substitutions for *L. europaeus* alleles obtained with *Mega 5* software using the HyPhy software package.

CHAPTER FOURTH: DISCUSSION

The taxonomic status of the Italian hare *Lepus corsicanus* has been uncertain since its first description by W. E. de Winton in 1898 (de Winton WE. *Annual Magazine of Natural History, London*, 1898, 1, 149–158). The distribution range of this species has shrunk severely over the last few decades owing to overhunting and restocking with nonindigenous Brown hares (*L. europaeus*) in central and southern Italy and Sicily. Recently, scanty populations of Italian hares were rediscovered, and samples for morphological and molecular analyses were collected.

Extensive human disturbance (overhunting and restocking) could have threatened, severely restricted and eventually eradicated the Italian hare from most of its former historical range. Historical distributions suggested that natural populations of Italian and Brown hares were allopatric with putative contact zones running from central Tuscany to the Gargano promontory (Palacios 1996). However, no data were available to document the effective degree of reproductive isolation or eventual gene flow among populations in contact. Moreover, hares can hybridize in nature, and the integrity of local Italian hare gene pools could have been disrupted by gene introgression owing to released, nonindigenous Brown hares (Rhymer & Simberloff 1996; Thulin *et al*. 1997).

Results obtained from mtDNA and nuclear DNA analysis (microsatellites and SNPs) confirm that *L. corsicanus* and *L. europaeus* species are distinct and genetically different from all other species studied.

The Italian hare and the European brown hare, which live in sympatric areas along the Italian peninsula often artificially created as a result of repopulation events, do not share any nuclear genotype, suggesting the absence of hybridization between the two species.

Results obtained from phylogenetic analysis show that *L. corsicanus* and *L. europaeus* possess a long history of independent evolution, and are reproductively isolated in nature. The Italian hare's adaptation to the Mediterranean ecosystems may explain the absence of gene flow between the two species.

Concerning Leu41 haplotype (found in two samples collected in 1999 in Calabria identified morphologically and genetically, through microsatellites analysis, as belonging to *L. europaeus*) which is placed in the phylogenetic tree in the same group of *L. corsicanus* haplotypes, we can interpret this phenomenon as an introgression of *L. corsicanus* mitochondrial genome in *L. europaeus* genome occurred in historical times as a result of an hybridization event. Recent

anthropogenic crossbreeding (occurred in breeding centre) can not be excluded.

Considering this phenomenon unique and isolated (only two hybrid individuals detected in the same area in nearly twenty years of sampling activity) the simple mitochondrial DNA analysis can be considered sufficient for a correct species identification of unknown samples, especially when compared to morphological observations.

Mitochondrial DNA, microsatellite loci and SNPs loci can be amplified and typed using non-invasive genetic procedures; the latter allow to identify species and individuals in the study areas using DNA extraction from faecal samples. This possibility makes feasible to organize monitoring plans that can describe the distribution of these two species, especially in sympatric areas of central and southern Italy.

Concerning only *L. corsicanus*, results showed a genetic divergence between individuals collected in Sicily and individuals from central and southern Italy, divergence originated with the separation of Sicily from the peninsula at the end of the last glaciation; graphs in Fig. 38 show this differentiation, in fact Sicilian populations do not share any mitochondrial haplotype with populations of the peninsula.

For this reason, Sicilian populations should be protected, and all translocations to and from the island should be strictly forbidden.

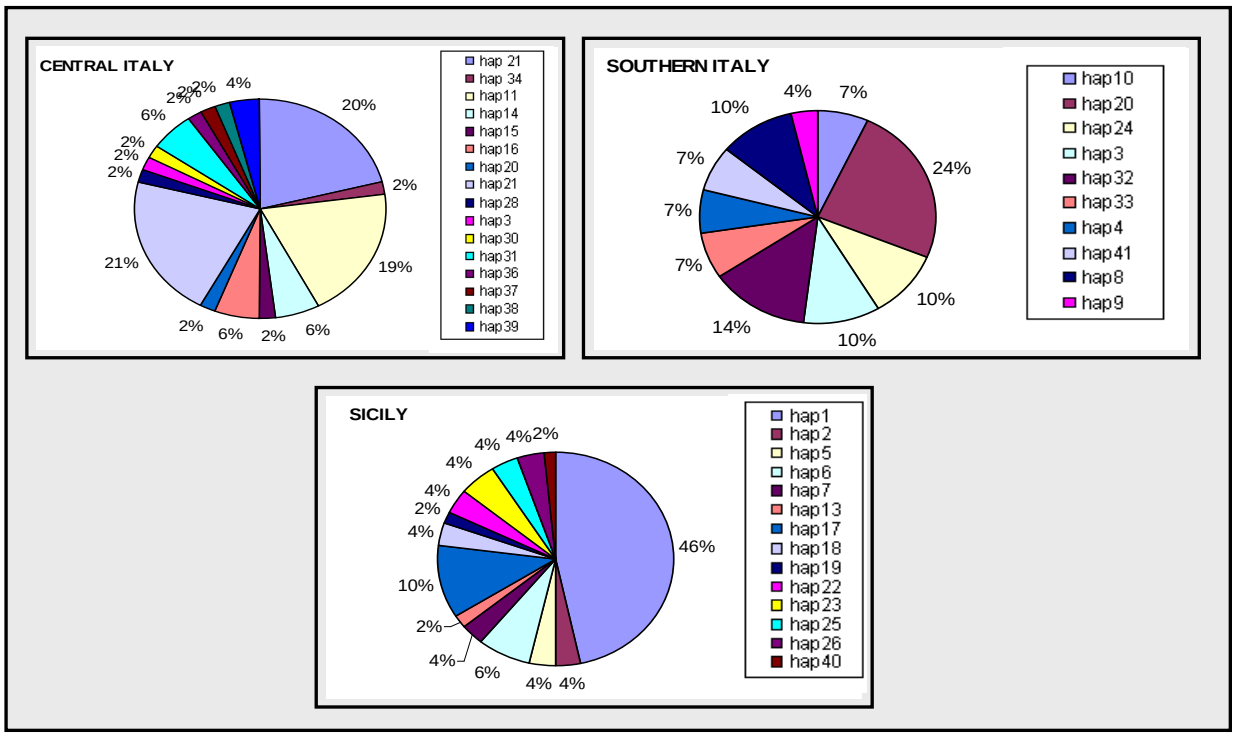


Fig. 38: *L. corsicanus* haplotypes distribution between Italian regions.

Low genetic variability was described among all populations of *L. corsicanus* populations; these results could be explained assuming poor differentiation among populations and/or a recent population decline.

Both markers also showed a close genetic relationship between *L. corsicanus* and *L. castroviejoi*, suggesting the hypothesis that the two taxa are very recently derived from common ancestors. It can be assumed that these two taxa are currently involved in a speciation process consequently to the fragmentation of their geographic range (Alves *et al*. 2008); additional analysis would be useful for a deeper evaluation of their taxonomic status.

In this project we analysed individuals from different areas of Africa, but we have chosen to not explore phylogenetic analysis because the number of samples was very small and, in spite of several molecular phylogenetic works on hares of the genus *Lepus,* no clear picture of phylogenetic relationships among African taxa within this genus is yet at hand.

Historically, many different species have been identified in Africa, very often based on poor morphological descriptions in terms of modern standards. Neighbour joining (NJ) and principal coordinate analyses (PCA) revealed relatively close relationships between *Lepus capensis* and the clades of African scrub hares (*L. saxatilis*) and brown hares (*L. europaeus*) (Slimen H.B., *et al*. 2007). However, further analyses are suggested including many more population samples from Africa, the Middle East, and Europe.

The presence of *L. timidus* mtDNA in populations of other hares species (as in the case of the Iberian hares), indicates widespread and ancient hybridization and genetic introgression events. The most likely hypothesis is that massive mitochondrial DNA introgression occurred as a result of hybridization events between these species, during the competitive replacement of arctic species with temperate species at the end of the last ice age (Melo-Ferreira *et al*. 2009).

Molecular markers associated with host-pathogen interactions are of great interest to understand the interplay between population dynamics and natural selection. Among these markers, MHC genes are of particular significance given their function in the adaptive immune system conferring protection against parasites and infectious diseases (Deter *et al.* 2008). The high level of polymorphism of these genes indicates that they are under some form of balancing selection and are adaptive in natural populations (Campos *et al.* 2010; Koutsogiannouli *et al.* 2009; Surridge *et al* 2008).

Concerning MHC analysis the overall results show a substantial high level of polymorphism and variability of the DQA locus, especially for *L. europaeus*. In general, this is in accordance with the results of our previous analyses on the brown hare, which examined the diversity of mtDNA and

microsatellite markers, and confirms the high genetic variability of this species. Italian hares showed six private alleles, not found in European brown hares, but very low values of heterozygosity.

The phylogeny of the exon 2 DQA alleles did not show a strong phylogeographic signal because alleles that belonged to a specific population did not group together and we found shared haplotypes between *L. corsicanus* and *L. europaeus*. This result contradicts the data obtained for mtDNA markers (Stamatis et al. 2009), which demonstrated a strong phylogeographic signal.

The absence of a phylogeographic signal is a characteristic feature of some MHC loci (Klein et al. 1998) and is thought to result from balancing selection (Hughes et al. 1996). In addition, when we included in the analysis alleles from other *Lepus* species i.e. *L. timidus, L. granatensis, L. saxatilis, L. capensis* and *L. californicus*, which mainly inhabit different areas of the world, in addition to alleles from a different Lagomorpha order (*Oryctolagus cuniculus*), these alleles were found to be scattered throughout the tree and mixed with the *L. europaeus* DQA alleles (Koutsogiannouli *et al.* 2009). Such a phenomenon has been observed for MHC loci in several organisms, such as fish, rodents, carnivores, ungulates and primates (Seddon & Ellegren 2002; Otting et al. 2002; Musolf et al. 2004; Ottova et al. 2005; Surridge et al. 2008), and may be derived from ancestral sequences that were present in a common ancestor and have persisted in the populations since their divergence. Pathogen recognition might provide the selection pressure required to maintain particular MHC sequences, and sharing similar allelic sequences may be evidence of the need for a specific immune response to a common pathogen. Furthermore, the bootstrap values for the phylogenetic tree were low, which implies a low phylogenetic signal. This is in accordance with the proposed antiquity of the MHC loci and the observed high level of nucleotide polymorphism.

Selection tests performed on our Italian hare and brown hare samples gave contrasting results when compared to those already present in literature (Campos *et al.* 2010; Koutsogiannouli *et al.* 2009; Surridge *et al* 2008), in which it is confirmed that different MHC loci are under positive selection; in fact, our tests did not reveal any evidence of selective pressure and all statistics values obtained are compatible with the null hypothesis of strict neutrality.

For us, this was a first approach to this type of analysis and tests are still ongoing; probably it will be necessary to conduct further analysis considering a higher number of samples and different statistical approaches.

CONCLUSIONS

Present-day distribution of the Italian hare is extremely fragmented in central and southern Italy. Populations survive at low density, mainly in protected areas and National Parks, where the species has managed to escape overhunting and competition with introduced Brown hares.

Although hybridization seems not to be a threat to the survival of *Lepus corsicanus* in central and southern Italy, strict prohibition of restocking with Brown hares should be enforced, at least in selected priority areas (especially in Sicily).

Despite the intensive release of Brown hares during the last 20 years, field surveys (Lo Valvo *et al*. 1997; Riga *et al*. 1998) have shown that hares of Sicily belong only to *L. corsicanus*. It is therefore possible that the peculiar conditions of Sicily are unsuitable for the Brown hare and that it cannot survive in the Mediterranean-type climate and habitats of the island. Although Sicilian populations of *L. corsicanus* are rather widespread and stable, conservation measures should be enforced in order to prevent the release of Brown hares in the island and to plan a controlled hunting and wise use of the Italian hare populations.

The aim of the National Action Plan (published in 2001) for *Lepus corsicanus* is to collect knowledges on the status and biology of the species to better promote its conservation. Genetics has provided and can continue to provide important informations for the conservation of the species.

The genetic analysis of non-invasive samples collected in central and south Italy is an useful tool to identify the species distribution areas; the identification of these areas, through the combination of genetic and environmental data, could allow the discrimination on a small scale of preferential or exclusive habitat for the Italian hare.

Key objectives in these areas to ensure the protection of the species would have to be: the reduction of hunting pressure, ecological corridors recovery between fragmented and separated distribution areas, and the quantitative and qualitative improvement of habitats.

The population dynamics study through the typing of non-invasive samples would also provide an opportunity to assess the status of single local communities and to assess, in concrete terms, which measures are necessary to prevent the extinction of local populations of the Italian hare.

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