Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Biodiversità ed Evoluzione

Ciclo XXIV

Settore Concorsuale di afferenza: 05/B1 - ZOOLOGIA E ANTROPOLOGIA

Settore Scientifico disciplinare: BIO/05 - ZOOLOGIA

TITOLO TESI

"Study of variability and genetic structure of European populations of *Myotis emarginatus* and *Myotis capaccinii* (Chiroptera, Vespertilionidae)"

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Esame finale anno 2012

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CHAPTER 1

GENERAL INTRODUCTION

1.1 CONSERVATION GENETICS

Biodiversity has been described as "... the variety and variability among living organisms and the ecological complex in which they occur. Diversity can be defined in terms of number of entities and their relative frequency. In the case of biodiversity, these entities are organized in different levels ranging from ecosystems to the chemical structures, which constitute the molecular basis of life. Therefore, the term includes ecosystems, species, genes and their relative abundance." (U.S. Congress Office of Technology Assessment, 1987).

Biodiversity conservation has become a topical issue, particularly in recent years, especially after the elaboration of the "Convention on Biological Diversity", adopted in 1992 in Rio de Janeiro (Brasil) and until now ratified by 188 countries.

Following the conclusion of the Rio convention, in the last 20 years, different branches of research on biodiversity have been developed. One is *Conservation Biology,* which is a multidisciplinary applied field drawing on ecology, wildlife biology, resource biology, evolutionary, taxonomy, molecular biology, population and conservation genetics.

The aim of *Population genetics* is to describe the genetic composition of populations and to understand the causes that determine changes (evolutionary forces). Every species is made up of many evolutionary units, the populations that contain a certain quantity of genetic variability on which evolution can act. Genetic variability within populations is often described by the frequency of allele frequencies at a number of *loci* that can vary in the course of generations due to mutations, natural selection, migration or genetic drift.

The second branch developing is *Conservation genetics,* which is the application of genetic techniques and analysis methods to preserve species and dynamic entities capable of coping with environmental changes. It deals with the genetic factors that affect extinction risk and genetic management regimes required to minimise these risks. There are 11 major 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 analyses in forensics.
- Use of molecular genetic analyses to understand aspects of species biology (mating, dispersal and migration patterns, reproduction systems) is important for conservation. Deleterious effects on fitness that can sometimes occur as a result of outcrossing (outbreeding depression).

It is important to remember that "the conservation of genetic variability is fundamental to healthiness of populations, because its decrement could increase the risk of extinction" (Frankham & Richard, 1995). It must evaluate the level of genetic connectivity between different populations, determining if there is communication with the same individual. Finally, the evaluation of phylogeographic patterns is also important in biodiversity conservation, identifying the actual distribution of a genetic lineages.

In the past years variability within natural species has been often been neglected, although genetic structure and population variability are a prerequisite for species survival, as a dynamic entity upon which evolutionary pressures act. Quantity of intraspecific genetic diversity depends on the numbers of individuals, geographic distribution, level of population isolation and to their specific genetic structure.

The global genetic biodiversity is rapidly declining as direct and indirect consequences of human activities. Environmental modifications caused by anthropization can reduce the size and structure of natural populations such that stochastic processes; environmental, demographic changes, or catastrophic events, could increase the risk of extinction. However even in the absence of these extreme events, genetic deterioration caused by a decrease in the number of individuals and population fragmentation can present a threat for the survival of species. The loss of genetic variability and consanguinity are in fact certain in few and isolated populations. In the short term these factors can reduce reproductive success, while in the long term they compromise the adaptation potentialities and increase the risk of population decrease.

In recente years, a variety of molecular markers for the study genetic variability have been developed, in particular since the advent of Polymerase Chain Reaction (PCR). These approaches have changed the study of ecological and evolutionary processes at all different levels.

These molecular markers have been detected using probes or specific primers. Molecular markers are not necessarily related to specific gene activities, because they could be located

both in expressed and non coding regions. Measurement of genetic variability obtained with molecular markers, allows for the identification of DNA sequence polymorphisms.

Microsatellite markers are the most useful tools for genetic analysis: they show huge level of polymorphism. They are distributed throughout the genome, they are co-dominant, easy to analyse and permit a high repetitiveness of analysis. Microsatellite markers can be transferred from species which are very similar.

Haplotypes found on mtDNA (mitochondrial DNA) could be also analysed, which are involved to evaluate if populations are geographically isolated or in contact among them evaluating statistics of Φ_{ST}.

Haplotype sequences also allow the evaluation of mitochondrial variability of each analysed population.

1.2 PHYLOGEOGRAPHY

Phylogeography is a discipline that bridges the fields of phylogeneties and population genetics, both in terms of an evolutionary timescale and in relation to focal groups (Avise *et al* 1987). Phylogenetic studies typically focus on ordinal, familial or genera interrelationships; phylogeographic studies examine relationships within a single species and population genetics are focused on the dynamics occurring within individual populations of a species.

The term 'phylogeography' was first used by Avise *et al.* in 1987 for a field that concerns the geographical distribution of genealogical lineages (Hewitt 2004b).

Since its inception (Avise et al., 1987), phylogeography has grown exponentially (Avise, 2000), reflecting its intuitive appeal and perceived success. This was possible thanks to the new techniques developed in the 1980's, such as PCR and the lowering costs of DNA sequencing.

Phylogeography makes use of molecular and geographical data to infer the role of historical factors in the distribution of current patterns of biodiversity (Avise 2000).

The most commonly used molecular marker in animal phylogeography was and still is mitochondrial DNA.

In fact it is used for the identification of the different populations of wild animals and in reconstructing species' phylogeny. Analyses of mitochondrial DNA were used for example in several taxonomic studies (Kohn and Wayne, 1997, Rodriguez, 2004, Salgueiro et al., 2007).

The mitochondrial DNA genome is presents in many copies in each cell and thus amplify more frequently than nuclear genes (Kohn and Wayne, 1997, Lucchini et al., 2002); has simple molecular structure; absence of recombination, due to an exclusively maternal inheritance; and finally high variability, which means a higher rate of mutation than the

nuclear genome (Howell et al., 1996; Parsons, 1997; Denver et al., 2000; Evelyne et al., 2001; Lambert et al., 2002).

In Europe, the geographic distribution of genetic diversity is explained in the context of climate changes during the Pleistocene. That is, assuming that the glacial and interglacial cycles caused certain profound changes in the distribution of communities and individual species.

Paleoecological and molecular evidence suggest that populations of species adapted to temperate climates survived at the glaciations in the southern refuge areas (both in the Mediterranean region and in Eastern Europe), acquiring specific genetic characteristics. During interglacial periods, these populations recolonized the central and northern Europe, following routes of dispersion that can be reconstructed using analysis of specific genetic markers.

Phylogeographic analyses show that each species has its own history. However, it is possible to identify several "models" of mammals' phylogeography in Europe:

1) model of the southern refuges, exemplified by the case of the hedgehog, *Erinaceus europaeus* (Santucci et al., 1998);

2) model characterized by successive phases of colonization, starting from speciation areas in Eurasia, as in the case of chamois, Rupicapra (Perez et al., 2002);

3) model characterized by dislocation to North of entire populations that disappear completely from the previous refuge areas, as happened in some bat species and ungulates species having a northern distribution (Flagstad & Røed, 2003).

Finally there are cases of apparent lack of phylogeographic structure, such as, for example, in the case of the wolf and other species with a high potential of dispersion and gene flow (Villa et al., 1999).

1.3 NON INVASIVE GENETICS

Endangered populations are complicated to study due to their low densities and limited observations (Dalèn *et al.*, 2004). Conservation and management of wildlife populations require information on parameters such as population size, demography, gene flow, and population structure but these parameters are difficult to obtain for species that are rare or elusive such as carnivores (Creel *et al*., 2003).

Recent developments in molecular genetics have created new methods such as ‘Non-invasive Genetics’ or ‘Non-invasive Genetic Sampling (NGS)’, that have found many applications in ecology, and can resolve some problems of conservation biology. They allow populations to be studied and make a census (Frantz *et al.*, 2003; Broquet *et al*., 2007, Puechmaille et al. 2007) analysing DNA extracted from biological traces such as hairs (Goossens *et al*., 1998;

Flagstad *et al*., 1999; Woods *et al*., 1999; Sloane *et al*., 2000), faeces (Taberlet *et al*., 1996, 1999; Gagneux *et al*., 1997; Kohn & Wayne 1997; Kohn *et al*., 1999, Vege et al., 2001, Puechmaille et al., 2007) and less direct sources of cells (urine and blood traces on snow (Valiere & Taberlet, 2000), sloughed skins (Amos *et al*., 1992; Bricker *et al*., 1996), chewed food material containing buccal cells (Sugiyama *et al*., 1993; Takenaka *et al*., 1993), and bird feathers (Smith *et al*., 1992; Segelbacher, 2002) or egg shells (Pearce *et al*., 1997)).

Non-invasive genetic sampling was introduced about 15 years ago (Taberlet & Bouvet, 1991; Taberlet & Bouvet, 1992; Hoss *et al*., 1992) and consists in a set of fields, laboratory and analytical techniques that allow to study the biology of natural populations analysing DNA extracted from biological traces left by individuals and then collected without having (even) to observe, disturb or capture them (Kohn & Wayne, 1997). Conservation biologists in particular have shown a deep interest in these techniques, which are now routinely used in forensic genetics and for investigating the biology and the genetic diversity of elusive, rare and/or endangered species avoiding any risks to impact their survival, their recapture rates or their population dynamics (Kohn & Wayne, 1997; Piggott & Taylor, 2003).

The chief molecular tools used in NGS are mitochondrial DNA (mtDNA) sequencing (Höss *et al*., 1992; Farrell *et al*., 2000) and above all microsatellite *multilocus* genotyping (Palsbøll 1999; Taberlet & Luikart, 1999). They allow species identification and the characterisation of the genetic identity of individuals and their molecular sexing. Many mammal conservation genetic studies using NGS have been recently published, providing abundant information on population parameters, identification, conservation and management strategies of elusive, rare and endangered species (Tikel, Blair & Marsh, 1996; Reed *et al*., 1997; Kohn *et al*., 1999; Bayes *et al*., 2000; Ernest *et al*., 2000; Lucchini *et al*., 2002; Waits, 2004; Boulanger *et al*., 2004; McKelvey & Schwartz, 2004; Fabbri *et al*., 2007; Puechmaille et al., 2007).

1.4 THE ORDER CHIROPTERA

The order of bats contains 18 families of approximately 1,200 species, though this number is likely to be an underestimation given the high number of cryptic species in bats (Simmons 2005; Miller-Butterworth et al. 2007), The order constitutes the largest and most widely distributed groups of mammals, and their diversity is second only to the rodents.

There were bats flying in the night skies long before there were humans (Wimsatt 1970) and modern bats radiated long ago following the Cretaceous-Tertiary boundary (65 mya; (Teeling *et al.* 2005)). Chiroptera means hand (*cheiro*) and wing (*ptera*). Bat wings are folds of skin supported by elongated arm, hand, and finger bones, and attached to the sides of the body. In most cases, bats' thumbs are relatively free of the wing membranes and bear claws, which are absent from the fingers.

They are the only mammals capable of true (meaning, flapping) flight, a feat only matched by the birds and extinct Pterosaurus; because the other so-called flying mammals (for example, squirrels, lemurs, and sugar gliders) glide, they do not fly; and are unique among the animal kingdom for being the only lineage to have acquired a sophisticated form of laryngeal echolocation (MacDonald 2006).

Indeed it is due to these remarkable traits of flight and a complex echolocation ability that this order is supposed to have successfully colonized so many global niches (Jones & Teeling 2006). The order Chiroptera, in fact, has been successful in colonising almost every part of the world, apart from some isolated islands, the Arctic Circle and Antarctic.

Bats are mammals and as such have body hair, a single lower jaw bone, three articulated inner-ear bones, replacement of juvenile (or first) teeth, produce milk to feed their young and have the ability to maintain a constant body temperature (Hill & Smit 1984). After a generally prolonged gestation period bats typically give birth to a single pup or twins in some cases.

Although some bats have remarkable faces and behaviour, wings are the most conspicuous features of the flying bats. Upon landing, bats immediately fold their wings so they appear to shrink in size. Small size is another distinctive feature of bats. The smallest species is *Craseonycteris thonglongyai* (Hill 1974) which weighs only 2 g and it is also the world's smallest mammal. The largest species is the *Acerodon jubatus* with a weight of up to 1.5 kg.

Living species of bats are classified in two suborders: the Megachiroptera, Fig. 1.1 (flying foxes and their relatives, family Pteropodidae), which are the fruit- and flower-visiting bats of the Old World tropics, and the Microchiroptera, which include all the other bats, Fig. 1.2.

Figure 1.1 ***Pteropus alecto***

Figure 1.2 ***Pipistrellus pipistrellus***

The two groups are easy to distinguish. The Pteropodidae have doglike faces, simple ears, and most have claws on their second fingers. Microchiroptera (18 families) lack claws on their second fingers, do not look like dogs, and their ears (and related structures) are more

complex. The teeth of megachiropterans tend to be more specialized than those of microchiropterans (excepting vampire bats). Microchiropteran specializations for flight, particularly in the shoulder girdles, are more complex than those of megachiropterans.

A wide variety of ear forms exists. Within the bats the possession of a tragus is a common feature. This is a cartilaginous structure set at the base of the ear opposite the pinna and is implicated in audition of certain frequencies. The eyes of many bat species are very small, with members of Phylostomidae and Pteropodidae as notable exceptions. Bats are not blind but possess visual capabilities used in light-sampling behaviour, social interactions, recognizing landmarks for foraging or migratory purposes even in the echolocating lineages. The species with well developed eyes typically feed on fruit and thus vision is likely used in foraging and assessing ripeness of fruit.

The hind-limbs of bats are unusual in mammals in that the femur is rotated 180° so that the knee is pointed backwards. Generally, the hind limbs of bats are adapted for their roosting or perching behaviour (hanging upside down by the feet) where tensile forces predominate.

The facial features of bats reflect their remarkable diversity. Bats are also diverse in their selection of roosts, places they spend the day, and by the social systems that develop in these places.

Absent only from very remote oceanic islands, the high Arctic, and the Antarctic, bats are extremely widespread. For the most part, heterothermic species are the bats of temperate regions.

Some species of bats migrate hundreds of miles (kilometres) to avoid inclement seasons, but there is detailed knowledge in only a few cases. Schreiber's long-fingered bats (*Miniopterus schreibersi*) in Australia, noctules (*Nyctalus noctula*) in Europe, or Brazilian free-tailed bats (*Tadarida brasiliensis*) in parts of the New World are species whose seasonal movements have been documented by band recoveries.

Bats can be virtually found in every habitat available from rainforests to deserts, mountain forests to seaside. Bats have two basic habitat requirements: roosts, or places to spend the day or hibernate, and places to feed. The actual selection of roosting or foraging habitat depends on the species and the time of year.

Roosts of bats can be divided into four broad categories: hollows, crevices, foliage, and "other." Hollows, situations where the roosting bat hangs free by its hind feet, may be inside trees, rocks (caves, mines), buildings, or even birds' nests. An unexpected discovery was of round-eared bats (genus Tonatia, family Phyllostomidae) roosting in hollows in the bases of arboreal termite nests. Crevices and cracks, situations where the bats' venter is against one surface (and its back may be close to the other), occur in rocks, trees (under bark or in wood),

and buildings. Bats roosting in foliage may hang from branches of trees, or among or under leaves. The "other" category includes bats roosting in unfurled leaves or in tents.

Among mammals, flight is a behaviour unique to bats. Two other behaviours, echo locating and hanging upside down, are associated with bats, but are not their characteristic. Not all bats echolocate and not all bats use echolocation the same way. Furthermore, the echolocation calls of many species of bats are ultrasonic, which, by definition, is beyond the range of human hearing. But many species of bats echolocate with sounds readily audible to people.

Bats also use vocalizations in many social situations, and their social calls are often quite audible to people.

The combination of small size and high metabolic rates means that bats consume enormous quantities of food. Consumption of large amounts of food often means that bats eat a variety of prey species, whether insects or fruit.

Insectivore is the most common form of feeding behaviour among bats, but the order contains many other modes of feeding, including: nectar or pollen (nectar ivory), fruit (frugivorous), meat (carnivorous on frogs, fish and other bats) and blood (sang ivory; Nowak 1994). Frugivorous is the second most common feeding habit.

While some insectivorous bats may eat more soft (e.g., moths, flies) than hard (beetles, bugs) prey, there is little evidence of specialization by prey species. Insectivorous bats should not be thought as consumers of mosquitoes. However, some smaller species, for example, Bodenheimer's pipistrelle (*Pipistrellus bodenheimeri*) from the Middle East, are known to eat regularly mosquitoes.

Insect-eating is a recurring lifestyle in bats. While most species of insectivorous bats hunt flying insects and use echolocation to detect, track, and assess their targets, others (gleaners) take prey from surfaces such as foliage or the ground.

Gleaning bats eat more than flying insects, consuming a wider range of prey, including walking insects and arthropods that do not fly.

Aerial-feeding bats tend to take smaller prey than gleaning bats. Among bats, it is rare to find aerial-feeding species taking vertebrate prey. The only known exception is the greater noctule (*Nyctalus lasiopterus*) from southern Europe. For at least part of the year, this 1.7 oz (50 g) bat preys on migrating birds.

Many species of animal-eating bats hunt along the water surface. Called "trawlers," these bats (often in the family Vespertilionidae, the plain-nosed bats) have enlarged hind feet with which they gaff small fish (*Myotis vivesi, Myotis rickettii*). Other trawling bats do not have such large hind feet, but still take the occasional fish and even mosquito larvae. This list includes

Daubenton's bat, pond bats (*Myotis dasycneme*), long-fingered bats (*Myotis capaccinii*), and large-footed myotis (*Myotis adversus*).

Other bats eat frogs. Most is known about the fringe-lipped bat (*Trachops cirrhosus*, family Phyllostomidae) of the New World tropics.

Throughout the tropics, some species of bats get food from plants. Included on the menu are fruits, seeds, leaves, nectar, and pollen. In the Neotropics, the plant-visiting bats belong to the family Phyllostomidae. In the Old World tropics, the bats are in the family Pteropodidae. The two families show remarkable convergence in structure and behaviour.

The most infamous bats are the blood-feeding vampires. There are three species: vampire bats, hairy-legged vampires (*Diphylla ecaudata*), and white-winged vampires (*Diaemus youngii*), all in the family Phyllostomidae. These bats only eat blood they obtain by making shallow bites on a prey's skin.

In their life histories, bats are long-lived with low reproductive output. In the wild, individually marked bats (little brown bats and greater horseshoe bats [*Rhinolophus ferrumequinum*]) have survived more than 30 years, and females have the capacity to produce one young per year.

Female bats roosting in nurseries with hundreds or even thousands of others use a combination of spatial memory, voice, and odour to recognize their own young. This ensures that her young receives enough milk and maximizes its chances of survival. The challenge of recognizing her young depends upon the female situation.

Gestation periods in bats range from 60–100 days, and in most bats, fertilization follows copulation. Most species of bats are monestrus, with females having one reproductive event per year. Some tropical species are diestrus, have two reproductive events per year, and females in a few species (e.g., lesser-crested mastiff bats, *Chaerephon pumila*) may bear up to five young per year (one per oestrous cycle).

Some species of bats extend the time between mating (typically polygynous) and birth. Sometimes fertilization follows copulation, but development or implantation of the fertilized egg is delayed, extending the gestation period (e.g., California leaf-nosed bats, *Macrotus californicus*).

The other approach, known from plain-nosed bats and horseshoe bats, is to delay fertilization. In this case, females store sperm in the uterus after copulation.

About their evolution, it is assumed that bats evolved from nocturnal, arboreal, insectivorous animals that lived in forests. The combination of their small size, delicate skeletons, and the forest conditions make the ancestors of bats unlikely candidates for fossilization. There are no

fossils of animals that are part bat, part something else, but it is speculated that a shrew-like animal would be a good candidate as a remote ancestor of bats.

The phylogenetic relationships of the different groups of bats have been the subject of much debate. The traditional subdivision between Megachiroptera and Microchiroptera reflects the view that these groups of bats have evolved independently of each other for a long time, from a common ancestor that was already capable of flight (Grzimek 2004). This hypothesis recognized differences between microbats and megabats and acknowledged that flight has only evolved once in mammals (Grzimek 2004). Most molecular biological evidence supports the view that bats form a single or monophyletic group (Simmons et al., 2008).

Genetic evidence indicates that mega bats originated during the early Eocene and should be placed within the four major lines of micro bats (Grzimek 2004, Teeling et al., 2005).

The Chiropteran phylogeny based on molecular evidence is controversial because micro bat paraphyly implies that one of two seemingly unlikely hypotheses occurred. The first suggests that laryngeal echolocation evolved twice in Chiroptera, once in Yangochiroptera and once in the rhinolophoids (Teeling et al., 2000;). The second proposes that laryngeal echolocation had a single origin in Chiroptera, was subsequently lost in the family Pteropodidae (all megabats), and later evolved as a system of tongue-clicking in the genus Rousettus (Springer et al., 2001).

Analyses of the sequence of the "vocalization" gene, FoxP2 was inconclusive of whether laryngeal echolocation was secondarily lost in the pteropodids or independently gained in the echolocating lineages (Li et al., 2007). However, analyses of the "hearing" gene, Prestin seemed to favour the independent gain in echo locating species rather than a secondary loss in the pteropodids (Li et al., 2008).

In the 1980s, a hypothesis based on morphological evidence was offered that stated that the Megachiroptera evolved flight separately from the Microchiroptera. The so-called flying primates theory proposed that when adaptations to flight are removed, the Megachiroptera are allied to primates by anatomical features that are not shared with Microchiroptera (Pettigrew, 1986). One example is that the brains of mega bats show a number of advanced characteristics that link them to primates (Pettigrew et al. , 1989; Ichida et al., 2000).

Although recent genetic studies support the monophyly of bats, (Simmons et al., 2008) debate continues as to the meaning of available genetic and morphological evidence (Pettigrew et al., 2008).

For the most part, bats interact little with people although many species exploit human structures as roosts or feed in rich patches of food people create.

In fact most of the 1,000 or so species of bats have little to do with people and vice versa. A few fruit-eating bats impact economically as pests of commercial crops, and vampire bats may be responsible for spreading rabies among livestock. On balance, other bats pollinate plants that are ecologically (and sometimes economically) important, while still others disperse seeds and play a vital role in reforestation. Insect-eating bats consume vast quantities of insects every year, including some agricultural pests.

Although bats are occasionally harvested as human food, and may be important economically as pollinators or agents of reforestation, they are rarely exploited economically. One important exception is the harvesting of bat guano, an activity that may disturb bats. In many parts of the world, there is a long tradition of harvesting bat guano as fertilizer.

1.5 GENUS MYOTIS

With about 100 species (Koopman, 1994; Simmons, 2005) distributed throughout the world (Fig. 1.3), except in the polar regions, the genus *Myotis* represents one of the most diverse and successful radiations among mammals.

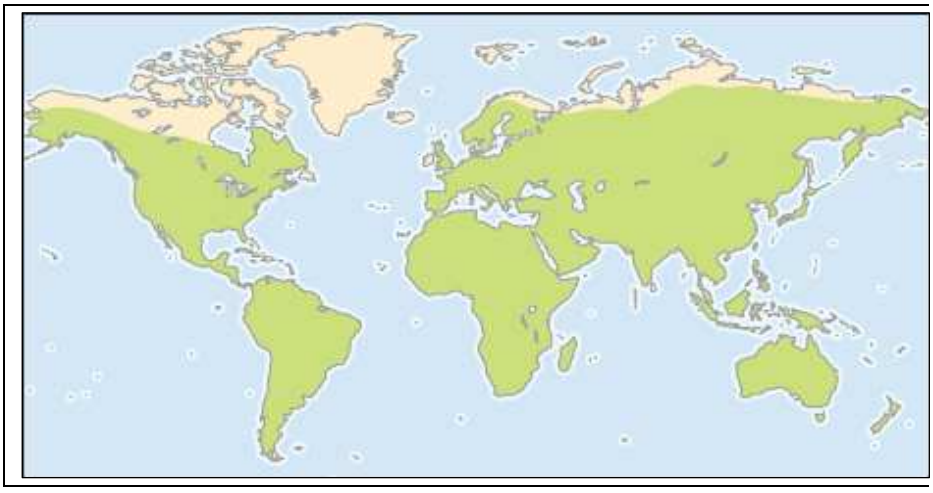


Figure 1.3 Distribution of genus Myotis, showed in green

Other distinctive feature of *Myotis* is a well developed tragus that reaches up from the base of the ear, and a nearly naked patagium, or flight membrane, that covers the relatively long tail. Myotis tails are commonly half as long as the body.

The Myotis = mouse-eared bats are especially noted for their wide distribution, and members of this genus are found al- most everywhere bats exist.

Given their nearly global distribution, it is not surprising that the habitat preferences of the Myotis vary greatly. While a large number of the temperate members inhabit caves during winter, their summer haunts can range from caves to woodlands, and riparian areas to deserts. A few species stay away from disturbed areas, but many will make use of cracks and crevices in human-made structures for roosts. Many species utilize tree hollows and loose bark for daytime roosts in the summer, with

various southern species even taking advantage of these spots for hibernation. A few more tropical species make use of vegetation for their roosts.

About behaviour, commonly, individuals awaken periodically during the winter. If the outside temperature is warm enough, they will travel outside of the hibernaculum, which may be a cave, attic, or tunnel, and look for food. Following hibernation, male bats in this subfamily will typically spend the summer alone, while females will group together in maternity colonies to bear and raise their young.

Mouse-eared bats generally return to the same summer and winter roosts, which may be as much as 125 mi (200 km) away from each other. Another change of roosts is occasionally made in summer or in winter. Roost changes are also typical of other Myotis species.

Most species begin mating in the fall, but very little is known about courtship behaviours. The little brown bat (*Myotis lucifugus*), which is one of the most well-known bats in the New World, engages in no courtship. Mating simply involves the male grasping the female by the nape of her neck during copulation, performed upside down. The two separate after copulation, often to find additional partners.

Vocalizations are used for communication and carry a variety of information. Echolocation behaviour is influenced by the presence of conspecifics. When bats hunt together, call duration decreases and call interval increases.

The bats in this group are almost exclusively insectivorous. Armed with echolocation capability, these skilled flyers are typically able to catch moths, beetles, flying ants, and other insects on the wing, but many will also glean leafhoppers, spiders, or other arthropods off of foliage. A few have expanded their diet to include fish or other vertebrates. Daubenton's bat (*Myotis daubentonii*) and *Myotis capaccinii* also occasionally eats fish.

Gestation averages about two months, with altricial young typically born in late spring or early summer. Litter size is commonly one or two pups, although some species produce more. During birth, most females turn right side up and catch the infant in the tail membrane. On average, females lactate for one to two months. During the same period, the young learn to fly from their mothers just three weeks after birth on average and begin to forage on their own. The young may become independent during the first year, or spend the winter with the family unit. They are often left in roosts with hundreds of other bats. Females of many species become sexually mature the first year, while males typically mature the following year. Compared to mammals of a similar size, bats can live a very long time. They average 10 years.

1.6 PHYLOGENETICS OF MYOTIS

The genus Myotis is an exceptional model for investigating speciation and diversification on a worldwide scale, considering its large distribution.

Because species have a rather undifferentiated morphology and often share many plesiomorphic characters (Menu, 1987), the taxonomic subdivision of that genus has been

difficult. Based on an extensive phenetic study of Eurasian and American species, Tate (1941) distinguished seven groups which he described as subgenera (*Selysius, Isotus, Paramyotis, Myotis, Chrysopteron, Leuconoe,* and *Rickettia*). Findley (1972) used numerical taxonomy on 48 cranial and external characters to classify most described species and retained only *Myotis, Selysius,* and *Leuconoe* as distinct subgenera. He also noticed that these three subgenera grouped species possessing a suite of related morphological traits which, supposedly, corresponded to three major modes of flight and food procurement. According to Findley (1972), the subgenus *Leuconoe* (type species *M. daubentonii*) is characterized by bats with relatively large feet, hairy legs, and a small plagiopatagium that typically forage on the water surface. Species of the subgenus *Selysius* (type species *M. mystacinus*) are usually smaller footed, with an enlarged uropatagium, and forage on aerial insect plankton. Species of the subgenus *Myotis* (type species *M. myotis*) are relatively larger animals with long ears, broad wings, and a more derived dentition (Menu, 1987) that are typically gleaners, catching their food on solid surfaces or even on the ground (Arlettaz, 1996). This subdivision into three major subgenera is currently widely accepted but it is entirely based on a limited suite of phenetic characters.

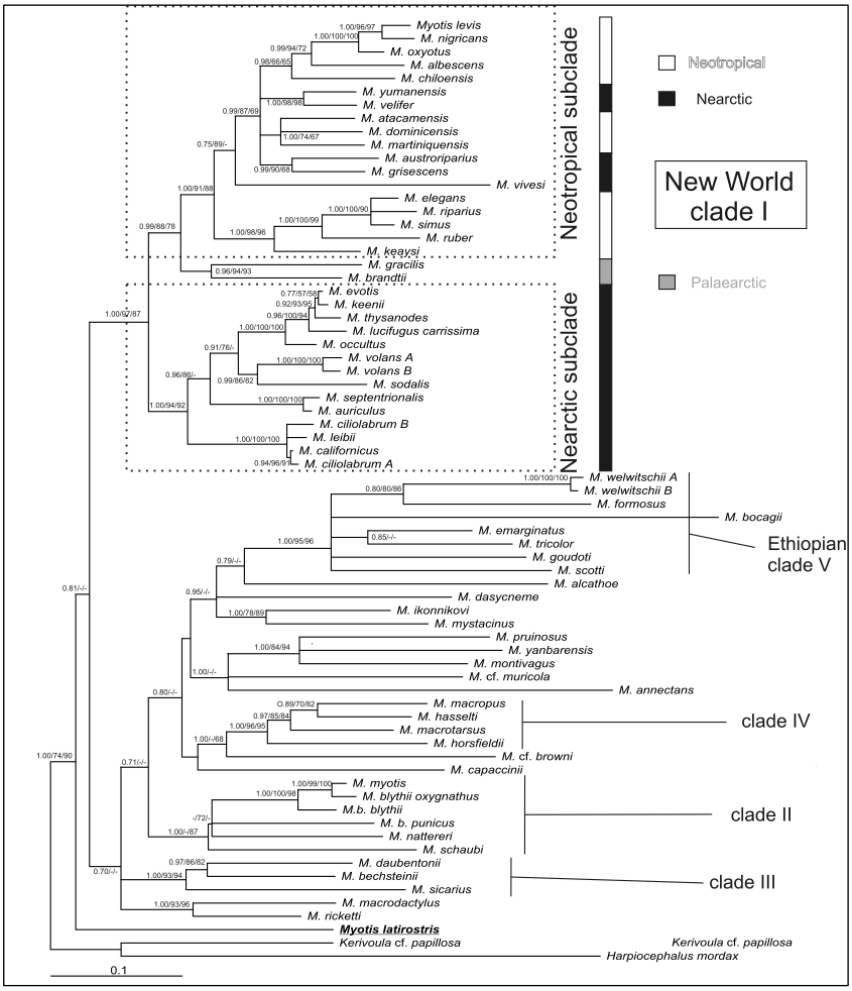


Figure 1.4 Phylogenetic relationships of Myotis species and two outgroup taxa obtained from CytB dataset

Several molecular studies (Hoofer and Van Den Bussche, 2003; Kawai et al., 2003; Ruedi and Mayer, 2001; Stadelmann et al., 2004a,b, Stadelmann et al. 2007), including one that explored relationships between external morphology and foraging behaviour (Fenton and Bogdanowicz, 2002) have shown that this current morphology-based subdivision of the genus Myotis into three subgenera (e.g., Findley, 1972; Koopman, 1994) does not reflect phylogenetic groupings, as already shown within the genus *Pipistrellus* (Barratt *et al.*, 1997), but rather represents adaptive convergences that produced the same ecomorphs independently through deterministic processes (Losos et al., 1998). Instead, the biogeographic evolution of this genus appears to include strongly imprinted phylogenetic relationships of current species. Indeed molecular studies have demonstrated that morphologically divergent species of Myotis, from the same continent tend to group into well supported clades (Ruedi and Mayer, 2001; Stadelmann et al., 2004a). Such biogeographic clades include one uniting all Ethiopian taxa (Stadelmann etal., 2004a) and another comprised of New World species (Hoofer and Van Den Bussche, 2003; Ruedi and Mayer, 2001) (Fig. 1.4).

In addition to these three classical subgenera, two rare South African species were regarded as belonging to a fourth subgenus, *Cistugo* (type species *M. seabrai*).

It was showed that the genus Cistugo fell basal to all vespertilionids, with a high genetic distance separating it from Vespertilionidae (Lack et al. 2010). Considering this, Cistugo should constitute a distinct family within Vespertilionoidea (Lack et al., 2010).

Finally, considering fossil records, the current prevailing opinion among palaeontologists is that the genus *Myotis* is an immigrant which invaded Europe, Africa, and the New World from another, yet unknown part of Asia (Findley, 1972; Menu, 1987).

1.7 LEGISLATION

Various international Agreements, ratified also by our country, have recognized the role of bats in ecosystem economy and the importance of their conservation in order to preserve biodiversity.

One of fundamental Agreements about bats is The Habitats Directive (92/43/EEC).

The Habitats Directive (together with the Birds Directive) forms the cornerstone of Europe's nature conservation policy. It is built around two pillars: the Natura 2000 network of protected sites and the strict system of species protection. All in all the directive protects over 1.000 animals and plant species and over 200 so called "habitat types" (e.g. special types of forests, meadows, wetlands, etc.), which are of European importance.

Bats are protected by Italian Law since 1939: the “Regio Decreto” of 5 June 1939, n. 1016, “Testo Unico delle norme per la protezione della selvaggina e per l’esercizio della caccia” states that “it is always forbidden to kill or capture bats from any species” (art. 38).

Nowadays, Italian bats are protected under the “Legge quadro in materia di fauna e attività venatoria” (L. 11 February 1992, n. 157), as well as under important international conventions signed by Italy (Bern Convention, 1979; Bonn Convention, 1979; Rio de Janeiro Convention, 1992); and, finally, under the “Habitats” Directive EEC/92/43. As part of the environment, bats are also protected by the Italian Law on the ‘environmental damage’ (L. 8 July 1986, n. 349).

Another important Act is the “Bat Agreement”, came into force in 1994 when it was ratified by a number of countries. Italy ratified the Agreement on 27th of May 2005 (GU n. 138, 16-6-2005 - S.O. n. 109) .

Under the Italian legal framework, it is forbidden to kill, capture, keep in captivity and trade bats; it is also forbidden to damage or disturb roosts and to disturb bats, especially during the mating season breeding period and when hibernating. Derogations to these prohibitions require a double authorisation, by the Ministry of Environment and by the local administration competent for the study area (regional, provincial or park administration). Both authorisations must be based on a technical opinion, which evaluates selectivity of methods and potential impact of the required activity on the conservation status of the affected population. The national legislation also calls the Ministry of Environment and the local administrations to monitor the conservation status of bats, and to regularly report these information to the European Community.

1.7 BATS IN ITALY

1.7.1 Summary Details of Resident Specie

Up to actual knowledge 37 bat taxa occur on the territory of Italy (Table 1.1). *Myotis dasycneme*, formerly included in the check-list of Italian bat species, is now classified as occasional as the only record available – a specimen captured in Trento – dates back to 1881; it is also worth mentioning that the occurrence of another species, *Rhinolophus blasii*, needs to be confirmed as it was not recorded after 1980.

Figura 1.5 ***Pipistrellus pygmaeus***

Figura 1.6 ***Plecotus macrobullaris***

Recent work (Kiefer and Veith, 2001; Spitzenberger et al., 2001, 2002; Chirichella et al., 2003; Mucedda et al., 2002) has described three new Plecotus species, all occurring in Italy: *P. macrobullaris* (Fig. 1.6), observed in the North of the country, and *P. sardus*, which is endemic to Sardinia.

The taxonomic status of Sardinian bats from the "*Myotis myotis* group" has been recently revised (Castella et al., 2000; Ruedi et al., 2001), proving that *Myotis punicus* occurs in the island. Russo and Jones (2000) showed that the cryptic species *Pipistrellus pipistrellus* and *P. pygmaeus* (Fig.1.5) both occur in Italy; and finally, a taxon from the "*Myotis mystacinus* group", named *Myotis aurascens* (Fig. 1.7) proposed as a good species by Benda and Tsytsulina (2000) is quoted for the country as a specimen recorded in Trentino but recent genomic analyses (Mayer & von Helversen 2001) suppose that *M. aurascens* really is not so separate from "*mystacinus*" group and other work is needed.

Figura 1.7 ***Myotis aurascens***

Generally speaking there is not a comprehensive national project to monitoring the trend of the bat population. Isolated historical data seem to confirm a general decrease of cave species, with some exceptions, but the data are scarce and widely distributed.

The main threats to bats in Italy come from loss of habitat, loss of roosting sites, pollution and use of pesticide in agricultural areas. The use of pesticides and agricultural intensification may be reducing prey abundance generally. Hedgerow and woods removal, conversion of dry grassland and river belt are reducing the connectivity and mosaic in the landscape.

Also some entrance in caves and disturbing of the maternal and hibernating colonies, renewal or demolition of many buildings with suitable roosts for bats and modern construction without access in attics and cellars.

	Taxa	Author	Italian name
1.	*Rhinolophus blasii*	(Peters, 1866)	Rinolofo di Blasius
2.	*Rhinolophus euryale*	(Blasius, 1853)	Rinolofo Eurìale
3.	*Rhinolophus ferrumequinum*	(Schreber, 1774)	Rinolofo maggiore
4.	*Rhinolophus hipposideros*	(Bechstein, 1800)	Rinolofo minore
5.	*Rhinolophus mehelyi*	(Matschie, 1901)	Rinolofo di Méhely
6.	*Barbastella barbastellus*	(Schreber, 1774)	Barbastello comune
7.	*Eptesicus nilssonii*	(Keyserling et Blasius, 1839)	Seròtino di Nilsson
8.	*Eptesicus serotinus*	(Schreber, 1774)	Seròtino comune
9.	*Hypsugo savii*	(Bonaparte, 1837)	Pipistrello di Savi
10.	*Myotis aurasces*	Kusjakin, 1936	Vespertilio dorato
11.	*Myotis bechsteinii*	(Kuhl, 1817)	Vespertilio di Bechstein
12.	*Myotis blythii*	(Tomes, 1857)	Vespertilio di Blyth
13.	*Myotis brandtii*	(Eversmann, 1845)	Vespertilio di Brandt
14.	*Myotis capaccinii*	(Bonaparte, 1837)	Vespertilio di Capaccini
15.	*Myotis dasycneme*	(Boie, 1825)	Vespertilio dasicnème
16.	*Myotis daubentonii*	(Kuhl, 1817)	Vespertilio di Daubenton
17.	*Myotis emarginatus*	(E. Geoffroy, 1806)	Vespertilio smarginato
18.	*Myotis myotis*	(Borkhausen, 1797)	Vespertilio maggiore
19.	*Myotis mystacinus*	(Kuhl, 1817)	Vespertilio mustacchino
20.	*Myotis nattereri*	(Kuhl, 1817)	Vespertilio di Natterer
21.	*Myotis punicus*	Felten, 1977	Vespertilio maggiore africano
22.	*Nyctalus lasiopterus*	(Schreber, 1780)	Nottola gigante
23.	*Nyctalus leisleri*	(Kuhl, 1817)	Nottola di Leisler
24.	*Nyctalus noctula*	(Schreber, 1774)	Nottola comune
25.	*Pipistrellus kuhlii*	(Kuhl, 1817)	Pipistrello albolimbato
26.	*Pipistrellus nathusii*	(Keyserling et Blasius, 1839)	Pipistrello di Nathusius
27.	*Pipistrellus pipistrellus*	(Schreber, 1774)	Pipistrello nano
28.	*Pipistrellus pygmaeus*	(Leach, 1825)	Pipistrello pigmeo
29.	*Plecotus auritus*	(Linnaeus, 1758)	Orecchione bruno
30.	*Plecotus austriacus*	(Fischer, 1829)	Orecchione grigio
31.	*Plecotus macrobullaris*	Kuzjakin, 1965	Orecchione alpino
32.	*Plecotus sardus*	Mucedda et al., 2002	Orecchione sardo
33.	*Vespertilio murinus*	(Linnaeus, 1758)	Seròtino bicolore
34.	*Miniopterus schreibersii*	(Kuhl, 1817)	Miniottero
35.	*Tadarida teniotis*	(Rafinesque, 1814)	Molosso di Cestoni

Table 1.1 List of bats species in Italy

1.8 STUDIED SPECIES

1.8.1 *Myotis emarginatus*

The Geoffroy's bat *Myotis emarginatus* is mainly present in southern, south-eastern and central Europe (Červerný, 1999) and is often recorded from northern Spain (Quetglas, 2002; Flaquer et al., 2004). It is also currently distributed from North Africa to Central Asia. Regarding Italy, all regions are included in the area (Fig. 1.8).

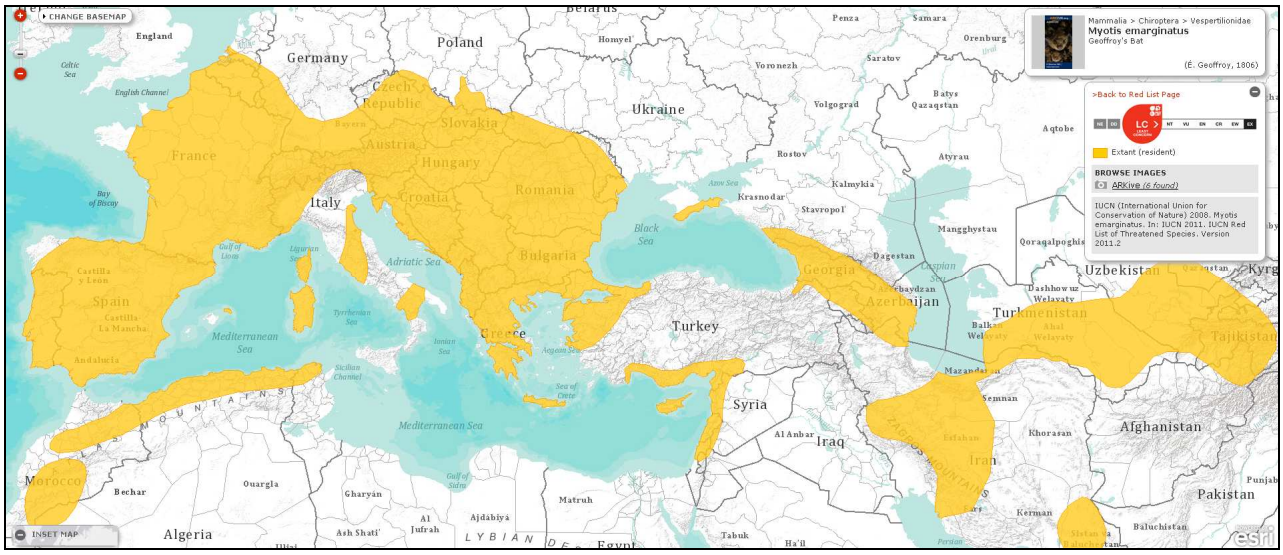


Figure 1.8 Distribution of *Myotis emarginatus*

Its biometrical parameters are the following: LTT 41-54 (58) mm; LCo (34) 38-46 (48) mm; AV 36-41 (43,5) mm; LO (14) 16-17 mm; LT 8-10 mm; AA 220-250 mm; Lcb 14-16 mm; FdC-M36-6,8 mm; P (5,9) 7-15 g.

Myotis emarginatus (Fig. 1.9) is a species mostly sedentary, longest movement documented is 160 km. (Schunger *et al.* 2004 in Hutterer *et al.* 2005), but may in fact move longer distances as winter roosts are not known in parts of its range where it occurs in summer.

Figure 1.9 The Geoffroy's bat *Myotis emarginatus*

Reported from sea level to about 1,800 m, it prefers areas of low or medium altitude, with mild climate, highest records in the Alps are 812 m (maternity colony) and 1,505 m (hibernaculum) (Spitzenberger 2002).

Thermophilic also in the choice of summer sites refuge, especially in the north of its areal, in order to use extremely hot attics of buildings, while at the South is often observed in cavities underground. Breeding colonies are also reported for tree cavities.

Generally roosts in summer with *Rhinolophus* species. It winters in underground sites. In Iran and the Caucasus, the species occurs in a variety of habitats, but in low numbers (M. Sharifi pers. comm. 2005).

Matings occur in the autumn, is not known whether occur even in winter.

Breeding colonies consisting mainly of 20-200 females, sometimes more conspicuous, up to maximum of 1,000 females (the male lead separate lives in this period). Births occur in June/July: only one young, exceptionally two. Females can breed already at the first year of life, but there is no evidence that the can give birth at the end of the year.

The maximum recorded age for this species is 18 years.

Its diet it is based mainly on diurnal Diptera (flies) and arachnids, which captures while they are on vegetation, on the walls of the stables or on the ground. However, it hunts, even in flight, generally within 5 m from the ground, sometimes on the water or under the lights, capturing various types of insects (Neuroptera, Diptera, Hymenoptera, Lepidoptera, Coleoptera)

In spite of the conservation value of this taxon – listed under Annexes II and IV of the Habitats Directive — few studies have focused on its habitat selection and foraging strategies. However the information available on *M. emarginatus* for aging preferences outside the Mediterranean highlights the fact that the species is mainly a forest forager (Krull et al., 1991; Demel et al., 2004). Its relatively low wing-load and aspect ratio values are both suited to slow, manoeuvring flight (Norberg and Rayner, 1987). Moreover, its specialisation in forest foraging is clearly related with the design of its echolocation call (Siemers and Schnitzler, 2004). It was confirmed that forest is a main foraging habitat for this species (Flaquer et al. 2008), including conifers which ranked first among habitats used by juveniles and proved important for adults too. Besides, in a typical Mediterranean landscape we observed that olive groves turned out to be an important foraging habitat for both adults and juveniles. These findings confirm the importance of this land-use type for Mediterranean bat populations (Davy et al., 2007), especially where traditional farming methods have created a complex mosaic of olive and deciduous woodland (Russo et al., 2002).

1.8.2 *Myotis capaccinii*

Myotis capaccinii (Fig. 1.11) (Bonaparte, 1837) is a Mediterranean troglophylous, cave-dwelling species (Papadatou et al. 2008) medium- sized species classed among the trawling bats (Guillén, 1999; Spitzenberger and Helversen, 2001).

Myotis capaccinii has a distribution spanning Algeria and Tunisia in northern Africa, and with Spain as its western border in Europe, extends into Iran and south-western Asia in the east (Koopman 1994). Although Corbet (1978) and Spitzenberger & Helversen (2001) stated that the species is monotypic, other authors recognize two subspecies (Ellerman & Morrison-Scott 1951, 1966; Koopman 1994). The suture zone between these two subspecies is considered to pass through south-eastern Europe. Specifically, *M. c. capacinnii* is recognized from north-western Africa to Yugoslavia and *M. c. bureschi* (Heinrich 1936) (terra typica: Karamlek, Stiranca Mountains: Bulgaria) from Bulgaria to Iran (Kumerloeve 1975; Deblase 1980; Harrison & Bates 1991) (Fig. 1.10).

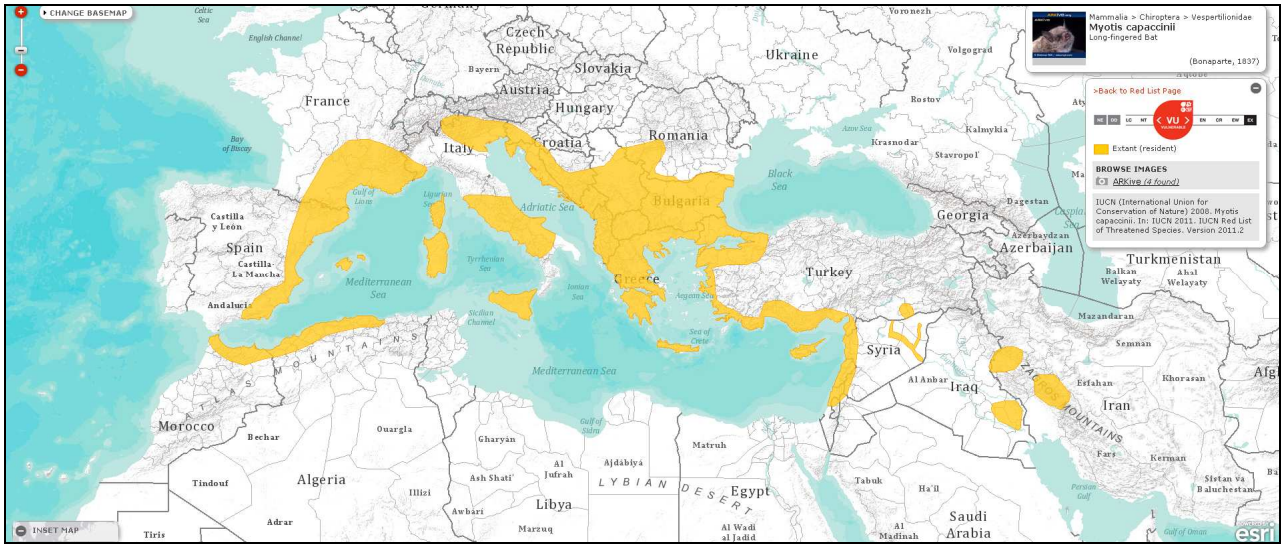


Figure 1.10 Distribution of *Myotis capaccinii*

Its biometrical parameters are the following: LTT (43) 47-53 (54) mm; LCo (34) 35-38 (42) mm; AV (37) 39-44 mm; LO 14-16 mm; LT 6,5-7,5 mm; AA 230-260 mm; Lcb 12,9-15 mm; FdC-$M^3$5,4-6 mm; P 6-15 g

Movements between summer and winter colonies are mostly within a distance of 50 km (maximum 140 km: Hutterer *et al.*2005). Breeding colonies usually consist of 100-1000 females, but in Albania was found a nursery with about 10,000 females. It gives birth in May-June: normally only one young, exceptionally two.

Figure 1.11 The Long-Fingered Bat (*Myotis capaccinii*)

Traditionally, it has been linked to inland waters, and several observations of hunting over water (usually calm) have been described (Ahlén, 1990; Kalko, 1990; Spitzenberger and Helversen, 2001). Although in Europe it is widely considered an endangered species (Hutson *et al*., 2001), there is little knowledge about its ecology, similarly to other Mediterranean bats (Russo and Jones, 2003). However, it is proved that it forages mainly on aquatic habitats (Almenar et al. 2006, Biscardi et al. 2007), where it takes prey directly from or above the water surface (Kalko 1990, Almenar et al. 2008). The large hind feet and pointed wings of *M. capaccinii* are well suited to capture prey while trawling through the water surface. This species is known to consume fish in the wild (Aihartza *et al*., 2003) (Fig 1.12).

Figure 1.12 *Myotis capaccinii* catching through the water surface

In suboptimal habitat conditions, the species would possibly be forced to change its behaviour (Goiti *et al*., 2003). The best-known species of trawling bats are able to exploit niches other than water, using different foraging strategies (mainly aerial hawking), i.e., *Myotis daubentonii* (Jones and Rayner, 1988; Arnold *et al*., 1998), *M. adversus* (Jones and Rayner, 1991), and *Noctilio albiventris* (Kalko *et al*., 1998).

Three putative factors might explain the observed selection behaviour of *M. capaccinii*: the water surfaces' acoustical proper- ties, the distribution of different kinds of water surfaces, and prey availability (Almenar et al.2006).

Lakes have been also noted as extensively occupied by the species (Russo and Jones, 2003).

Myotis capaccinii is able to cover long distances to reach its foraging sites as are other trawling species (Arnold *et al*., 1998; Barclay *et al*., 2000). Recorded average and maximum distances are high compared to other bats with similar wing shapes (Jones *et al*., 1995). The maximum straight line distances between roost and the farthest foraging fixes recorded on one night for Italian *M. capaccinii* were about twice those recorded in Spain (Almenar et al.,

2006), averaging 3.7km per night with maximum values of 9.9 km (Almenar et al. 2008).

As observed elsewhere (Almenar et al., 2006; Levin et al., 2006), the species foraged and commuted almost exclusively along linear water habitats. Riparian vegetation, also used by other, less specialized bats (Racey, 1998), is certainly far more important for water habitat specialists such as *M. capaccinii*. The considerable distances travelled require the protection of long stretches of rivers and associated riparian vegetation around roosts. Aquatic habitats are usually arranged in a linear pattern, or scattered along the landscape, so *M. capaccinii* should be able to reach distant places, especially if available prey is not evenly distributed throughout these habitats. This ability allows the species to exploit vast areas around its main caves.

Myotis capaccinii preferred higher water quality. Pollution by micro-contaminants is a factor of concern for another trawling species, *M. dasycneme* (Reinhold et al., 1999), whereas eutrophication has no effect – or even positive effects – on *M. daubentonii* (Racey et al., 1998). *M. capaccinii* mainly preyed upon chironomids.

Piscivory in *M. capaccinii*, also known for Spain and Israel (Aihartza et al., 2003; Levin et al., 2006), should be regarded as a generalized strategy of this species. Based on a limited sample (eight droppings from four bats), Aihartza et al. (2003) found that fish occurred in 100% of droppings, whereas we observed it with a much lower frequency. As in Spain (Aihartza et al., 2003), *M. capaccinii* caught cyprinids.

Remarkably, *M. capaccinii* also occurs on Mediterranean islands where water habitats are scarce (e.g. the Ionian island of Petala; Hanak et al.,2001)

1.8.3 Threats on *Myotis emarginatus* and *Myotis capaccinii*

Typically, 3 main threats are recognised as affecting bats: pesticide contamination, roost disappearance or disturbance, and alteration of foraging areas (Hutson et al. 2001, Racey & Entwistle 2003).

M. emarginatus, locally, can be rare or common. The species experienced a significant decline from the 1960s to the 1990s, but in recent time the numbers in several regions have increased and the species has spread into new areas. It lives in large colonies (up to 1,200 individuals in Austria in one maternity colony).

The species experienced a significant decline in at least parts of its range from the 1960s to the 1990s, but now it is expanding in central Europe and is stable or not significantly declining elsewhere. The range is still large and the species is not specialized to restricted habitat, although it has a very specialized diet. It has assessed as Least Concern by IUCN Red List .

Because in Europe the species is mainly associated with agricultural landscapes, therefore all

agricultural activities can affect populations of this species. Loss of and disturbance to roost sites in buildings (including remedial timber treatment in attics) and underground sites are also threats.

The abandoning of the land also poses a threat. Although in some cases — depending on the initial landscape structure — agricultural abandonment may temporarily increase landscape heterogeneity and its potential for biodiversity (Mac Donald et al., 2000), it will often finally lead to landscape homogenisation and have detrimental effects on animal diversity (Russo, 2007). Juveniles proved to be less mobile than adults, moving on average 1.6 km less than adults to reach foraging sites (Flaquer et al., 2008). In that sense it is recommend to protect forest areas and traditionally farmed olive groves at least into a 2 km buffer from the main roost.

Although conifers represented an important foraging habitat for *M. emarginatus*, it has to remark that artificial conifer woodland planted in areas where this habitat is non-native may have a negative impact on other Mediterranean bats foraging in treed habitats such as *R. euryale* (Russo et al., 2002), so caution is required when planning reforestation in programs implemented to improve the landscape for bats.

Factors such as large-scale wildfires, also frequent in the Mediterranean, pose a threat to this species since they lead to significant loss of preferred habitat. Finally, a study highlighted (Flaquer et al. 2008) that *M. emarginatus* tends to avoid urban areas for foraging, a fact which should be considered carefully even in Mediterranean parks, where expanding urbanisation is not rarely still a problem in some territories in spite of the legal protection status.

In the African part of the range, instead, cave habitat where the species roosts is being destroyed by fires and vandalism. The species is also collected for traditional medicine practices in North Africa.

It is protected by national legislation in most range states. There are also international legal obligations for its protection through the Bonn Convention (Eurobats) and Bern Convention in the range states where these apply. It is included in Annex IV of EU Habitats and Species Directive, and there is some habitat protection through Natura 2000.

Protection of roosts and promotion of awareness about the lack of medicinal value of the species is required.

M. capaccinii is Vulnerable (Hutson et al. 2001, 2008) and its populations in western Europe are especially threatened (Guillén 1999).

Locally it can be abundant. Generally, the population is fragmented, but these "fragments"

may constitute robust parts of the overall population. Declines have been reported in many range states. In Spain, the population has declined by 30-50% in the last 10 years to fewer than 10,000 individuals. Only 30 colonies are known that comprise more than 20 individuals (Palomo and Gisbert 2002). At least six important colonies are threatened by the construction of buildings nearby and five colonies have disappeared over the last 10 years. In France the population has declined to very low numbers (an estimated 3,800 individuals). Colonies have been lost in the western part of the range in the last 15 years (S. Aulagnier pers. comm. 2006). Colonies in central Romania known from the 1960s have disappeared, and the species is now restricted to the south. The species is almost absent in winter and probably hibernates in Bulgaria (Z. Nagy pers. comm. 2006). The Bulgarian population is estimated at c.20,000. In Croatia there are still some large colonies but these are threatened by pollution of karstic water bodies (F. Spitzenberger pers. comm. 2006), and the species is listed as Endangered in the Croatian Red Book of Mammals (Tvrtkovic 2006). In Turkey it has a decreasing population and is considered vulnerable; it is most often encountered in small groups, very occasionally up to several hundred individuals (A. Karatas pers. comm. 2005). The species is naturally rare in Iran (M. Sharifi pers. comm. 2005) and north Africa (S. Aulagnier pers. comm. 2006). The size of colonies is smaller in the western part of the range (several hundreds of individuals in summer) than in the eastern part (up to several thousands in winter).

Myotis capaccinii strictly depends on underground shelters (Papadatou et al. 2008) and the alteration or disturbance of breeding roosts has resulted in local extinctions (Almenar et al. 2007). Guillén (1999) proposed that disappearance of foraging habitat and roosts is the main cause of regression for the species in western Europe. Médard & Guibert (1990) suggested that the alteration of Mediterranean rivers has been a major cause of its regression in France. The effects of pesticides have not been examined for *M. capaccinii* but are thought to be deleterious, as for other species (e.g. Clark et al. 1988, Gerell & Lundberg 1993, Guillén et al. 1994, Clark 2001). In fact, its typical prey (chironomids) are known to accumulate toxic compounds (Reinhold et al. 1999).

For cave-dwelling bats, conservation strategies that focus on the protection of roosts are feasible as long as direct threats by human activities are correctly identified. Areas of the landscape suitable for future protective actions can easily be identified, need relatively low resource investment, and raise few socioeconomic disputes. On the other hand, more accurate information is needed before protection of foraging habitat can be implemented into bat conservation strategies. Because cave-dwelling bats usually use a large area around the roost (e.g. Adam et al. 1994, Russo et al. 2002, 2005, Goiti et al. 2006) and limitation of human

activities over vast land extensions is seldom possible, conservation guidelines should be as precise as possible. Comprehensive information about key habitat features and actual foraging ranges is therefore necessary for the optimisation of conservation measures applicable to foraging areas.

It is protected by national legislation in most range states. There are also international legal obligations for its protection through the Bonn Convention (Eurobats) and Bern Convention in the range states where these apply. It is included in Annex II (and (IV) of EU Habitats and Species Directive, and hence requires special measures for conservation including designation of Special Areas for Conservation. Some habitat protection through Natura 2000. In Spain, fences are in place to protect several known colonies. Measures needed include protection of colonies (these measures should avoid the blocking of any cave entrances with gates and control of tourist access) and improvement of water quality.

1.9 GENETIC APPLICATION ON BATS

The use of genetic methodologies to answer conservation-related questions makes sense intuitively. DNA is the basic unit of evolutionary change and is moulded by processes that leave characteristic 'signatures' that can accumulate over time, thereby recording the evolutionary history of a species or population. Thus, we can use DNA as a tool to infer the historical perturbations that have impacted a species or population and explain their current distribution and genetic diversity. We can then use this information to predict the response of a species or population to future pressures such as environmental change, habitat loss and population decline (Loeschcke et al. 1994).

Bats have major cultural importance, especially in Asia. But perhaps the greatest significance of bats to humans stems from their insectivorous diet. A single vespertilionine, like one of genus Myotis, can eat thousands of insects a night, and many of these arthropods are seen as pest species. For instance, bats help control populations of mosquitoes, flies, moths, beetles, and other insects. While decreases in fly or mosquito populations are readily seen as a benefit for all humans, unchecked populations of beetles, moths, and other pest species can cause significant damage to agricultural crops and forests.

In addition, many vespertilionine bats are very susceptible to pesticides and other chemicals, making them good as bio-indicators. These bats carry rabies and other zoonotic diseases of concern to humans.

Following previous considerations, conservation of bats has a high priority. Genetic approaches can be very useful to identify critical situations and develop actions to preserve and protect colonies. In fact during the last twenty years many studies about bat populations have been carried out to investigate their biology, distribution range, size, phylogeography

and possible hybridation among different bat species (Ruedi et al, 2001, Stadelmann et al., 2004).

Other studies regard the use of DNA barcoding, that can aid conservation and research by assisting field workers in identifying species, by helping taxonomists determine species groups needing more detailed analysis, and by facilitating the recognition of the appropriate units and scales for conservation planning.

Knowledge about the genetic structure of bat populations, could be useful also to find solutions for bat disease, like White Nose Syndrome (WNS). It is a poorly understood disease associated with the deaths of more than a million bats (Blehert et al., 2009). The condition, named for a distinctive fungal growth around the muzzles and on the wings of many affected animals, was first identified in a cave in Schoharie County, New York, USA, in February 2006 (Blehert et al., 2009). Objectives in that case, are not only to identify and understand the causes of WNS, but also to try to mitigate its impact, particularly on the most severely affected species. These actions need to well-understand the population structure and study the impact the population decline is likely to have on the genetic health of the populations.

Another important point is the need, with the increasing attention paid to biodiversity and conservation, new to apply new methodologies necessary to study species, particularly elusive and endangered ones, as bats. Bats are difficult to catch mainly because of their small size, high vagility and nocturnal lifestyle (O'Shea, Bogan & Ellison 2003), thus it's important the use of non-invasive sampling for population genetic analysis (Puechmaille et al. 2007).

Before this study, only one paper concerning these two species has been published, that is about the genetic structure of *M. capaccinii* in Eurasian transition (Bilgin et al, 2008). However there weren't any work about *M. emarginatus* population structure, in fact previous papers about this species concerned only ecological aspects (Flaquer et al. 2008) and phylogenetics relationships (Ruedi et al., 2001, Stadelmann et al., 2004, Stadelmann et al, 2007) with other Myotis species.

M. capaccinii's mitochondrial DNA (mtDNA) analyses indicated that between Anatolia and Europe there could be a suture zone similar to those recorded in other animal species, including bats, suggesting the association of more than one refugium with the region (Bilgin et. al 2008). Contrary to most of the other species where a suture zone was seen in Anatolia, for *M. capaccinii* the geographical location of the genetic break was in south-eastern Europe. This mitochondrial differentiation was not reflected in the nuclear microsatellites, however, suggesting that the lack of contact during the ice ages did not result in reproductive isolation.

Hence taxonomically, the two mitochondrial clades cannot be treated as separate species (Bilgin et al. 2008).

Regarding *M. emarginatus*, it is interesting to notice that all molecular reconstructions placed it and another non Ethiopian species (*Myotis formosus*) within the African radiation (Stadelmann et al., 2004, Stadelmann et al, 2007). Their phylogenetic position in this Ethiopian clade suggests that they are derived from two African ancestors that secondarily colonized Eurasia (Stadelmann et al., 2004)

1.10 AIMS OF THE THESIS

The main purposes of this conservation genetic study on these two bat species are:

- Evaluating phylogeographic connection between different colonies of the two species.
- Studying of population structure across species range.
- Investigating population structure in Italy
- Developing a NGS methods to analyze genetic population structure.
- Considering previous *M. capaccinii* results on population structure, verify if the high values of differences discovered in Eurasian Transition could be confirmed using a larger dataset.
- Verifying and evaluating genetic differences between *M. emarginatus* from African Mediterranean coast and European individuals of same species.
- Verifying eventual taxonomic implication of our results
- Using new markers, such as intron primers, develop a more reliable phylogeography of these two species (Internship at UCD, University College Dublin).

CHAPTER 2

MATHERIALS AND METHODS

In this study all the genetic analyses were performed at the Laboratory of genetics, Italian Institute for Environmental Protection and Research (ISPRA).

2.1 SAMPLING COLLECTION LOCALITIES

In this study, we collected 895 samples from 32 colonies of *M. emarginatus* and 17 of *M. capacinii*, using both invasive and non invasive sampling procedures. Most of these colonies were found in historically known Italian locations (caves, mines or attics) and were sampled in the years 2009-2010 (Figure 2.1, Table 2.1-2.4). Some of these locations are classified as European Union Site of Community Importance (Directive 92/43/CEE, 1992): Agliè (cod. IT1110047), Venaria (cod. IT1110079 - La Mandria Regional Park). More precisely we collected 687 samples from Northern to Southern Italy, included Sardinia. Other 208 samples (Fig. 2.2) were obtained in 2010 and 2011 by different foreign groups, Spain and Moroccan samples from "Estation Biologica de Dognana", Greek samples from "University of Leeds", Turkish from "Istanbul Univeristy" and Iranian samples by "Natural History Museum of Genève".

COUNTRY	REGION	COLONY	SAMPLING	SPECIES	N° SAMPLES
ITALY	LOMBARDIA	CAMPO DEI FIORI	INVASIVE	*M. emarginatus*	35
ITALY	LOMBARDIA	LAVENO MONBELLO	INVASIVE	*M. emarginatus*	7
ITALY	LOMBARDIA	PAVIA	INVASIVE	*M. emarginatus*	16
ITALY	FRIULI	CORMONS	INVASIVE/NON INVASIVE	*M. emarginatus*	4/20
ITALY	TRENTINO-ALTO ADIGE	GARGAZZONE	INVASIVE	*M. emarginatus*	20
ITALY	TRENTINO-ALTO ADIGE	MERANO	INVASIVE	*M. emarginatus*	15
ITALY	TRENTINO-ALTO ADIGE	SAN SIGISMONDO	INVASIVE	*M. emarginatus*	20
ITALY	PIEMONTE	TRINITA	INVASIVE/NON INVASIVE	*M. emarginatus*	8/43
ITALY	PIEMONTE	TETTI PESIO	NON INVASIVE	*M. emarginatus*	17
ITALY	PIEMONTE	VENARIA REALE	NON INVASIVE	*M. emarginatus*	58
ITALY	PIEMONTE	BRICHERASIO	NON INVASIVE	*M. emarginatus*	37
ITALY	PIEMONTE	PASSERANO	NON INVASIVE	*M. emarginatus*	35
ITALY	PIEMONTE	REVELLO	NON INVASIVE	*M. emarginatus*	40
ITALY	PIEMONTE	PAPERIA	NON INVASIVE	*M. emarginatus*	40
ITALY	PIEMONTE	RACCONIGI	NON INVASIVE	*M. emarginatus*	27
ITALY	PIEMONTE	CRISSOLO	INVASIVE	*M. emarginatus*	30
ITALY	EMILIA-ROMAGNA	TERRA DEL SOLE	INVASIVE/NON INVASIVE	*M. emarginatus*	13/20
ITALY	TOSCANA	VAL D'ARNO	INVASIVE	*M. emarginatus*	20
ITALY	TOSCANA	PARCO VILLA DEMIDOFF	INVASIVE	*M. emarginatus*	20
ITALY	TOSCANA	TENUTA DI SAN ROSSORE	NON INVASIVE	*M. emarginatus*	25
ITALY	MARCHE	ESANATOGLIA	INVASIVE	*M. emarginatus*	9

COUNTRY	REGION	COLONY	SAMPLING	SPECIES	N° SAMPLES
ITALY	LAZIO	VILLA DOMIZIANO	INVASIVE	*M. emarginatus*	15
ITALY	SARDEGNA	CONCA ZIO STEVANEDDU	INVASIVE	*M. emarginatus*	8
ITALY	SARDEGNA	CASA PORTO FERRO	INVASIVE	*M. emarginatus*	8
SPAIN	JEREZ	JEREZ DE LA FRONTERA	INVASIVE	*M. emarginatus*	12
SPAIN	CORDOBA	CORDOBA	INVASIVE	*M. emarginatus*	10
SPAIN	LA CORUNA	LA CORUNA	INVASIVE	*M. emarginatus*	2
SPAIN	GRANADA	ARENAS DEL REY	INVASIVE	*M. emarginatus*	12
SPAIN	LA RIOJA	LA RIOJA	INVASIVE	*M. emarginatus*	2
MOROCCO	AZROU	DI`OUM-ER-RBIA	INVASIVE	*M. emarginatus*	10
MOROCCO	TETUOAN	OULED ALI MANSOUR	INVASIVE	*M. emarginatus*	13
TUNISIA		FEIDJA NATIONAL PARK	INVASIVE	*M. emarginatus*	10

Table 2.1: list of *M. emarginatus*'s colonies sampled

TOTAL SAMPLES	INVASIVE	NON INVASIVE
681	319	362

Table 1.2: resume of *M. emarginatus* 2009-2011 sampling

COUNTRY	REGION	COLONY	SAMPLING	SPECIES	N° SAMPLES
ITALY	LOMBARDIA	LIERNA	INVASIVE	*M. capaccinii*	16
ITALY	PIEMONTE	ISOLA BELLA	NON INVASIVE	*M. capaccinii*	46
ITALY	LAZIO	FONDI	INVASIVE	*M. capaccinii*	5
ITALY	SARDEGNA	GROTTA SU COLORU	INVASIVE	*M. capaccinii*	7
ITALY	SARDEGNA	GROTTA SA ROCCA ULARI	INVASIVE	*M. capaccinii*	3
GREECE	GRECIA	KOUFOVOUNO	INVASIVE	*M. capaccinii*	20
GREECE	GRECIA	MARONIA	INVASIVE	*M. capaccinii*	20
SPAIN	ALMERIA	PULPI	INVASIVE	*M. capaccinii*	12
SPAIN	HUESCA	ALCAMPELL	INVASIVE	*M. capaccinii*	10
SPAIN	VALENCIA	NAQUERA/ROTOVA	INVASIVE	*M. capaccinii*	10
SPAIN		SERON	INVASIVE	*M. capaccinii*	2
SPAIN	MALLORCA	COVA SANT MARTI	INVASIVE	*M. capaccinii*	15
TURKEY	BALIKESIR	BOGAZPINAR	INVASIVE	*M. capaccinii*	7
TURKEY	BALIKESIR	NUSRET DUDENI	INVASIVE	*M. capaccinii*	8
TURKEY	SARIHIDIR	NEVSEHIR	INVASIVE	*M. capaccinii*	28
TURKEY	INSUYU MANGARASI	BURDUR	INVASIVE	*M. capaccinii*	2
IRAN	IRAN		INVASIVE	*M. capaccinii*	3

Table 2.2: list of *M. capaccinii*'s colonies sampled

TOTAL SAMPLES	INVASIVE	NON INVASIVE
214	168	46

Table 2.3: resume of *M. capaccinii* 2009-2011 sampling

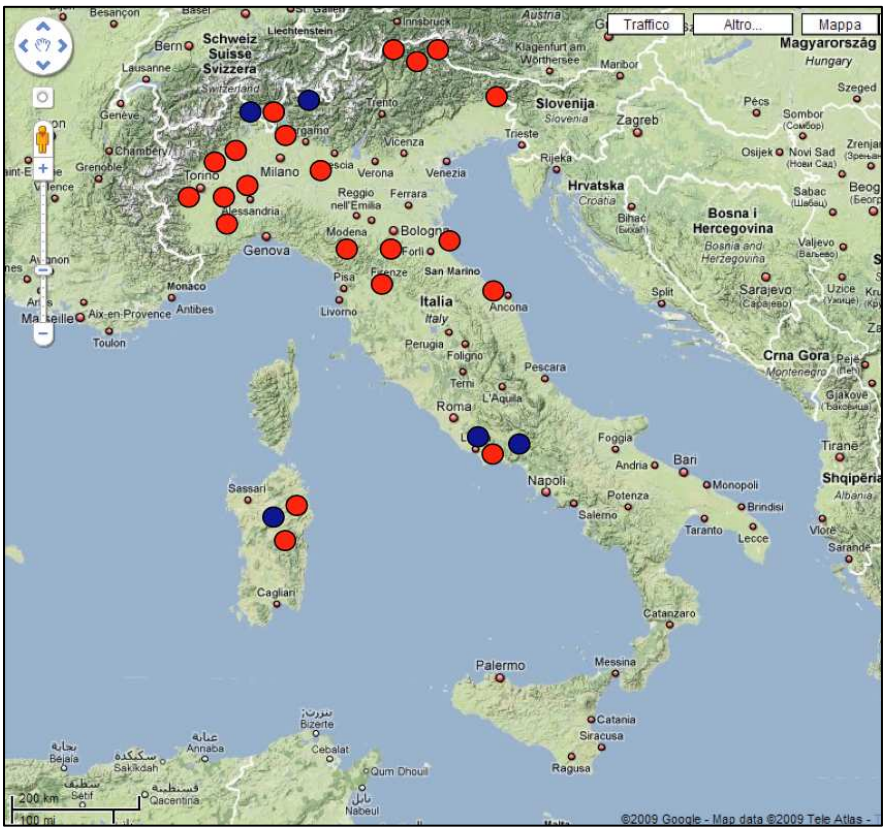


Figure 2.2: map of sampled Italian colonies. Red spots represent *M. emarginatus* colonies, while blues *M. capaccinii* ones

Figure 2.3: map of the European colonies. Red spots represent *M. emarginatus* colonies, while blues *M. capaccinii* ones

To exclude stress and nuisance to the animals, sampling was conducted during the post-reproductive seasons (Agnelli et al. 2004). In fact the catch at the roosts are, in general, delicate operations, especially when there is a large colony. For specific single roost, it is also good to limit at one or at least few catches per season. We want also to reduce the stress to the mothers, exclude severe damage to the puppies and in general prevent a strong negative reaction that should also lead the abandonment of the site

2.2 SAMPLE COLLECTION AND PRESERVATION

2.2.a TISSUE COLLECTION

This sampling involved 28 Italian colonies, 24 of *M. emarginatus* and 4 of *M. capaccinii*. For each individual sex was morphologically determined and 3 mm small punches of the wing membrane were obtained using a sterile biopsy (Fig. 2.3 and 2.4) (Worthington Wilmer and Barratt 1996), fixed in 90% ethanol and then stored at − 20°C until DNA extraction.

Figure 2.4 and 2.4: taking a biopsy sample from a captured bat

For the capture we used specific methods as described above.

2.2.1 Capture

Capture of bats allows positive species identification (see Nagorsen and Brigham, 1993, for identification key), age and sex determination, the collection of mass and other mesurable data, and an assessment of reproductive condition (Anthony, 1988; Racey, 1988). However, this obviously requires some handling and disturbance to the animal and not all species or sexes are equally catchable, if catchable at all.

The two most common methods of capture involve the use of mist nets or harp traps, although several other methods (e.g., hand nets, funnels) were used in the past (e.g., LaVal and LaVal, 1977; Youngson and Mckenzie, 1977; Fenton and Bell, 1979; Kunz and Kurta, 1988). Many of these other techniques require sampling at or in roost sites, and are not recommended because they tend to be disruptive to the bats and may cause them to abandon the roost.

Conservation of bats and critical habitats, as well as minimization of disturbance must be considered for all potential sampling protocols.

2.2.2 Mist Nets

Mist netting is the most common method used to capture bats (Kunz and Kurta, 1988). Catching bats in mist nets depends on careful selection of netting sites (Fig. 2.5). Productive netting sites (i.e. areas of high bat activity) can be determined by direct observation of bats or by using bat detectors (see below).

The major advantages of using mist nets to sample bats are that they are relatively inexpensive, highly portable and easy to use and set up. The disadvantages are that they have certain biases associated with them, in terms of which species can be caught, and they require constant monitoring to ensure that bats do not chew their way out, become badly entangled or cause injury to themselves. A further disadvantage is the recent difficulty in obtaining suitable mist nets from suppliers.

The success of mist nets at a location decreases if a net is set up at the same location more than once (Kunz and Brock, 1975). In addition, certain species are adept at avoiding mist nets or fly at heights that make their capture difficult, even though they may be present in a study area. For example, *Lasiurus blossevilli*, *L. cinereus* and *Eptesicus fuscus* tend to fly higher than the location of most mist nets and gleaning species such as *Myotis evotis*,*Plecotus townsendii*, and *Antrozous pallidus* seem better able to detect and avoid mist nets, particularly now that monofilament nets are unavailable. Setting nets higher in the canopy can increase the success of capturing these high flying species, and numerous designs for canopy netting is described in Kunz (1996). Also, juveniles may be more susceptible to capture than older age classes creating a biased interpretation of population composition. In addition, environmental factors may influence the effectiveness of mist netting. The presence of wind may decrease capture success by causing the mist net to billow and thus become more detectable (Nyholm, 1965). Rain also adheres to mist nets, rendering them more "visible" to bats.

2.2.2.1 Equipment

Mist nets used for capturing bats are usually black, 6 to 36 m in length, 2 m high, have four shelves, a mesh size of 36 mm and are constructed from 50 or 70 denier/2 ply nylon (Fig. 2.6; Kunz and Kurta, 1988). Unfortunately, recent restrictions by the Japanese manufacturers and a government trade ban by Japan have made mist nets very difficult to obtain and monofilament nets, the most effective ones for capturing bats, are no longer available. Nets less than 12 m in length tend to be easier to handle, especially for one person. Poles made of 3 m lengths of aluminium tubing are often used to support the nets.

The tubing should have a wall thickness of about 1.6 mm and should be at least 2.5 cm in diameter. Thin-walled electrical conduit is inexpensive and readily available and makes excellent mist net poles. Connectors (e.g., 20-30 cm long solid aluminium shafts that fit the inside diameters of poles) can be made to join lengths of pole to make sections of the necessary length. To keep mist nets in place, guy lines can be attached to the poles and anchored to vegetation or rocks.

Figure 2.5. Example of mist net placement. Note that the net is placed in the vegetation such that a potential flight corridor is covered by the net.

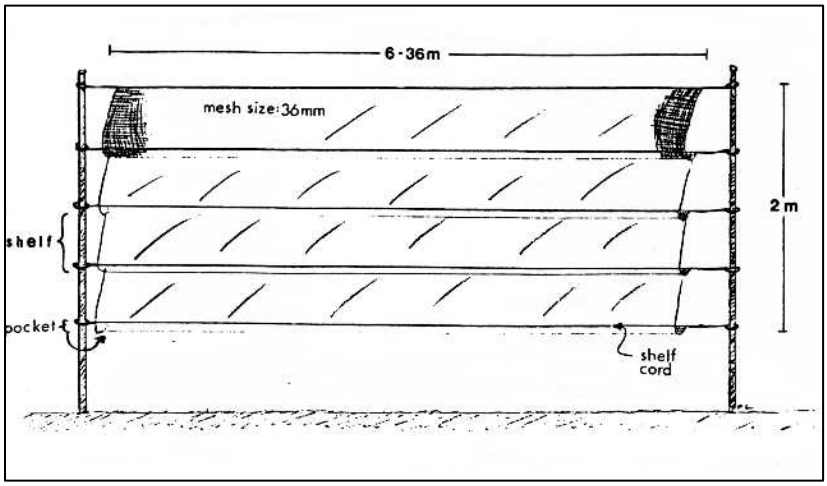


Figure 2.6: Mist net components and dimensions.

2.2.3 Harp Traps

Harp traps, specifically designed for capturing bats, were first described by Constantine (1958) and later modified by Tuttle (1974). Unlike mist nets, harp traps may be set up and left unattended. Similar considerations as those for setting mist nets are used for the placement of harp traps (Fig. 2.7). Harp traps may be hoisted off the ground by ropes or positioned outside

the entrances to buildings, caves, or mines. As for mist nets, trapping success tends to decrease with each successive night in the same location (Kunz and Anthony, 1977).

The major advantages of using harp traps to sample bats are that they are less labour intensive, they do not require constant supervision (thus several can be set up per night) and they can be used to catch species that tend to avoid mist nets (such as *Myotis ciliolabrum* and *Myotis evotis*, Holroyd *et al.*, 1994). Disadvantages include the small area sampled by the trap (only about 2 m^2 as opposed to several times that for each mist net used), its limited portability, which may limit its use to areas accessible by roads, and its greater cost (approximately $500 CAN). A collapsible, 7 kg harp trap described by Tidemann and Woodside (1978) which takes 30 minutes to set up or dismantle at least partly solves the portability problem.

2.2.3.1 Equipment

Harp traps (Fig. 2.8) consist of two 2 m by 1.8 m frames of aluminium tubing. Vertically strung across each frame is a bank of 6 - 8 pound (3 - 3.5 kg) monofilament fishing line. Lines are strung 2.5 cm apart. The two frames are spaced 7 to 10 cm apart. Attached to the bottom of the frame is a canvas bag, lined with polyethylene. The trap works on the principle that a flying bat can not easily detect or avoid the bank of lines and will become trapped between the monofilament lines and fall into the holding bag below. The bats drop into this bag and are unable to crawl out over the slippery polyethylene. If a bat manages to fly straight through the first set of lines, it is blocked by the second set. The degree of tension on the lines may have to be increased if bats are able to fly straight through without becoming trapped, or decreased if they simply 'bounce off'.

Figure 2.7. Examples of harp trap placement, a) along a forest trail, (b) at the entrance to a tunnel.

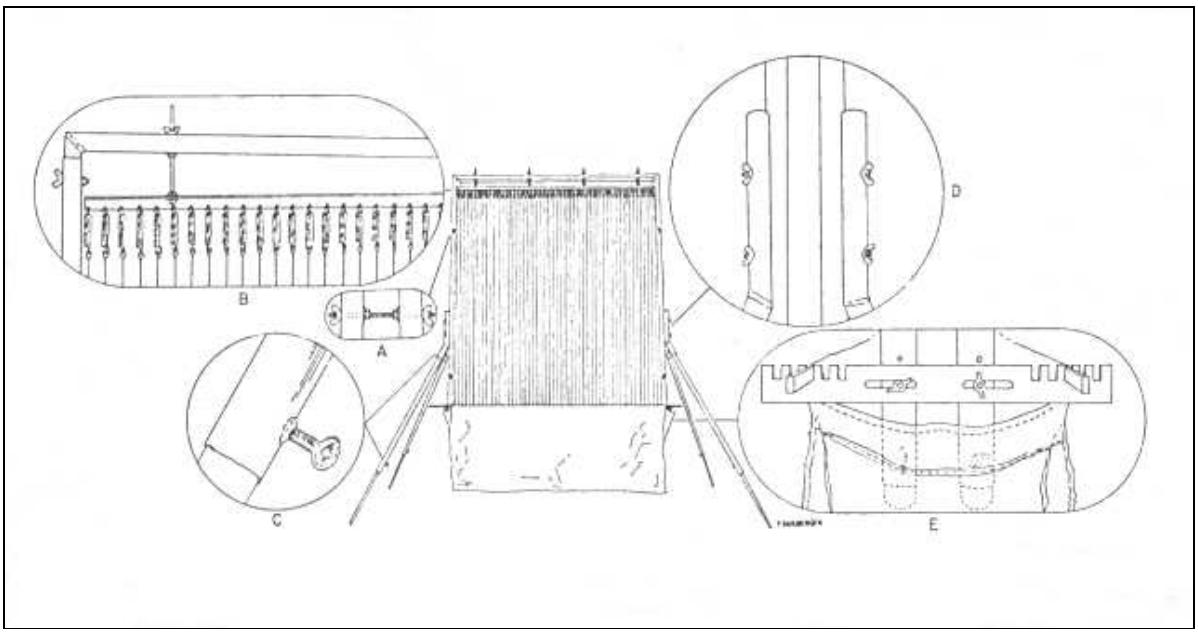


Figure 2.8. Harp trap design and detail. (design from Tuttle 1974, drawn by Tom Swearingen).

2.2.b NON-INVASIVE SAMPLING

Non-invasive procedures were used to sample eleven colonies collecting a total of of 362 samples of *M. emarginatus* and 46 of *M. capaccinii* thus minimizing the invasiveness and expand much more the extension of the collecting sample. Faecal pellets were collected using sterilized equipment soon after the animals defecated and immediately preserved in sample vials containing ethanol 90% and stored at ambient temperature during day of collection, and – 30°C in the laboratory.

2.3 DNA MARKERS USED IN THE ANALYSES

2.3.1 Nuclear DNA: Microsatellites

Microsatellites 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 analyzed 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.

Two models have been hypothesized to explain the main mutation mechanisms that could generate microsatellites:

-DNA slippage: it occurs during replication when the nascent DNA separates and reassociates itself temporarily from the DNA template. During replication of non-repetitive sequences the possible disassociation of the sister chromatid does not usually generate mutations because the nascent DNA can reassociated only and exactly in the complementary point of the DNA template. Instead, during tandemly repeat DNA replication, the single strand nascent DNA can pair in another point of the DNA template. When replication continues, the nascent DNA is found to be longer or shorter than the template (Hancock, 1995).

-DNA recombination: it can vary microsatellite length through asymmetrical crossing-over or gene conversion. Asymmetrical crossing-over occurs very frequently between tandemly repente DNAs that do not align themselves precisely giving rise to the deletion of a DNA fragment from a chromatid and its insertion into another chromatid. It may occur between two chromatids of the same chromosome or between two different chromosomes. Gene conversion, to answer to DNA damages, produces the unidirectional transfer of a DNA sequence from one allele to another one (Hancock, 1999).

As microsatellites show a high polymorphism rate and a high-quality result reliability, they are considered very popular genetic markers among molecular biologists. In fact these markers are important for map building since the distribution of this sequence repeats within the genome is random and act as landmarks for the organization of the DNA (Mellersh & Ostrander, 1997).

2.3.2 Mitocondrial DNA: CytB and CR

Every eukaryotic cell contains at least one copy of the entire nuclear genome housed in its nucleus. In contrast, every cell contains as many as several thousand mitochondria.

This organelle has been found to play a central role in numerous cellular functions such as metabolism (oxidative phosphorylation), apoptosis, and aging (Boore, 1999). It has been known for many years that mitochondria are semi-autonomous, possessing their own genome and the machinery for replication, transcription, and protein synthesis (Saccone et al., 2000).

Mitochondria descended from free-living bacteria that became symbiotic with eukaryotic cells about 1.5 billion years ago. The original model of mitchondrial evolution held that the nucleus originated in an Archaebacterium and then the symbiosis began with a eubacterial progenitor of the modern mitochondrion (Margulis, 1971). The conventional “endosymbiosis theory” has been modified over the years and the revision has been labeled the “hydrogen hypothesis” (Martin and Muller, 1998; Muller and Martin, 1999).

In animals, mtDNA (Fig. 2.9) is usually small (15 to 20kb) and encodes 37 genes. Variations in the size of animal mtDNAs are due primarily to duplications rather than the presence of additional genes. The typical mitochondrial gene complement includes 13 protein subunits of the enzymes involved in oxidative phosphorylation, the two rRNAs of the mitochondrial ribosome, and the 22 tRNAs necessary for the translation of the proteins encoded (Boore, 1999).

In most multicellular organisms, mtDNA is inherited from the mother (maternally inherited). Mechanisms for this include simple dilution (an egg contains 100,000 to 1,000,000 mtDNA molecules, whereas a sperm contains only 100 to 1000), degradation of sperm mtDNA in the fertilized egg, and, at least in a few organisms, failure of sperm mtDNA to enter the egg. Whatever the mechanism, this single parent (uniparental) pattern of mtDNA inheritance is found in most animals, most plants and in fungi as well.

It has been reported that mitochondria can occasionally be inherited from the father in some species such as mussels (Hoeh et al:, 1991; Penman et al., 2002).Paternally inherited mitochondria have additionally been reported in some insects such as fruit flies (Kondo et al., 1992), honeybees (Meusel and Moritz, 1993), and periodical cicadas (Fontaine et al:, 2007).

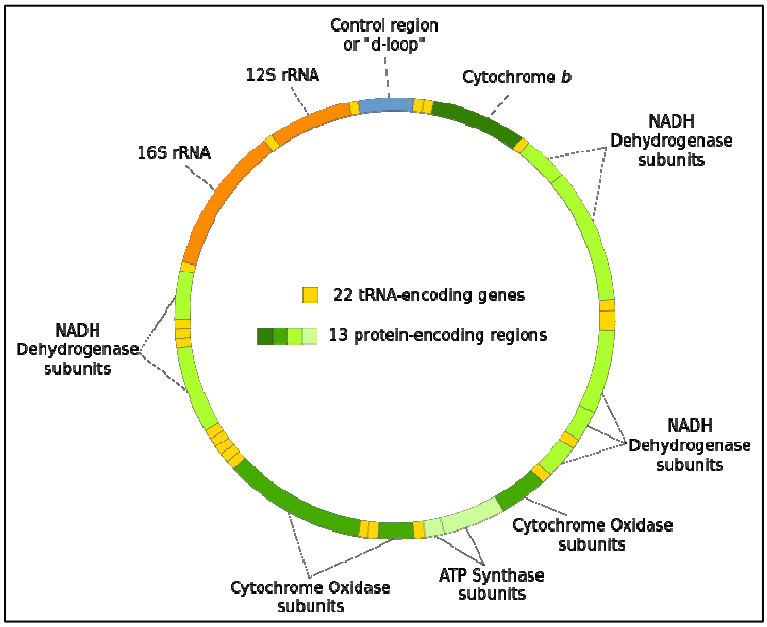


Figure2.9: mitocondrial DNA, indicated Control Region and Cytochrome b

In this study we used two different mtDNA markers, Cytocrome B and Control Region.

- **Cytochrome b/b6** is the main subunit of transmembrane cytochrome bc1 and b6f complexes (Howell, 1989; Esposti et al, 1993). In addition, it commonly refers to a region of mtDNA used for population genetics and phylogenetics. In the mitochondrion of eukaryotes and in aerobic prokaryotes, cytochrome b is a component of respiratory

chain complex III (EC 1.10.2.2) also known as the bc1 complex or ubiquinol-cytochrome c reductase. In plant chloroplasts and cyanobacteria, there is an analogous protein, cytochrome b6, a component of the plastoquinone-plastocyanin reductase (EC 1.10.99.1), also known as the b6f complex. These complexes are involved in electron transport and the generation of ATP and thus play a vital role in the cell. Cytochrome b/b6 (Howell, 1989; Esposti et al, 1993) is an integral membrane protein of approximately 400 amino acid residues that probably has 8 transmembrane segments. In plants and cyanobacteria, cytochrome b6 consists of two subunits encoded by the petB and petD genes. Cytochrome b/b6 non-covalently binds two heme groups, known as b562 and b566. Four conserved histidine residues are postulated to be the ligands of the iron atoms of these two heme groups.

Cytochrome b is commonly used to determine phylogenetic and phylogeographic relationships between organisms due to its sequence variability. It is considered to be most useful in determining relationships within families and genera. Comparative studies involving cytochrome b have resulted in new classification schemes and have been used to assign newly described species to a genus, as well as deepen the understanding of evolutionary relationships (Castresana, 2001).

-**The mitochondrial control region** (CR) is a noncoding segment located between the genes for tRNAPhe and the tRNAPro. The control region and mtDNA D-loop are sometimes used synonymously in the literature (Aquadro CF et al., 1983); specifically the control region includes the D-loop along with adjacent transcription promoter regions. For this reason, the control region is also known by the acronym DLP, standing for **D**-**L**oop and associated **P**romoters (Michikawa et al., 1999). Although this region is highly variable at the sequence level, this variation is mainly confined to two hypervariable domains (HVI and HVII) on the 5’ and 3’ sides of the CR (e.g. Vigilant et al.,1991).

2.3.3 Nuclear Introns

A nuclear genetic marker (BGN intron 4; Lyons et al. 1997) was amplified for a representative subset of the acquired samples. The X-linked gene BGN encodes fix a protein which plays a role in assembly of collagen fibrils and muscle regeneration. Preliminary results had shown that there was low genetic diversity for the species at this intron and therefore a representative subsample would capture the majority of the haplotypes present in Europe. A 600bp intron fragment was amplified spanning intron 4**.** This gene was chosen as the sex chromosomes are thought to evolve faster that the autosomes due to their smaller effective population size. In addition, intronic regions mil have higher variability than the more

conserved exons. BGN has already been shown to be informative within mammalian lineages (Murphy et al. 1999; Gaines al. 2005; Roca et al. 2005).

For this study we amplified other five introns: ACPT-4; ABHD11-5; ACOX2-3; COPS7A-4; and ROGDI- 7, already used in a previous study about *Myotis nattereri* complex (Salicini et al., 2011). In addition this markers are biparentally inherited and thus would complement data from the maternally inherited mtDNA data. Primer sequences and sources are listed in Table 2.5.

Primer name	Sequence (5' - 3')	Reference
BGN -F 11	CTCCAAGAACCACCTGGTG	Lyons *et al.* (1997)
BGN-R	TTCAAAGCCACTGTTCTCCAG	Lyons *et al.* (1997)
ABHD11-F1	CTGCTCACCAACCTGGTGGAGGT	Igea *et al.* (2010)
ABHD11-R1	TTVGGCACRGTCTGCATCTGGGC	Igea *et al.* (2010)
ACOX2-F1	CCTSGGCTCDGAGGAGCAGAT	Igea *et al.* (2010)
ACOX2-R1	GGGCTGTGHAYCACAAACTCCT	Igea *et al.* (2010)
ACPT-F1	GAYTTTGACCGSACVCTGGAGAG	Salicini *et al.* (2011)
ACPT-R1	AGYAGYTCVYGGTATCGRGGACA	Salicini *et al.* (2011)
COPS-F1	TACAGCATYGGRCGRGACATCCA	Igea *et al.* (2010)
COPS-R1	TCACYTGCTCCTCRATGCCKGACA	Igea *et al.* (2010)
ROGDI-F1	CTGATGGAYGCYGTGATGCTGCA	Igea *et al.* (2010)
ROGDI-R1	CACGGTGAGGCASAGCTTGTTGA	Igea *et al.* (2010)

Table 2.5 - **Sequences of nuclear intron primers used in the present study**

PCR reactions were carried out in 25µl simplex reactions consisting of 2µl DNA (lOng/pl), 2.5pl 10X PCR buffer without $MgCI_2$, 0.75µl $MgCI_2$(1.5mM), 0.5µl dNTPs (0.2mM). 0.5µl of each primer (0.4pM), 0.2 µi (1 U) Platinum Taq DNA polymerase High Fidelity and 18.05pl ddH_20. All reagents were supplied by Invitrogen (Life Technologies). PCR amplifications were carried out on a Biometra T3000 rhermocycier. The PCR program used is listed in Table 2.5. Amplified fragments were approximately 600bp in length. PCR products were purified using Exo-SAP (Roche) following the manufacturer's protocol: 4.5pi ddH_20,0.45µl SAP, 0.05 µl Exo and reactions were placed on a thermocycler program for 15mins. at 37°C followed by 15 mins. at 80°C. Amplified regions were sequenced directly using the same primers used in the PCR.

2.4 ANALYSIS PROCEDURES

2.4.1 DNA extraction

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 scats and tissues were used. Negative (no scat or tissue material added to the extractions) and positive (samples with known genotypes)

controls were always used to check possible contaminations during both extraction processes. Regarding non invasive samples we analyzed 10 or 15 samples for each colonies, about European colonies not all of their samples have been analyzed yet, because we obtained them only at the ending of 2011. They will be used for future analysis.

2.4.2 Manual extraction

20 scat samples and 68 tissue samples were manually extracted using a kit with its own protocol developed by Zymo Research, we used manual extraction only for few samples in order to verify if the protocol is working with them. Excremental DNAs were extracted in a separate room only dedicated to low-DNA-content samples to avoid contaminations among them. The used solutions are characterized by the presence of:

WLCB: White cell lysis buffer produces the cell disruption, that is a necessary for releasing biological molecules from inside a cell.

SDS: the SDS (Sodium Dodecyl Sulphate) reagent is an anionic detergent that denatures secondary and non-disulfide-linked tertiary protein structures. It applies a negative charge to each protein in proportion to its mass.

Zymo Research protocol (summary)

Preparation of the samples:

- a piece of tissue (50 mg) or of scat material (80 mg) is cut and transferred into an "eppendorf" test tube of 2.0 ml containing 175 μl WLCB, 20 ul Proteinase K and 5 μl SDS respectively; flamed sterilized scalpels and forceps are used.

Digestion of the samples:

- in rotation at 57°C for 30 minutes or overnight for tissue, 30 minutes for scats.

Collecting DNA:

- centrifuge at room temperature for 1 minute at 10000 rpm; - add 800 μl of ***Zymo Lysing Buffer*** and wait and vortex for 5 minutes; - centrifuge at room temperature for 1 minute and transfer the mixture to a *Zymo spin Column* in a collection tube. Centrifuge again for 1 minute and discard the collection tube with the flow through.

DNA is now bound to filter in the Zymo spin Column. Each pellet is washed twice, first with 200 μl of ***Pre Wash Buffer*** ,centrifuged at room temperature for 1 minute; second with 500 μl of ***gDNA Wash Buffer***, and centrifuged at room temperature for 1 minute. After the spin Column is transferred to a new 1,5 ml eppendorf tube.

We added 250/300 μl of **TE** to the spin column and incubate it for 10 minutes at room temperature. Then we centrifuge at the top speed for 30 seconds to elute the DNA. The eluted DNA can be preserved in freezer at -20°C.

2.4.3 Automated extraction

207 scat samples and 285 tissue samples were extracted in an automatic manner by the ***MULTIPROBE IIEX*** robot (Perkin Elmer) and using the Zymo Research kit stool and tissue extraction. 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.

Manual phase:

Preparation of the samples:

- a piece of tissue (50 mg) or of scat material (80 mg) is cut and transferred into an "eppendorf" test tube of 2.0 ml containing 175 ul WLCB, 20 µl Proteinase K and 5 µl SDS respectively; flamed sterilized scalpels and forceps are used.

Digestion of the samples:

- in rotation at 57°C for 30 minutes or overnight for tissue, 30 minutes for scats.

Collecting DNA:

- centrifuge at room temperature for 1 minute at 10000 rpm; - add 800 µl of ***Zymo Lysing Buffer*** and wait and vortex for 5 minutes; - centrifuge at room temperature for 1 minute.

Automated phase:

- link the multiblock with Zymo 96 plate filter to the robot's platform containing a vacuum pump system to aspirate liquid solutions and trap the DNAs. Select "Vacuumelutiononly" program for the software's folder.
- add the mixture to Zymo 96 plate and the software activates the vacuum for 10 minutes;
- add 250 µl of ***Pre Wash Buffer*** and the software activates the vacuum for 10 minutes;
- add 500 µl of ***gDNA Wash Buffer*** and the software activates the vacuum for 10 minutes;
- add 300 µl of TE in each spot of Zymo 96 plate; plate is centrifuge for 30 seconds at maximum speed.

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

2.4.4 DNA amplification

DNA amplification is a necessary procedure to obtain sufficient DNA quantity to carry out molecular analyses. 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 chemically 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 uses single stranded DNA as a template and, by the action of DNA polymerase enzyme, it synthesizes a complementary strand over and over again, until extensive quantities are produced. Every PCR consists of a cycle, repeated many times, made up of the following steps: denaturation of the DNA sample at temperatures up to 90-95°C; binding 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 and ends in the complete replication of both strands of the target sequence (Fig 2.10).

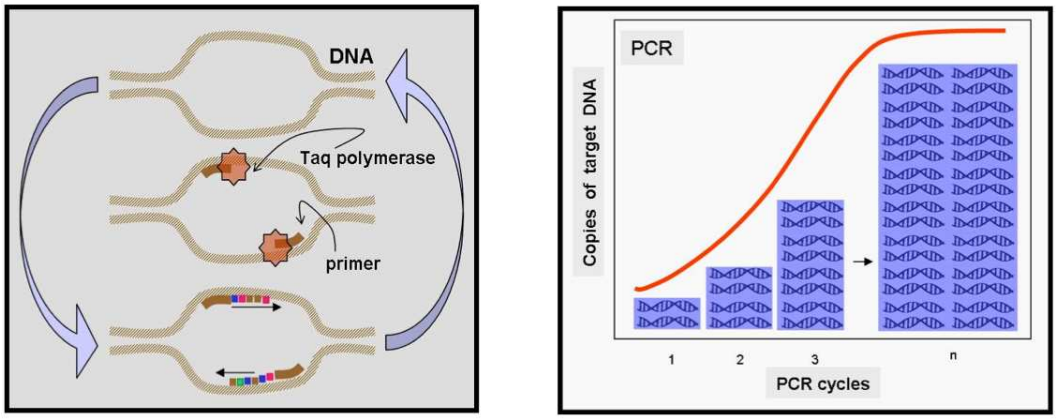


Figure 2.10 different phases of the Polymerase Chain Reaction and exponential amplification of target DNA

2.5.1 Microsatellite Amplification

As repeated sequences of microsatellites are flanked by unique sequences, it is possible to design PCR primers (Forward and Reverse) that selectively amplify microsatellite *loci*. Genotyping analysis is done to identify the molecular weight of the alleles present at each *locus* via electrophoresis.

Since there is actually no data about *M. emarginatus*, initially have been tested on 30 tissues samples of the Campo dei Fiori colony, a set of 15 microsatellites developed for *Myotis myotis* (Castella & Ruedi, 2000). Of these 9 works only six are informative (A13, G30, D15, E24, H29 and H19).

Regarding *M. capaccinii*, we used microsatellites identified by previous work (Bilgin et al., 2008).

First step, after identifying the informative microsatellites, all tissue samples were genotyped, after that we started the analysis of fecal samples. It is important to remember that when you amplify DNA from feces, researchers must resolve genotyping errors, particularly ADO and FA (Taberlet et al. 1996).

As non-invasively collected samples usually provide low target DNA concentration and low target DNA quality (Taberlet *et al*., 1999), to delete those lacking enough DNA to complete the genotyping and to impede possible problems during further laboratory procedures, all the DNA samples were initially screened using a multiple-tube approach (Taberlet *et al*., 1996; Gagneux *et al*. 1997; Lucchini *et al*., 2002). The screening consisted in amplifying each sample four times at 2 microsatellite *loci* (D15 and E24) chosen, for their high PCR success and their low dropout and false allele rates, among the first 6 microsatellite used for the individual identification (Lucchini *et al*., 2002; Fabbri *et al*., 2007).

Only the samples with positive PCRs major than 50 % passed the screening and they were amplified four times at the other 4 microsatellite *loci*, always using a multiple-tubes approach by which the samples heterozygote at least in 2 replicates or homozygote at least in 4 replicates at a given *locus* were scored as reliable at that *locus* and genotypes were recorded; while all the other heterozygote, homozygote and uncertain genotypes (due to failure of one amplification or to allelic dropout) were additionally replicated four times. All samples that could be not reliably typed at all *loci* after 8 amplifications were discarded.

Microsatellites were PCR-amplified following a previous protocol developed in ISPRA genetic lab (Randi & Lucchini, 2002; Lucchini *et al*., 2002; Fabbri *et al*., 2007) separately in 10 µl of volume, using 2 µl of DNA solution, 1 µl of PCR Buffer 10X (1,5 mM of MgCl2), 2 µg of BSA (Bovine Serum Albumin), 0,4 µl of dideossinucleotides (dATP, dCTP, dTTP, dGTP) 2,5mM, 0,15 µl of each primer 10 µM, 0,25 units of Taq and 4,25 µl of PCR water.

Cycling conditions were optimized for each primer pair and for tissue or scat samples, the number of cycles varied from 30 to 45, starting from the following general PCR program:

94°C x 2'→(94°C x 15''→ 57°C/60°C x 30''→ 72°C x 30'') for 30-45 cycles → 72°C x 10' → 4°C x 10' → 15°C

2.5.3 Analysis of microsatellites in automated capillary sequencers

Microsatellite analysis consists in separating the 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 automatic 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. Electrophoresis is programmed through a particular computer software that activates and controls all operation

performed by the automated sequencer. The capillary sequencer does not use radioactive markers but fluorescent marker systems (*fluorescent dyes*) that are incorporated in the DNA during PCR amplification or sequencing, using primers labelled with a fluorescent dye or incorporating a labelled nucleotide in the DNA. 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 repats) in an image file and in an electropherogram in which the molecular weights of the alleles are precisely determined by the use of internal standards.

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. 2.11).

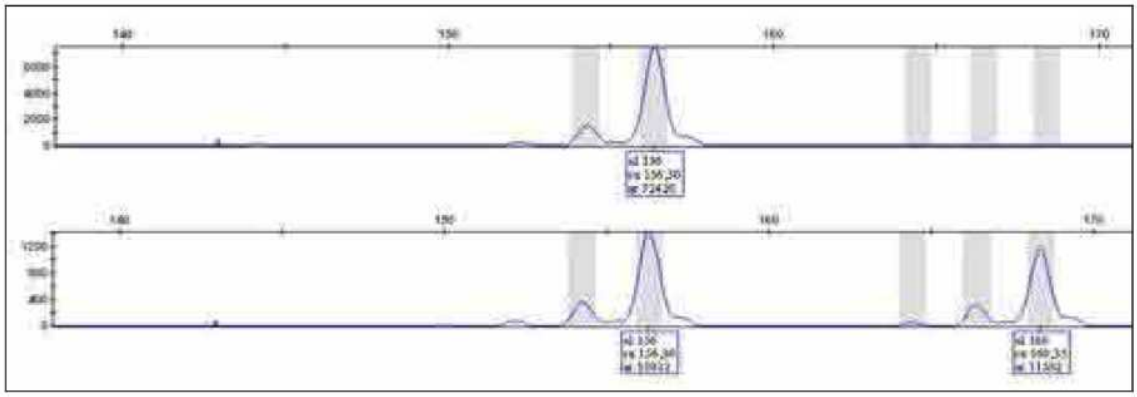


Figure 2.11 example of electropherograms, the single peak stands for a homozygous sample at a given *locus*, the double peaks indicate a heterozygous sample at a given *locus*

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.

2.6 MICROSATELLITE DATA ELABORATION

The software used to manually or automatically correct the results of the automated analyses is ***GeneMapper v.3.0*** of the Applied Biosystems (ABI). 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 specific 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.

2.6.1 Data reliability: RelioType

In this study *multilocus* genotypes were detected using a multiple-tube approach by which the same DNA samples were amplified independently several times *per locus* and the results of each replicate were compared. In this way it was possible to detect eventual dropouts or false amplification. The necessary number of replicates to obtain a reliable *multilocus* genotype was computed using the software ***RelioType*** **(Miller, Joyce & Waits, 2002).** It is a program for assessing how reliable an observed multilocus genotype is and for directing further replication if it is not sufficiently reliable. It is based on the model developed by Miller, Joyce and Waits (2002). The program requires two input files: a first file with allele counts from the population which the program converts into allele frequencies and a second file containing the genotyping data. The software calculate for each *multilocus* genotype of the second input file a probability of reliability using the allele frequencies contained in the first input file. The estimation of reliability assumes that false alleles do not exist in the data set, which is a clearly unrealistic assumption. One simple way to catch false alleles is to require that all alleles are observed multiple times.

2.6.2 Multilocus genotype comparison: Gimlet

When using the multi-tube approach, it is useful to easily construct consensus genotypes and to rapidly calculate the error rates. ***Gimlet v. 1.3.2*** (**G**enetic **I**dentification with **M**ulti**L**ocus **T**ags) (Valière, 2002) is a software dedicated for geneticists who work on individual identification using molecular tags in diploid species. This software allows to easily construct consensus genotypes from a set of PCR repetitions for each samples choosing the alleles that

appeared the most at each *locus* (an allele is retained in the consensus if its score is above a threshold set by the user), and to rapidly calculate the error rates (allelic dropouts (ADO) and the false alleles (FA)) comparing the repeated genotypes and the consensus. The program can be also used to compare the different genotypes to reference ones already analysed. A genotype is identified when its *multilocus* genotype matches completely with a reference genotype. Moreover the program possesses also an option for pooling several genotypes that match themselves. The regrouping is conducted as an identification where all genotypes are potential reference genotypes

In both cases the software indicates the pairs of genotypes where only one allele (for one or two *loci*) or two alleles (for one *locus*) is (are) different between the genotypes. In this way it is possible to re-check these genotypes by re-looking at the electropherograms or by repeating PCRs at doubt *loci* because, considering very low PID, it is improbable to find 2 identical genotypes differing only for 1 allele on 12.

2.6.3 Species detection: Structure

In this study, population assignment was performed using a Bayesian clustering procedure implemented in ***Structure v. 2.1*** (Pritchard *et al*., 2000; http://pritch.bsd.uchicago.edu; Falush *et al,*. 2003).

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. The model assumes 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). This method can be used to detect the presence of cryptic population structure and to perform assignment testing. Pritchard *et al.*'s model assumes Hardy-Weinberg (HWE) and linkage (LE) equilibrium among the unlinked *loci*. Departures from HWE and LE lead the population to be split into subpopulations, to which individuals are assigned. The number of contributing populations can be estimated and, for a given number of populations, their gene frequencies and the admixture proportions for each individual are all jointly estimated. In this way the sampled population is subdivided into a number of different subpopulations that effectively cluster the individuals. Then, individuals of *a-priori* known or unknown origin may be assigned probabilistically to the subpopulations.

The model does not assume a particular mutation process, and it can be applied to most of the commonly used genetic markers including microsatellites, SNPs and RFLPs, provided that they are unlinked. In this study I performed the analyses using the "admixture" model which

assumes that each individual may have ancestry in more than one parental population and that allele frequencies of a K population can be obtained independently from the others.

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). The difference 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 clustering 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 are considered as *burnings*, they are deleted from the results interpretation that is based only on the following permutations.

2.6.4 Genetic population study: GeneAlex

GeneAlex 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.

In this study GeneAlex was 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 (*N*P) 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.

The software was also utilized for assignment tests and Principal Coordinate Analysis (PCA), in fact for each sample the expected genotype frequency at each *locus* is calculated and log-transformed 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 analyses and the further assignments occur.

To simplify the graphic visualization of Structure and GeneAlex results the program ***Genetix v.4.2*** (Belkhir *et al.,* 2001; **http://www.University-montp2.fr/-genetix/genetix.htm**) was used. It can describe in three dimensions all the variability analyzed in GeneAlex by Principal Coordinate Analysis and the different Structure clusterings.

2.6.5 Adegenet package

Adegenet (Jombart, 2008) is a package written using the R languages, it is devoted to the multivariate analysis of genetic markers data. These data can be codominant markers (e.g. microsatellites) or presence/absence data (e.g. AFLP), and have any level of ploidy. 'adegenet' defines two formal (S4) classes:

- genind: a class for data of individuals ("genind" stands for genotypes-individuals).
- genpop: a class for data of groups of individuals ("genpop" stands for genotypes-populations)

Both types of objects store information from molecular markers in a matrix, that can be directly analyzed using multivariate methods such as Principal Component Analysis, Correspondance Analysis, etc.. Moreover, this package offers methods for manipulating and analyzing information coming from genetic markers. We used it to obtain a clearest graphic representation of PCA, previously elaborate with GenAlex and Genetix.

2.7 SEQUENCES DATA ELABORATION

Tissue and faecal samples were analyzed at the level of CytB using two universal primers L3 and H4 (Irwin et al., 1991) for *M. emarginatus*, MyocaF and MyocaR (Bilgin et al., 2008) for *M. capaccinii* . The HVII was PCR-amplied using the primers L16517 (Fumagalli et al., 1996) and sH651 (Castella et al., 2001), a shorter version of the primer H00651 (Kocher et al.,1989).

The amplification was performed on a final volume of 10 µl, and with 1 ul of DNA previously extracted, 0,4 µl of dNTPs, 0,15 µl of each primers, 1 µl buffer 10X, 2 µl BSA, 0,25 units of taq and H_2O to bring up the volume. The amplification reaction included started with an initial denaturation at 95 °C for 3 min, followed by 35 cycles consisting of 45s at 94°C, 45s at 50°C and 1min at 72°C. The cycles ended with one final extension of 7 min at 72 °C. The sequencing was carried out in both directions 5 'and 3' using the respective primers. The PCR sequencing has provided for 25 cycles in a volume of 10 microliters: 1 ul of PCR product, 5.7 ul of H_2O, 0.3 ul of primer, 1 ul of fluorescent dye (ABI Big Dye) and 2 ml of 5 × buffer (supplied with the fluorescent dye). The parameters of the sequencing reaction for each cycle

were 10 s of denaturation at 96 ° C, 5 s annealing at 50 ° C and 4 min extension at 68 ° C, followed by 7 minutes final extension at 72 ° C. The sequencing reactions were purified by precipitation in ethanol.

The purified sequences were analyzed in an ABI 3730 automatic sequencer following the protocols of the supplier (Applied Biosystems Inc., Foster City, CA). The ambiguous bases were corrected manually using Seqscape V 4.1 (Applied Biosystems Inc., Foster City, CA) and aligned with CLUSTAL X (Thompson et al. 1997) before further data analysis. We used BioEdit for the aligned, which implement the CLUSTAL X algorithm, Network for building a phylogenetic network between different haplotypes and GENEIOUS and its plug-in (MODELTEST, PAUP and MRBAYES) to develop trees.

2.7.1 BioEdit

BioEdit (Hall, 1999) is a biological sequence editor that runs in Windows 95/98/NT/2000/XP and is intended to provide basic functions for protein and nucleic sequence editing, alignment, manipulation and analysis. BioEdit is not a powerful sequence analysis program, but offers many quick and easy functions for sequence editing, annotation and manipulation, as well as a few links to external sequence analysis programs. Sequence lengths and numbers are limited only by available system memory.

2.7.2 MEGA

The objective of the *MEGA* (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2011) software was to provide tools for exploring, discovering, and analyzing DNA and protein sequences from an evolutionary perspective. *MEGA 5* is specifically designed to reduce the time needed for ordinary tasks in data analysis and to provide statistical methods of molecular evolutionary genetic analysis in an easy-to-use computing workbench. We used MEGA to built the NJ unroot Tree of different colonies, using distance matrix of Fst and φp.

2.7.3 ARLEQUIN

The goal of Arlequin (Excoffier et al., 2005) is to provide the average user in population genetics with quite a large set of basic methods and statistical tests, in order to extract information on genetic and demographic features of a collection of population samples.

The statistical tests implemented in Arlequin have been chosen such as to minimize hidden assumptions and to be as powerful as possible. Thus, they often take the form of either permutation tests or exact tests, with some exceptions.

Arlequin can handle several types of data either in *haplotypic* or *genotypic* form. The basic data types are:

DNA sequences

RFLP data

Microsatellite data

Standard data

Allele frequency data

By *haplotypic form* it means that genetic data can be presented under the form of haplotypes (i.e. a combination of alleles at one or more loci). This haplotypic form can result from the analyses of haploid genomes (mtDNA, Y chromosome, prokaryotes), or from diploid genomes where the gametic phase could be inferred by one way or another. Note that allelic data are treated here as a single locus haplotype.

By *genotypic form*, it means that genetic data is presented under the form of diploid genotypes (i.e. a combination of pairs of alleles at one or more loci). Each genotype is entered on two separate lines, with the two alleles of each locus being on a different line.

Using Arlequin were calculated the following indices :

• haplotype diversity (h), the probability that two haplotypes randomly taken in the population are different (Nei, 1987);

• nucleotide diversity (π), the probability that two randomly chosen homologous nucleotides are different (Tajima, 1983; Nei, 1987).

The two parameters h and π are measures of genetic diversity but can also be used to study recent demographic events that have affected a population or species (Grant and Bowen, 1998; Benzie et al., 2002).

Arlequin 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 **FST** for Microsatellites and its analogue φpt, for mtDNA, which estimates the proportion of the genotypic variance among populations, relative to the total variance.

The software ARLEQUIN allowed to perform appropriate statistical analysis to investigate the 'demographic history' of the populations of *M. emarginatus* and *M. capaccinii* object of this study. The conducted analyses were:

• Mismatch Distribution, or the distribution of nucleotide differences between pairs of individuals; Rogers and Harpending (1992) proved that the increase or decrease of the size of a population directly affect a specific type of molecular data, precisely the frequency distribution of the number sites where the nucleotide pairwise combinations of sequences differ. The distribution resulting from the analysis is compared with that which would be an ideal model of sudden population expansion.

• Sum of squares of standard deviations (SSD), which allows to test the validity of the expansion by means of bootstrap replicates which in turn will compare the expected

distribution between the SSD and the SSD between the observed and expected simulated distributions (Schneider and Excoffier, 1999);

• Theta0 (θ0) and theta1 (θ1), which correspond to the size of each the study population before and after the event of expansion;

In addition to the Mismatch Distribution we made two more analysis, even those made with the software ARLEQUIN: the D test of Tajima (Tajima, 1989) and Fs test of Fu (Fu, 1997), able to measure how the patterns of molecular diversity observed differ from those expected at neutral equilibrium mutation

2.7.4 NETWORK

Network (http://www.fluxus-engineering.com) is used to reconstruct phylogenetic networks and trees, infer ancestral types and potential types, evolutionary branching and variants, and to estimate dating.

The algorithms are designed for non-recombining bio-molecules. Successful applications include mtDNA, Y-STR, amino acid, RNA, virus DNA, bacterium DNA, some effectively non-recombining autosomal DNA, and non-biomolecule data such as linguistic data. By contrast, recombining bio-molecules will deliver high-dimensional networks which will be difficult to interpret.

The Network software was developed to reconstruct *all possible shortest least complex* phylogenetic trees (all *maximum parsimony* or *MP* trees) from a given data set. Two different network-building options are included which can be used independently of each other.

The *reduced median* or *RM* (Bandelt et al. 1995) network algorithm RM requires binary data (example: at nucleotide position 16092 each taxon must have either T or C). To allow interpretation of complex data, a reduction parameter is available. If the reduction threshold r is set to a sufficiently high number, RM will yield a full median network containing all MP trees.

The *median-joining* or *MJ* (Bandelt et al. 1999) network algorithm allows *multi-state* data (example: at nucleotide position 16092 there can be A, C, G, T, and ambiguities such as N). For larger data sizes, the parameter epsilon can be set low to calculate sparse networks quickly, or incrementally increased to calculate higher-resolution networks at the cost of longer run times and increased network complexity. If epsilon is set to a sufficiently high number, MJ will yield a full median network (software and memory limits permitting). Optionally, MJ allows external rooting of the network using an outgroup.

In this study we used the MJ network algorithm, as it is recommended by Network User Manual.

2.7.5 MODELTEST

MODELTEST (Posada and Crandall, 1998) is a simple calculator written in ANSI C and compiled for the Power Macintosh and Windows 95/NT using Metrowerks CodeWarrior and for Sun machines using gcc. It is designed to compare different nested models of DNA substitution in a hierarchical hypothesistesting framework. MODELTEST calculates the likelihood ratio test statistic $\delta = -2\log \Lambda$ and its associated P-value using a $\chi 2$ distribution with q degrees of freedom in order to reject or fail to reject different null hypothesis about the process of DNA substitution. It also calculates the AIC estimate associated with each likelihood score.

The output of MODELTEST consists of a description of the likelihood ratio tests performed, and their associated P-values. The program interprets the resulting P-values and chooses the model that fits the best data among those tested following the likelihood ratio test and/or AIC criteria, using a default individual alpha value of 0.01 (for maintaining an overall alpha value of 0.05, the standard Bonferroni correction -alpha/number of tests- results in a individual alpha value of 0.01), or another value specified by the user. The program also calculates the AIC values, indicating the smallest

2.7.6 PAUP

PAUP (**P**hylogenetic **A**nalysis **U**sing **P**arsimony) (Swofford,LD, 2003) is a computational phylogenetics program for inferring evolutionary trees (phylogenies), written by David L. Swofford. Originally, as the name implies, PAUP only implemented parsimony, but from version 4.0 (when the program became known as PAUP*) it also supports distance matrix, and likelihood methods. Version 3.0 ran on Macintosh computers and supported a rich, user-friendly graphical interface. Version 4.0 added support for Windows (graphical shell and command line) and Unix (command line only) platforms. However, the graphical user interface for the Macintosh version requires Classic, which is no longer supported by Mac OS X 10.5 and later. There is a command line version of PAUP* for Intel-based Macs.

2.7.7 MRBAYES

MrBayes (Huelsenbeck, J. P. and. Ronquist F, 2001; Ronquist, F. and. Huelsenbeck J. P, 2003)is a program for the Bayesian estimation of phylogeny. Bayesian inference of phylogeny is based upon a quantity called the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations. The conditioning is accomplished using Bayes's theorem. The posterior probability distribution of trees is impossible to calculate analytically; instead, MrBayes uses a simulation technique called Markov chain Monte Carlo (or MCMC) to approximate the posterior probabilities of trees.

2.7.8 GENEIOUS

Geneious Pro (Drummond et al., 2011) is a revolutionary bioinformatics software platform that is both ultra-powerful and easy to use, it is developed by Biomatters Ltd (**http://www.geneious.com/**). Scientists, researchers and students are able to search, organize and analyze genomic and protein information via a single desktop program that provides publication ready images to enhance the impact of your research.

Complex data such as entire bacterial genomes or human chromosomes can be stored, visualized and analyzed using a single desktop application that unites pipelines and tools to ensure analyses are easily repeatable.

Geneious features sequence alignment, Contig assembly, primers, cloning, BLAST, phylogenetics, automatic publication updates and more. This basically means it provides sophisticated genome & protein research tools that aim to speed up the analysis process. This software has been used to elaborate CytB and CR trees, using specific plung-ins that allow to use MODELTEST and PAUP respectively.

The model used in building the neighbour-joining trees was choosed running MODELTEST**,** phylogenetic analyses of *M. emarginatus* and *M. capaccinii* were made using GENEIOUS software (Biomatter LTD) and its PAUP* plugin . The model used in building the neighbour-joining trees was choosed running MODELTEST. We made also a Bayesian analysis with MrBayes, using thse following parameters: The Bayesian tree was obtained by running two Markov chains of one million generation each and all default settings. The general time reversible model of DNA evolution (the closest model to Tamura-Nei implemented in MrBayes) with gamma-shaped rate of variation and a proportion of invariable sites (Yang 1993) was selected as the evolutionary model, as done in another similar work (Ruedi et al., 2008). Supports of nodes were assessed either as posterior probabilities (Bayesian tree, with the initial 2000,000 generations considered as burn-in), or as bootstraps (NJ tree, 100 000 pseudoreplicates).

2.7.9 TREE CONSTRUCTION METHODS

2.7.9.1 NEIGHBOUR JOINING

Neighbour joining is a bottom-up clustering method for the creation of phenetic trees (phenograms), created by Naruya Saitou and Masatoshi Nei (1987). Usually used for trees based on DNA or protein sequence data, the algorithm requires knowledge of the distance between each pair of taxa (e.g., species or sequences) to form the tree (Didelot, 2010).

Neighbour joining starts with a completely unresolved tree, whose topology corresponds to that of a star network, and iterates over the following steps until the tree is completely resolved and all branch lengths are known:

1. Based on the current distance matrix calculate the matrix *Q* (defined below).
2. Find the pair of taxa in *Q* with the lowest value. Create a node on the tree that joins these two taxa (i.e., join the closest neighbours, as the algorithm name implies).
3. Calculate the distance of each of the taxa in the pair to this new node.
4. Calculate the distance of all taxa outside of this pair to the new node.
5. Start the algorithm again, considering the pair of joined neighbours as a single taxon and using the distances calculated in the previous step.

The Q-matrix

Based on a distance matrix relating the *r* taxa, calculate *Q* as follows:

$$Q(i,j) = (r-2)d(i,j) - \sum_{k=1}^{r} d(i,k) - \sum_{k=1}^{r} d(j,k)$$

where $d(i,j)$ is the distance between taxa i and j.

Neighbour joining is based on the minimum-evolution criterion, i.e. the topology that gives the least total branch length is preferred at each step of the algorithm. However, neighbour joining may not find the true tree topology with least total branch length because it is the agreeded algorithm that constructs the tree in a step-wise fashion. Even though it is sub-optimal in this sense, it was extensively tested and found a tree that is quite close to the optimal tree. Nevertheless, it has been largely superseded by phylogenetic methods that do not rely on distance measures and offer superior accuracy under most conditions.

The main virtue of neighbour joining in comparison to other methods is its computational efficiency. That is, neighbour joining is a polynomial-time algorithm. It can be used on very large data sets for which other means of analysis (e.g. minimum evolution, maximum parsimony, maximum likelihood) are computationally prohibitive.

Unlike the UPGMA algorithm for tree reconstruction, neighbour joining does not assume that all lineages evolve at the same rate (molecular clock hypothesis) and produces an unrooted tree. Rooted trees can be created by using an outgroup and the root can then effectively be placed on the point in the tree where the edge from the outgroup connects.

Furthermore, neighbour joining is statistically consistent under many models of evolution. Hence, given data of sufficient length, neighbour joining will reconstruct the true tree with high probability.

2.7.9.2 BAYESIAN ANALYSIS

Bayesian inference in phylogeny generates a posterior distribution for a parameter, composed of a phylogenetic tree and a model of evolution, based on the prior for that parameter and the likelihood of the data, generated by a multiple alignment. The Bayesian approach has become more popular due to advances in computational machinery, especially, Markov chain Monte Carlo algorithms (Geyer, 1991). Bayesian inference has a number of applications in molecular phylogenetics, for example, estimation of species phylogeny and species divergence times.

2.7.9.3 MAXIMUM PARSIMONY

Parsimony is a non-parametric statistical method commonly used in computational phylogenetics for estimating phylogenies. Under parsimony, the preferred phylogenetic tree is the tree that requires the least evolutionary change to explain some observed data.

Parsimony is part of a class of character-based tree estimation methods which use a matrix of discrete phylogenetic characters to infer one or more optimal phylogenetic trees for a set of taxa, commonly a set of species or reproductively-isolated populations of a single species. These methods operate by evaluating candidate phylogenetic trees according to an explicit optimality criterion; the tree with the most favourable score is taken as the best estimate of the phylogenetic relationships of the included taxa. Maximum parsimony is used with most kinds of phylogenetic data; until recently, it was the only widely-used character-based tree estimation method used for morphological data.

Estimating phylogenies is not a trivial problem. A huge number of possible phylogenetic trees exist for any reasonably sized set of taxa; for example, ten species gives over two million possible unrooted trees. These possibilities must be searched to find a tree that best fits the data according to the optimality criterion. However, the data themselves do not lead to a simple, arithmetic solution to the problem. Ideally, we would expect the distribution of whatever evolutionary characters (such as phenotypic traits or alleles) directly following the branching pattern of evolution. Thus we could say that if two organisms possess a shared character, they should be more closely related to each other than to a third organism that lacks this character (provided that character was not present in the last common ancestor of all three, in which case it would be a symplesiomorphy). We would predict that bats and monkeys are more closely related to each other than either is to a fish, because they both possess hair—a synapomorphy. However, we cannot say that bats and monkeys are more closely related to one another than they are to whales because they share hair, because we believe the last common ancestor of the three had hair.

However, the phenomena of convergent evolution, parallel evolution, and evolutionary reversals (collectively termed *homoplasy*) add an unpleasant wrinkle to the problem of estimating phylogeny. For a number of reasons, two organisms can possess a trait not present in their last common ancestor: If we naively took the presence of this trait as evidence of a relationship, we would reconstruct an incorrect tree. Real phylogenetic data include substantial homoplasy, with different parts of the data suggesting sometimes very different relationships. Methods used to estimate phylogenetic trees are explicitly intended to resolve the conflict within the data by picking the phylogenetic tree that is the best fit to all the data overall, accepting that some data simply will not fit. It is often mistakenly believed that parsimony assumes that convergence is rare; in fact, even convergently-derived characters have some value in maximum-parsimony-based phylogenetic analyses, and the prevalence of convergence does not systematically affect the outcome of parsimony-based methods (Sober, 1983).

Data that do not perfectly fit a tree are not simply "noise", they can contain relevant phylogenetic signal in some parts of a tree, even if they conflict with the tree overall.

CHAPTER 3

RESULTS

A total set of 239 scat and 353 tissue samples were analyzed in this study (Table 3.1).

COUNTRY	REGION	COLONY	SAMPLING	SPECIES	N°SAMPLES
ITALY	LOMBARDIA	CAMPO DEI FIORI	INVASIVE	*M. emarginatus*	35
ITALY	LOMBARDIA	LAVENO MONBELLO	INVASIVE	*M. emarginatus*	7
ITALY	LOMBARDIA	PAVIA	INVASIVE	*M. emarginatus*	16
ITALY	FRIULI	CORMONS	INVASIVE/NON INVASIVE	*M. emarginatus*	4/20
ITALY	TRENTINO-ALTO ADIGE	GARGAZZONE	INVASIVE	*M. emarginatus*	20
ITALY	TRENTINO-ALTO ADIGE	MERANO	INVASIVE	*M. emarginatus*	15
ITALY	TRENTINO-ALTO ADIGE	SAN SIGISMONDO	INVASIVE	*M. emarginatus*	20
ITALY	PIEMONTE	TRINITA	INVASIVE/NON INVASIVE	*M. emarginatus*	8/43
ITALY	PIEMONTE	TETTI PESIO	NON INVASIVE	*M. emarginatus*	17
ITALY	PIEMONTE	VENARIA REALE	NON INVASIVE	*M. emarginatus*	16
ITALY	PIEMONTE	BRICHERASIO	NON INVASIVE	*M. emarginatus*	10
ITALY	PIEMONTE	PASSERANO	NON INVASIVE	*M. emarginatus*	10
ITALY	PIEMONTE	REVELLO	NON INVASIVE	*M. emarginatus*	10
ITALY	PIEMONTE	PAPERIA	NON INVASIVE	*M. emarginatus*	10
ITALY	PIEMONTE	RACCONIGI	NON INVASIVE	*M. emarginatus*	12
ITALY	PIEMONTE	CRISSOLO	INVASIVE	*M. emarginatus*	30
ITALY	EMILIA-ROMAGNA	TERRA DEL SOLE	INVASIVE/NON INVASIVE	*M. emarginatus*	13/20
ITALY	TOSCANA	VAL D'ARNO	INVASIVE	*M. emarginatus*	20
ITALY	TOSCANA	PARCO VILLA DEMIDOFF	INVASIVE	*M. emarginatus*	20
ITALY	TOSCANA	TENUTA DI SAN ROSSORE	NON INVASIVE	*M. emarginatus*	25
ITALY	MARCHE	ESANATOGLIA	INVASIVE	*M. emarginatus*	9
ITALY	LAZIO	VILLA DOMIZIANO	INVASIVE	*M. emarginatus*	15
ITALY	SARDEGNA	CONCA ZIO STEVANEDDU	INVASIVE	*M. emarginatus*	8
ITALY	SARDEGNA	CASA PORTO FERRO	INVASIVE	*M. emarginatus*	8
SPAIN	JEREZ	JEREZ DE LA FRONTERA	INVASIVE	*M. emarginatus*	2
SPAIN	CORDOBA	CORDOBA	INVASIVE	*M. emarginatus*	2
SPAIN	LA CORUNA	LA CORUNA	INVASIVE	*M. emarginatus*	2
SPAIN	GRANADA	ARENAS DEL REY	INVASIVE	*M. emarginatus*	2
SPAIN	LA RIOJA	LA RIOJA	INVASIVE	*M. emarginatus*	2
MOROCCO	AZROU	DI`OUM-ER-RBIA	INVASIVE	*M. emarginatus*	2
MOROCCO	TETUOAN	OULED ALI MANSOUR	INVASIVE	*M. emarginatus*	2

Table 3.4: list of *Myotis emarginatus* samples analyzed

COUNTRY	REGION	COLONY	SAMPLING	SPECIES	N°SAMPLES
ITALY	LOMBARDIA	LIERNA	INVASIVE	*M. capaccinii*	16
ITALY	PIEMONTE	ISOLA BELLA	NON INVASIVE	*M. capaccinii*	46
ITALY	LAZIO	FONDI	INVASIVE	*M. capaccinii*	5
ITALY	SARDEGNA	GROTTA SU COLORU	INVASIVE	*M. capaccinii*	7
ITALY	SARDEGNA	GROTTA SA ROCCA ULARI	INVASIVE	*M. capaccinii*	3
GREECE	GRECIA	KOUFOVOUNO	INVASIVE	*M. capaccinii*	20
GREECE	GRECIA	MARONIA	INVASIVE	*M. capaccinii*	20
SPAIN	ALMERIA	PULPI	INVASIVE	*M. capaccinii*	2
SPAIN	HUESCA	ALCAMPELL	INVASIVE	*M. capaccinii*	2
SPAIN	VALENCIA	NAQUERA/ROTOVA	INVASIVE	*M. capaccinii*	2
SPAIN		SERON	INVASIVE	*M. capaccinii*	2

Table 3.5: list of *Myotis capaccinii* samples analyzed

3.1 MICROSATELLITES

Most of the samples collected until September 2010 were manually and automatically extracted using the Zymo extraction kit (Zymo research). The automatic extraction was carried out using the ***MULTIPROBE IIEX*** robot (Perkin Elmer). *Myotis emargiantus* samples were analyzed using the following set of seven microsatellites: A13-G30-D15-E24-H19-H29 (Castella and Ruedi, 2000), while *Myotis capaccinii* samples were analyzed using the following seven microsatellites: A13-D15-E24-G25-H19-H29-F19 (Castella and Ruedi, 2000, Bilgin, 2008)

3.1.2 NON-INVASIVE ANALYSES

All the 239 scat samples were initially submitted to a preliminary quality screening test by PCR at 2 microsatellite *loci*, using a multiple tube approach (Taberlet *et al*., 1996; Gagneux *et al*.1997).

A total of 109/193 (56,5%) samples of *M. emarginatus*, after the multiple tubes procedure, showed to possess enough DNA to complete the analyses so they were amplified at the other 4 microsatellite *loci*, necessary for the individual genotype identification, after the second screening eleven samples were discarded. In total, 98 faecal samples (51% of total analyzed samples or 90% of screening test positive samples) obtained a complete and reliable genotyping.

On the contrary 10/46 samples of *M. capaccinii* (21,7%) passed the first screening step, and were then analyzed at the other 5 microsatellites. None of them was discarded (Table 3.3).

SPECIES	SAMPLES ANALYZED	SAMPLES PASSED FIRST SCREENING	SAMPLED GENOTYPIZED	% SUCESS
M. emarginatus	193	109	98	56,5%
M. capaccinii	46	10	10	21,70%

Table 6.3: summary of NGS analysis

The 98 reliable genotypes from *Myotis emarginatus* were submitted to the regrouping procedure carried out by the software Gimlet v.1.3.2 (Valière, 2002) to determine if the same genotype of an individual was present several times or if there was a match between non-invasive and invasive genotypes. This procedure allows us to detect that on a total of 181 *Myotis emarginatus* samples 97 different unique genotypes were identified. For *Myotis capaccinii* we followed the same procedure detecting no matches and identifying 10 unique genotypes.

These unique genotypes, detected through genotyping at 6 microsatellite loci for *M. emarginatus* and at 7 for *M. capaccinii*, were analyzed with all the 353 tissues samples using an admixture model implemented in ***Structure v. 2.1*** (Pritchard *et al*., 2000; Falush *et al*,.

2003) to establish their belonging population considering their microsatellite allele frequencies.

We calculated with the software Genalex, the PI of all the loci. Using the eight colonies of *M. emarginatus* it varied between 9.89 and 1.75 * 10-6 * 10-7, these values are significant, considering the population of *M. emarginatus* in Italy around 100000 individuals, these values must be allowed if they do not exceed 0.00001.

We calculated the different genotyping errors with the software Gimlet. *Myotis emarginatus* samples showed the dropout average among loci value of 31%, and false alleles (FA) values of 0.0%. If we look at the values through the different loci we have values ranging from 10% (at locus) to 46% (at locus), while values of the false alleles are equal to 0.0%. In the case of *Myotis capaccinii* average values of ADO and FA among loci are both equal to 0.0%.

	A13	G30	D15	E24	H19	H29	Mean
POSITIVE PCR	76%	71%	95%	60%	90%	55%	74%
ADO	20%	46%	10%	43%	26%	43%	31%
FA	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 3.4: rates of positive *Myotis emarginatus* PCRs, allelic dropouts (ADO) and false alleles (FA) observed using replicated PCRs of 6 microsatellite *loci* in genotyped excremental DNA samples. The 6 *loci* considered are the *loci* used for the individual identification.

	A13	D15	E24	G25	H19	H29	F19	Mean
POSITIVE PCR	38%	100%	88%	94%	94%	100%	60%	85%
ADO	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
FA	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 3.5: rates of positive *Myotis capaccinii* PCRs, allelic dropouts (ADO) and false alleles (FA) observed using replicated PCRs of 7 microsatellite *loci* in genotyped excremental DNA samples. The 7 *loci* considered are the *loci* used for the individual identification

All FA rates showed a value of 0 in both species, in *Myotis capaccinii* also ADO values are 0, probably because in that case we analyzed only 10 samples, in fact the ADO pattern increases at the samples number increase.

The fact that *locus* G30, E24 and H29 in *M. emarginatus* presented the highest ADO rate and A13 in *M. capaccinii* showed the lowest amplification success, could be due to the length of its amplified DNA sequence and to the high molecular weight of its alleles.

The highest values of genotyped samples (genotyping success) were obtained in monospecific colonies, as it is showed in table 3.6. In the polyspecific colonies there are from three to five different species over *Myotis emarginatus*, in case of *Myotis capaccinii* there are two other species This means that the more species are in colony the more difficult will be to pick non invasive samples related to the species under study.

COUNTRY	REGION	COLONY	SAMPLING	SPECIES	N° SAMPLES	N° GENOTYPES	%
ITALY	FRIULI	CORMONS	POLYSPECIFIC	*M. emarginatus*	20	8	40%
ITALY	PIEMONTE	TRINITA	POLYSPECIFIC	*M. emarginatus*	43	12	28%
ITALY	PIEMONTE	TETTI PESIO	MONOSPECIFIC	*M. emarginatus*	17	12	70%
ITALY	PIEMONTE	VENARIA REALE	MONOSPECIFIC	*M. emarginatus*	16	12	75%
ITALY	PIEMONTE	BRICHERASIO	MONOSPECIFIC	*M. emarginatus*	10	6	60%
ITALY	PIEMONTE	PASSERANO	MONOSPECIFIC	*M. emarginatus*	10	4	40%
ITALY	PIEMONTE	REVELLO	MONOSPECIFIC	*M. emarginatus*	10	8	80%
ITALY	PIEMONTE	PAPERIA	MONOSPECIFIC	*M. emarginatus*	10	9	90%
ITALY	PIEMONTE	RACCONIGI	MONOSPECIFIC	*M. emarginatus*	12	8	67%
ITALY	EMILIA-ROMAGNA	TERRA DEL SOLE	POLYSPECIFIC	*M. emarginatus*	20	0	0%
ITALY	TOSCANA	TENUTA DI SAN ROSSORE	MONOSPECIFIC	*M. emarginatus*	25	18	72%
ITALY	PIEMONTE	ISOLA BELLA	POLYSPECIFIC	*M. capaccinii*	46	10	22%

Table 3.6: colonies where NGS samples were collected. The percenteage column shows the amount of genotyped samples

The lowest value of identified genotypes was detected in Terra del sole's colony, this could be explained considering that in this colony there are five different species over *Myotis emarginatus* and due to that probably during samples recovering some errors occurred.

Considering both monospecific and polyspecific colonies, the global genotyping success values are respectively 70% genotyped samples for monospecific colonies and 23% in polyspecific ones (Table 3.7 and Fig. 3.1).

	SAMPLES	GENOTYPES	%
MONOSPECIFIC COLONIES	110	77	70%
POLYSPECIFIC COLONIES	129	30	23%

Table 3.7: global resume of NGS samples genotyped in monospecific and polyspecific colonies respectively

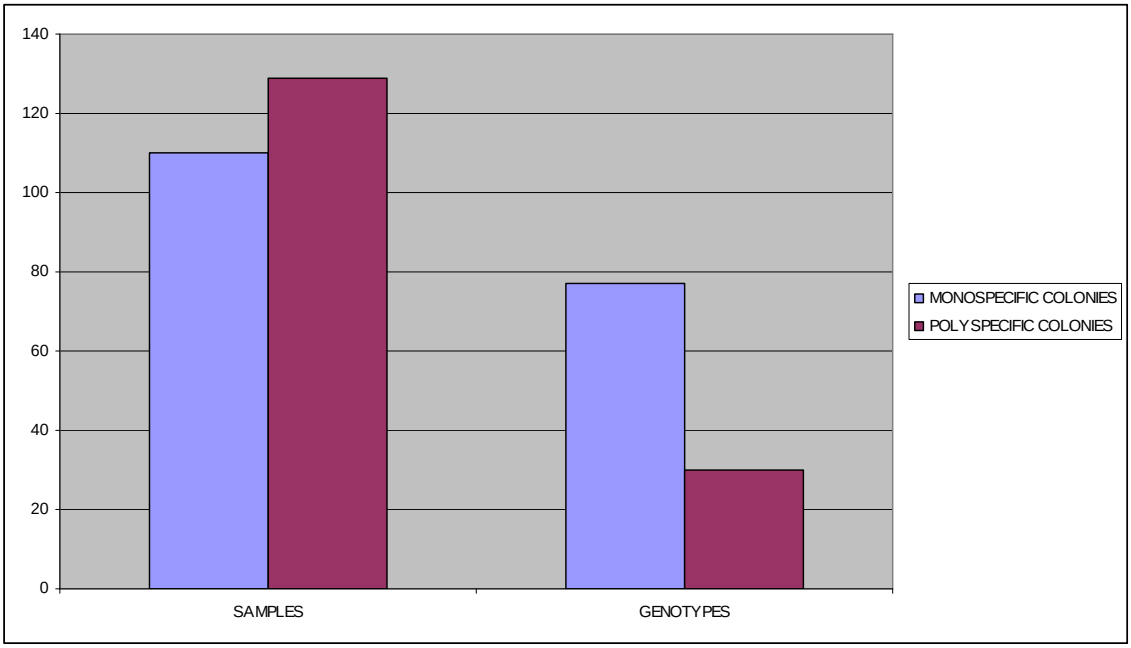


Figure 3.1: graph of NGS samples collected and genotyped

The 107 distinct non-invasively detected bats genotypes in this study were also used to estimate some of the chief parameters of Population Genetics using the programs GeneAlex v. 6.0 (Peakall & Smouse, 2005; 2006) and Genetix v.4.2 (Belkhir *et al.*, 2001).

All *loci* in *Myotis emargiantsu* were polymorphic in the Italian bats population, showing high values of both observed and expected heterozygoses (**HO** = 0,59-0,74; **HE** = 0,62-0,80) and a mean number of alleles per *locus* of 10 ranging from 6 (D15) to 13 (A13) (Table 3.8).

	A13	G30	D15	E24	H19	H29	Mean Value
Na	13	11	6	12	10	8	10
Hobs	0.8454	0.6392	0.6701	0.9175	0.4021	0.7216	0.6993
Hexp	0.8675	0.8373	0.6943	0.8504	0.7490	0.7593	0.7930

Table 3.8: values of *Myotis emarginatus* Ho and He for each locus

The heterozygosis values, ranged from a minimum of 0.5833 to a maximum of 0.8148, as can be seen from Table 3.9. In *Myotis capaccinii*, in the only non-invasively sampled colony, the value is 0.5714 Hobs

COLONY	Hexp	Hobs
CORMONS	0.6914	0.5833
TRINITA	0.7656	0.7639
TETTI PESIO	0.7459	0.7778
VENARIA REALE	0.7465	0.6389
BRICHERASIO	0.7431	0.6667
PASSERANO	0.6823	0.6667
REVELLO	0.7005	0.6458
PAPERIA	0.7346	0.8148
RACCONIGI	0.7370	0.6458
SAN ROSSORE	0.7770	0.7037
ISOLA BELLA	0.4914	0.5714

Table 3.9: values of Ho and He for each colnies

3.1.3 *Myotis emarginatus*

These are the first results regarding *Myotis emarginatus's* population genetics structure. For these species we used tissue samples and individuals that we previously genotyped using NGS samples. All tissue samples were analyzed following the same procedures used for non-invasive samples, but replicating the analyses twice per sample per locus. We run the software GIMLET obtaining ADO and FA values for all loci equal to 0,0, probably because these samples were collected from alive animals and put immediately in alcohol to preserve them (Table 3.10).

	A13	G30	D15	E24	H19	H29	Mean
POSITIVE PCR	100%	100%	100%	100%	100%	100%	100%
ADO	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
FA	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 3.10 rates of positive *Myotis emarginatus* PCRs, allelic dropouts (ADO) and false alleles (FA) observed using replicated PCRs of 6 microsatellite *loci* in genotyped tissue DNA samples. The 6 *loci* considered are the *loci* used for the individual identification

3.1.3.1 Genetic structure of *Myotis emarginatus*'s populations

We obtained other 243 genotypes from the 243 tissue samples which were added to the non-invasive ones for a total of 340 *Myoti emargiantus* unique genotypes. They were used to elaborate a PCA graph by software GenAlex (Fig. 3.2)in order to evaluate the relationship among colonies. In a further analysis we put together all samples from Sardinia and Morocco, because of the limited number of these samples.

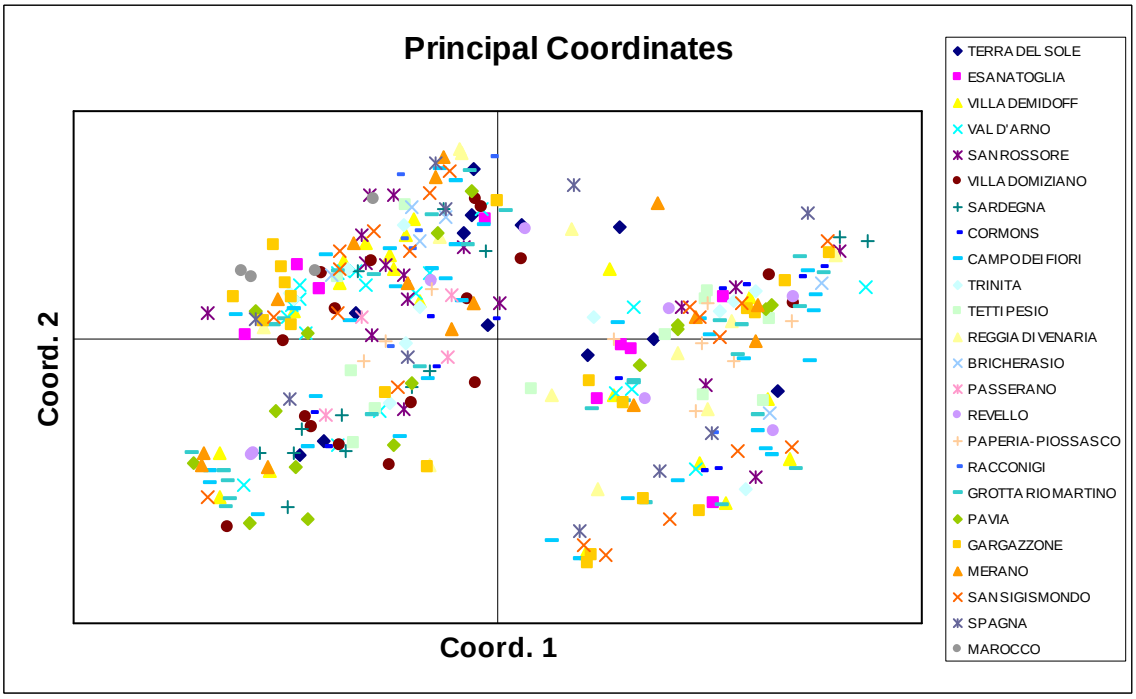


Figure 3.2: PCA graph of 24 *Myotis emarginatus* colonies

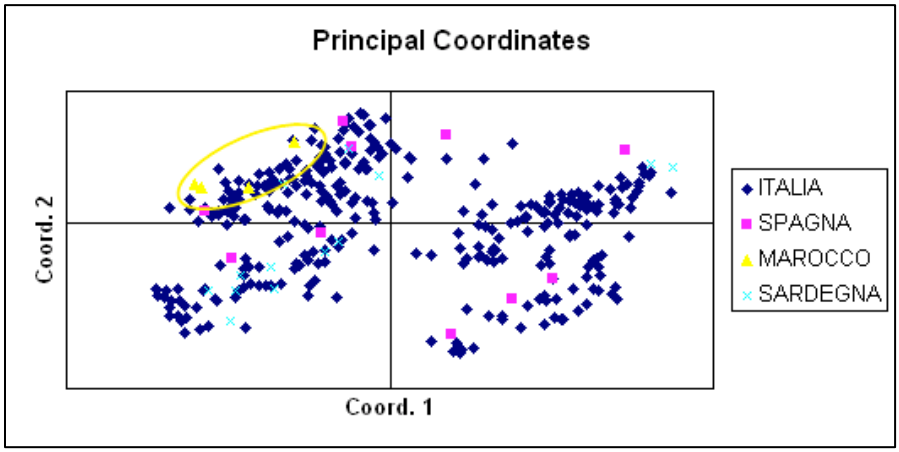


Figura 3.3: PCA graph of 24 *Myotis emarginatus* colonies regrouped in 4 geographic groups

Successively we run a FCA analysis with GENETIX (Belkhir *et al.*, 2001) using the same regrouping method in order to better visualize the clustering.

Results from GENETIX were more clear, in fact the four Moroccan samples are quite far away from other European samples. In this case all three axes can explain all the global diversity (Fig. 3.4).

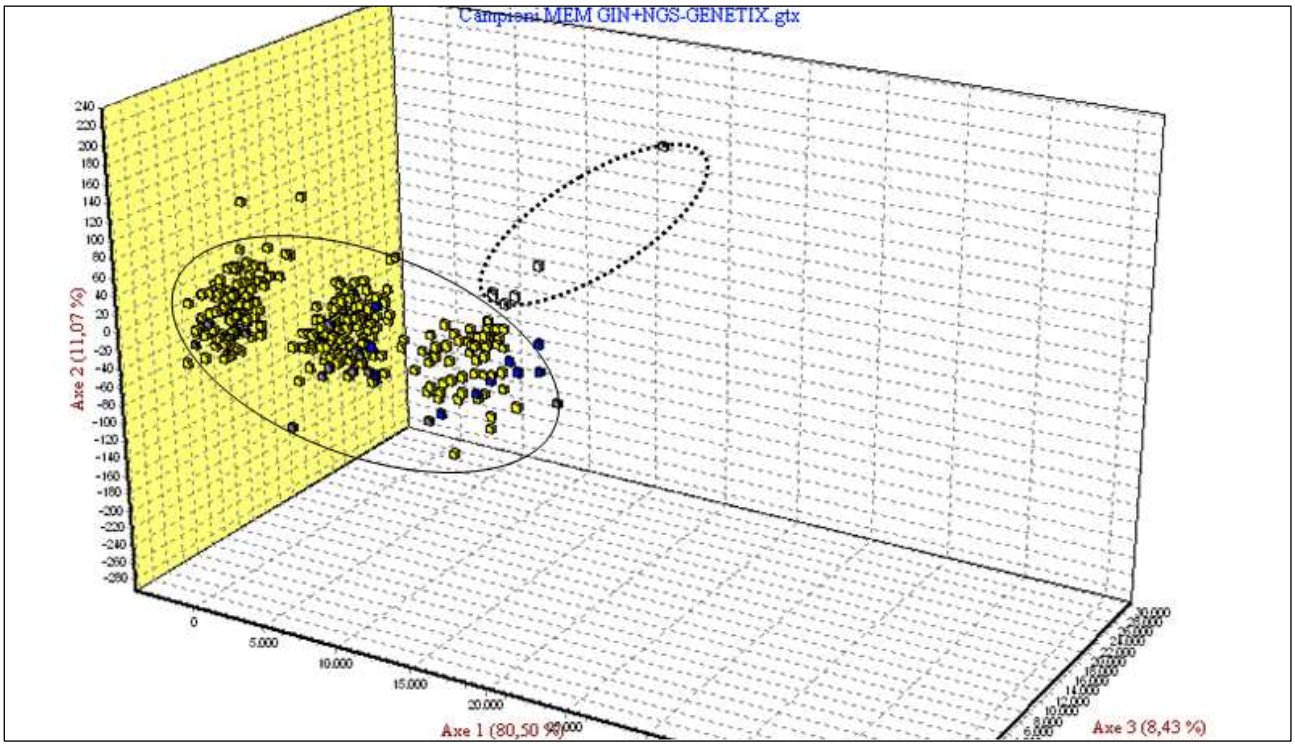


Figure 3.4: FCA graph obtained using GENETIX, the Moroccan samples are coloured in white, others are in blue (Spain), yellow (Italy) and grey (Sardinia)

To verify if the four Marocco samples would have some influence on the global genetic structure, we used software STRUCTURE (Pritchard *et al*., 2000; Falush *et al*,. 2003).

We select K=2 after verify result with Structure Harvester. The best value of Delta was obtained with K=2 as showed in Figure n. 3.5 and Table 3.10.

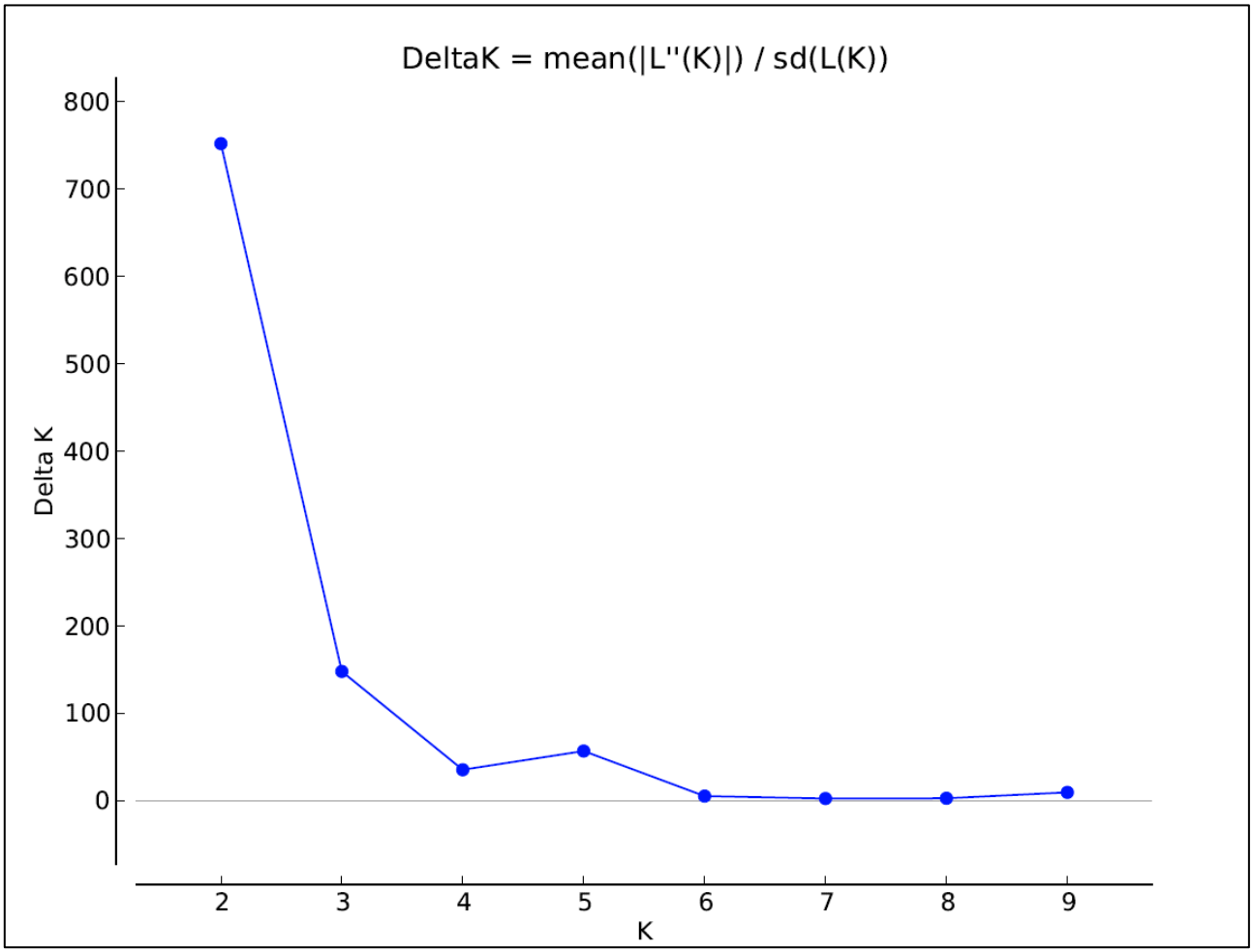


Figure 3.5: Estimate number of population (K) derived from the structure clustering analyses. The magnitude of Delta K was calculated using the method described by Evanno et al. (2005) and implemented in Structure Harvester

K	Reps	Mean LnP(K)	Stdev Ln'(K)	Ln'(K)	|Ln"(K)|	Delta K
1	5	-7292.790000	0.056765	-	-	-
2	5	-6917.288889	0.257121	375.501111	193.372222	752.067567
3	5	-6735.160000	0.665582	182.128889	98.308889	147.703547
4	5	-6651.340000	0.698570	83.82000	24.520000	35.100278
5	5	-6543.000000	1.166190	108.34000	65.960000	56.560233
6	5	-6500.620000	1.918854	42.380000	9.540000	4.971718
7	5	-6448.700000	5.259753	51.920000	11.500000	2.186415
8	5	-6385.280000	2.156850	63.420000	5.300000	2.457288
9	5	-6316.560000	3.516817	68.720000	32.400000	9.212877
10	5	-6280.240000	4.074678	36.320000	-	-

Table 3.10: Evanno Table, K = populations; Reps = replicates; Mean LnP(K) = log probability of the data; Stdev LnP(K) = standard deviation of log probability of the data; Ln'(K) = $LnP(K)_n$ - $LnP(K)_{n-1}$; $|Ln''(K)| = Ln'(K)_n - Ln'(K)_{n-1}$; Delta K = [Ln''(K)]/Stdev LnP(K)

In this study 130000 simulations were used (30000 as *burnings*) to carried out the assignment. Resuls show that 196 samples were assigned to one population and other 146 to a second one. The plot showed a non specific population genetic pattern, but it is possible to identify two big groups which characterized the entire population. A genetic component may be described as characteristic of Morocco, as the four samples from that area show all the same component,

and a second component might be characteristic of populations in Europe and not identified in Moroccans samples (Fig. 3.6).

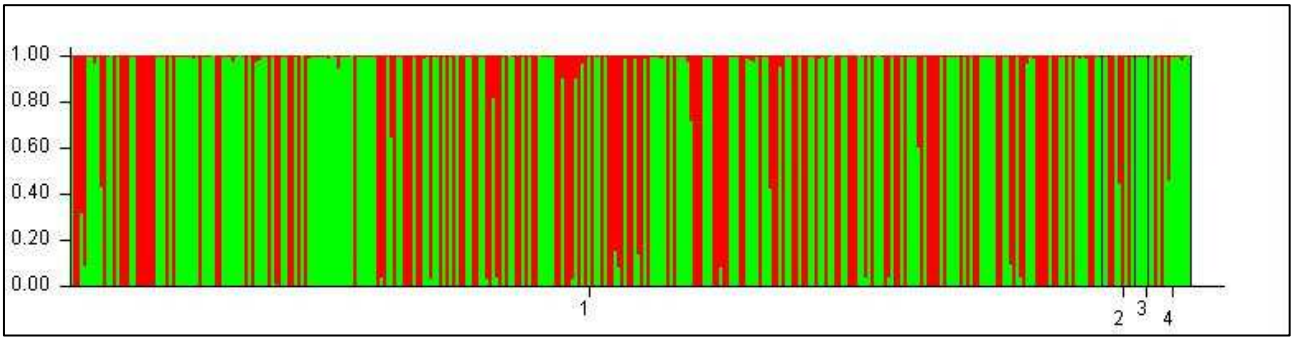


Figure 3.6: Genetic clusters obtained with two groups. Each individuals is represented by one vertical line with K segments coloured proporzionally according to their belonging to a genetic cluster. Black lines separate individual bats from different populations, classified as following: 1, Italy, 2: Sardinia, 3 Spain and 4 Morocco

3.1.3.2 Population genetics analysis and microsatellite variability

The 269 distinct *Myotis emarginatus* genotypes invasively and non-invasively detected in this study were also used to estimate some of the chief parameters of Population Genetics using the programs GeneAlex v. 6.0 (Peakall & Smouse, 2005; 2006) and Genetix v.4.2 (Belkhir *et al.*, 2001).

All *loci* were polymorphic in the European bat population, showing high values of heterozygosis (**HO** = 0,32-0,82; **HE** = 0,70-0,86) and a mean number of alleles per *locus* of 11,17 ranging from 7 (D15) to 14 (A13) (Table 3.11).

	A13	G30	D15	E24	H19	H29	Mean Value
Na	14	13	7	13	11	9	11.17
Hobs	0.8279	0.4139	0.6469	0.8424	0.4388	0.3252	0.5825
Hexp	0.8646	0.8040	0.7011	0.8601	0.7763	0.7524	0.7931

Table 3.11: values of *Myotis emarginatus* Ho and He for each locus, Na = number of allele observed

Most of the analyzed samples were in Hardy-Weinberg equilibrium (Table 3.12) without excesses or a defects in heterozygous or homozygous in comparison with the expected values over all loci . The exceptions are the colony of Trinità, Tetti Pesio, Passerano, Paperia and Morocco.

COLONY	He	Ho	N_{Amean}	Np	Fis	Fst
TERRA DEL SOLE	0.7470	0.5032	6,000	1	0.36971*	0.02885
ESANATOGLIA	0.6975	0.4907	5,500	0	0.35097*	0.02727
VILLA DEMIDOFF	0.7904	0.6167	7,667	1	0.24409*	0.02025
VAL D'ARNO	0.7759	0.5637	7,500	0	0.29786*	0.02265
SAN ROSSORE	0.7745	0.7255	7,000	2	0.09342*	0.02102
VILLA DOMIZIANO	0.7716	0.5306	7,667	0	0.33977*	0.02181
SARDINIA	0.7034	0.5053	7,667	0	0.31978*	0.02713
CORMONS	0.6992	0.5712	6,500	1	0.22781*	0.02902
CAMPO DEI FIORI	0.7654	0.5020	7,500	0	0.35780*	0.02580
TRINITA	0.7691	0.7639	6,167	0	0.05024	0.02129

COLONY	He	Ho	N_{Amean}	Np	Fis	Fst
TETTI PESIO	0.7459	0.7778	6,333	1	0.00081	0.02241
REGGIA DI VENARIA	0.7465	0.6389	6,000	0	0.18650*	0.02238
BRICHERASIO	0.7431	0.6667	5,167	1	0.19192*	0.02255
PASSERANO	0.6823	0.6667	4,167	0	0.16522	0.02551
REVELLO	0.7005	0.6458	4,833	0	0.14398*	0.02462
PAPERIA-PIOSSASCO	0.7346	0.8148	5,833	0	-0.05075	0.02297
RACCONIGI	0.7370	0.6458	5,833	0	0.18879*	0.02285
GROTTA RIO MARTINO	0.7559	0.5050	7,833	0	0.34804*	0.02440
PAVIA	0.7559	0.5104	6,500	0	0.35328*	0.02193
GARGAZZONE	0.7518	0.4803	6,667	0	0.38364*	0.02345
MERANO	0.7681	0.6111	5,833	0	0.23725*	0.02133
SAN SIGISMONDO	0.7777	0.5833	7,000	1	0.27382*	0.02087
SPAIN	0.6421	0.5037	5,333	1	0.26660*	0.02849
MOROCCO	0.4769	0.5694	3,000	0	-0.03797	0.03766

Table 3.12: genetic diversity in 24 *Myotis emarginatus* nursery colonies genotyped at 6 unlinked microsatellite *loci.* Ho = observed heterozygosity, He = expected heterozygosity, NAmean = mean number of alleles per *locus* (direct count), NP = number of private alleles. Departures from Hardy–Weinberg equilibrium were assessed for the population from average multilocus FIS values, FST = effect of subpopulations or individual diversity coefficient within the population

The global AMOVA (Table 3.14) indicated that, using all the loci, the percent of variation attributable to population differences was 97,49%. The Fst values for each locus were very close to zero (Table 3.14), also indicating the weak differentiation in microsatellites. These values also showed that, for each locus, within population differentiation comprised at least 95% of all the observed differentiation.

	Fst	Within population variance	Among population variance
GLOBAL	0.02504	97,49%	2,51%
A13	0.01524	98,47%	1,53%
G30	0.02710	97,28%	2,71%
D15	0.01399	98,40%	1,60%
E24	0.00867	99,13%	0,87%
H19	0.04351	95,65%	4,35%
H29	0.04401	95,60%	4,40%

Table 3.14: the results of global and locus-by locus AMOVAs for microsatellites based on Fst , and the distribution of variance

ARLEQUIN was used also to calculate the overall level of nuclear genetic differentiation among European populations of *M. emarginatus*, which was relatively low (FST= 0.024), but nevertheless significant ($P < 0.001$). Not all pairwise FST values between colonies were significant at the 5% level and ranged between -0.011 (Revello — Tetti Pesio) and 0.2059 (Morocco — Passerano; Table3.15. Only samples from Morocco showed a significant Fst value ($P \leq 5\%$).

STR variability along the transect of Italian populations of *M. emarginatus* does not follow a pattern of IBD ($R2 = 0.163$, $P = 0.16$), suggesting that nuclear gene flow at this scale is not simply a function of geographical distance, Because this would require continuous sampling.

	TERRA DEL SOLE	ESAIATOGLIA	VILLA DEMIDOFF	VAL D'ARNO	SAII ROSSORE	VILLA DOMIZIANO	SARDINIA	CORMONS	CAMPO DEI FIORI	TRINITA	TETTI PESO	REGGIA DI VENARIA	BRICHERASIO	PASSERANO	REVELLO	PAPERIA-PIOSSASCO	RACCONIGI	GROTTA RIO MARTINO	PAVIA	GARGAZZONE	MERAINO	SAII SIGISMONDO	SPAIN	MOROCCO
TERRA DEL SOLE	0.00000																							
ESAIATOGLIA	0.05514	0.00000																						
VILLA DEMIDOFF	0.01384	0.02192	0.00000																					
VAL D'ARNO	0.01828	0.02378	0.01031	0.00000																				
SAII ROSSORE	0.00410	**0.05502**	0.01180	0.02007	0.00000																			
VILLA DOMIZIANO	0.00450	**0.06744**	0.00793	**0.02899**	0.02439	0.00000																		
SARDINIA	0.01446	**0.09164**	0.01485	**0.04394**	**0.03822**	-0.00398	0.00000																	
CORMONS	0.01741	**0.09918**	0.02289	**0.04393**	**0.03542**	0.01827	0.03378	0.00000																
CAMPO DEI FIORI	0.00558	0.02699	0.00140	**0.02911**	**0.02243**	0.01203	0.02026	0.01564	0.00000															
TRINITA	0.01584	0.04184	0.00311	0.02442	0.00861	0.02133	0.03029	0.02753	0.01746	0.00000														
TETTI PESO	0.00563	**0.05055**	0.02188	0.02585	**0.02606**	0.02767	**0.04545**	0.01057	0.00866	0.01768	0.00000													
REGGIA DI VENARIA	-0.00499	0.02101	0.00802	0.01964	0.01703	0.02532	0.03452	0.02281	-0.00161	0.00587	-0.00961	0.00000												
BRICHERASIO	0.00325	**0.05915**	0.00779	0.02996	0.00454	0.01098	0.04314	0.03169	0.01924	0.00326	0.01610	0.00999	0.00000											
PASSERAIO	0.04802	**0.11964**	0.01095	0.04976	0.03719	0.02000	0.03023	**0.08246**	0.06198	0.02284	**0.07198**	0.04693	0.03480	0.00000										
REVELLO	-0.00934	**0.05569**	0.02095	0.02651	0.03054	0.01853	0.02960	0.01983	0.00583	0.02510	-0.01103	0.00732	0.00611	0.07688	0.00000									
PAPERIA-PIOSSASCO	0.00438	**0.04826**	0.00880	**0.03741**	0.00443	0.02328	**0.04328**	0.01406	0.00289	0.00410	0.00699	-0.00079	0.00228	0.02917	0.00016	0.00000								
RACCONIGI	0.01266	0.05765	0.03723	0.03005	**0.03789**	0.03518	**0.06096**	**0.06634**	**0.04467**	0.03498	0.03531	0.00781	0.02817	0.04526	0.05792	**0.05449**	0.00000							
GROTTA RIO MARTINO	0.01978	0.02340	0.00273	0.01715	**0.02299**	0.01872	**0.03209**	0.02734	-0.00088	0.01676	0.00535	0.00366	0.02069	0.07245	**0.00182**	0.00532	**0.06496**	0.00000						
PAVIA	0.00928	0.02514	0.00132	0.00636	0.01671	0.00332	0.00977	0.02969	0.00133	0.01029	0.01207	0.01251	0.03466	**0.04226**	-0.00331	0.01702	0.04048	0.00413	0.00000					
GARGAZZONE	0.03475	0.02887	0.01212	**0.03110**	**0.03571**	**0.04796**	0.06734	**0.04959**	**0.02930**	0.00255	0.02061	0.02396	0.05798	0.07501	0.03601	0.02634	**0.05751**	0.02342	0.02426	0.00000				
MERAINO	-0.00476	0.03071	0.00846	0.02554	**0.02725**	0.02403	**0.03833**	**0.04248**	0.01921	0.01324	0.02437	0.01610	0.01440	0.03832	0.02168	0.02148	0.01993	**0.02693**	0.02420	0.00748	0.00000			
SAII SIGISMONDO	-0.00033	0.02102	0.00400	0.01955	0.02279	0.02087	**0.02613**	0.01045	-0.00217	0.01025	-0.00086	-0.00146	0.01631	0.06203	-0.00630	0.01063	0.02876	0.00417	0.00385	0.01309	0.00549	0.00000		
SPAIN	0.02839	**0.08648**	**0.04666**	**0.06425**	**0.03239**	0.03584	0.03545	0.03867	0.01844	0.03618	0.03237	0.01732	0.05425	**0.11971**	0.04335	0.03572	0.05828	0.03325	0.02617	**0.06814**	**0.05204**	0.02922	0.00000	
MOROCCO	**0.13983**	**0.12852**	**0.11882**	**0.11639**	**0.07419**	**0.13632**	**0.14873**	**0.22930**	**0.13297**	**0.10322**	**0.14577**	**0.11590**	**0.13205**	**0.20594**	**0.13073**	**0.16155**	**0.10745**	**0.13406**	**0.09272**	**0.13528**	**0.10074**	**0.10440**	**0.09175**	0.00000

Table 3.15: estimates of population differentiation among the 24 nursery colonies of *Myotis emarginatus*

The NJ tree representing the nuclear genetic relationships among all European populations is also consistent with a major subdivision between Italian, Spain and other Moroccan samples (Fig. 3b), In particular, most of all Italian colony, except for San Rossore, cluster in one big group. These analyses of nuclear variation clearly indicate that bats from Italy are more closely related to each other than to populations found southerly, in Spain and Morocco. In turn, all of those analyses suggest that the Moroccan population (Dì Oum Erbia and Ouled ali Mansour) is distinct from those of the European continent.

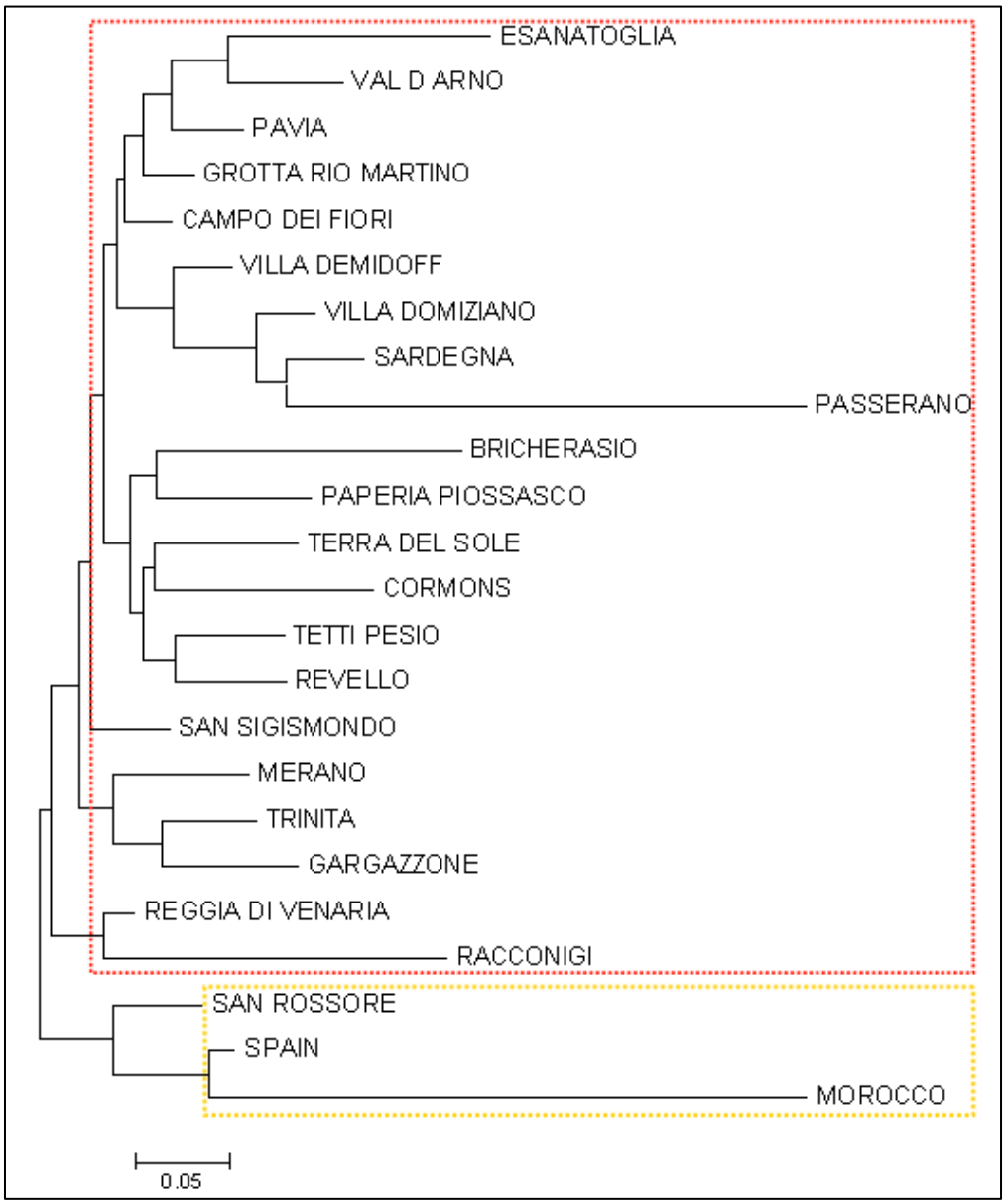


Figura 3.7: Unrooted NJ trees of linearized pairwise FST among nursery colonies derived from STR loci

3.1.2 *Myotis capaccinii*

This is the second work about *Myotis capaiccinii*, and the first considering almost the entire areal of this species. We used 79 tissues samples and 10 individuals that we previously genotyped too. All tissue samples were analyzed following the same procedures used for non-invasive samples, but replicating the analyses twice per sample per locus Populations analyses were also the same used in *Myotis emarginatus*. ADO and FA values from GIMLET in tissue samples resulted 0,0 for all loci (Table 3.16) .

	A13	G25	D15	E24	H19	H29	F19	Mean
POSITIVE PCR	100%	100%	100%	100%	100%	100%	100%	100%
ADO	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
FA	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%

Table 3.16: rates of positive *Myotis capaccinii* PCRs, allelic dropouts (ADO) and false alleles (FA) observed using replicated PCRs of 7 microsatellite *loci* in genotyped excremental DNA samples. The 7 *loci* considered are the *loci* used for the individual identification

3.1.2.1 Genetic structure of *Myotis capaccinii*'s populations

The 79 analysed tissues produced 79 genotypes which were added to the N non-invasive ones for a total of 89 *Myoti capaccini* unique genotypes. They were used to elaborate a PCA graph with GenAlex in order to evaluate the relationship among colonies. In a further analysis we put together all samples from Sardinia and Spain, because also for this species we had only a few samples from those regions. In the first PCA we regrouped samples in their six native colonies. It showed that these six colonies could be easily separated from each other. We then run a FCA analysis with GENETIX (Belkhir *et al.*, 2001) using the same regrouping method in order to better visualize the clustering. It showed a very clear genetic structure: all sample clustered in six different groups, corresponding to the different colonies (Fig.3.8).

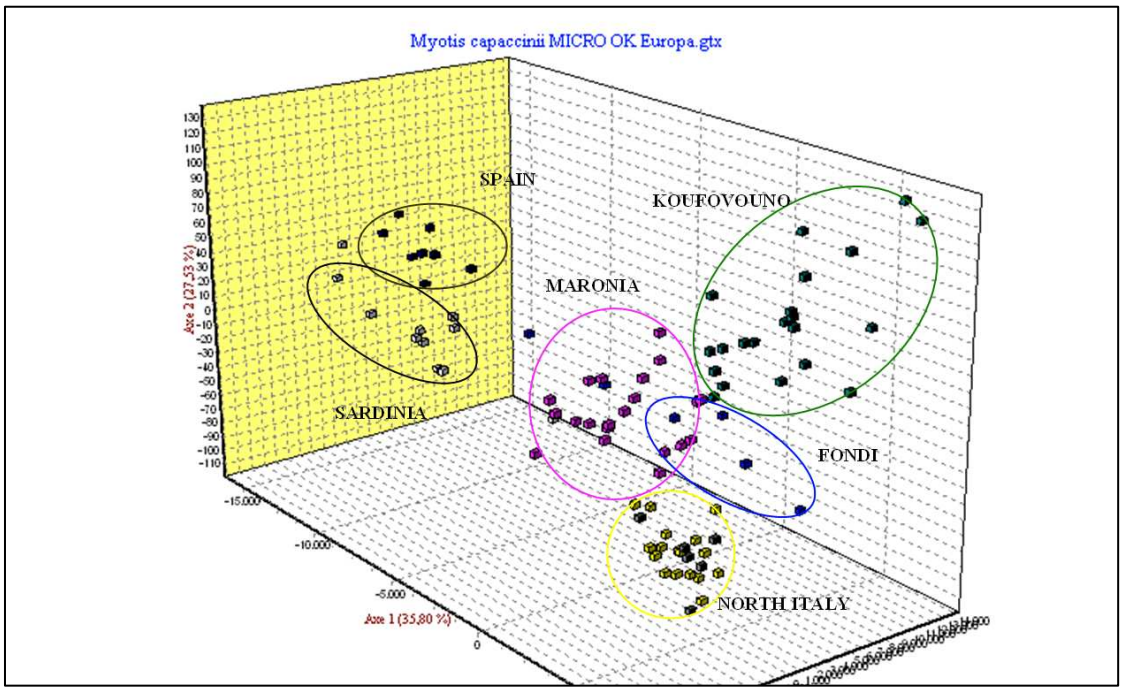


Figure 3.8: FCA graph obtained using GENETIX, all the seven colonies regrouped in six well defined groups

The only exception is represented by the two colonies found in northern Italy, which cluster in one group, probably due to the fact that this two colonies are not so far away (around 60 Km) one from each other.

At this point we carried out another analysis using the Adegenet Package, based on R language, in order to draw another PCA, which could confirm our previous results. Surprisingly this new PCA showed a more important role played by the colony of Fondi, in fact in this graph Fondi would seem to be a point of connection between two different genetic lineage, one composed by Spain and Sardinia and other two described by North Italy and Greece clusters (Fig. 3.9).

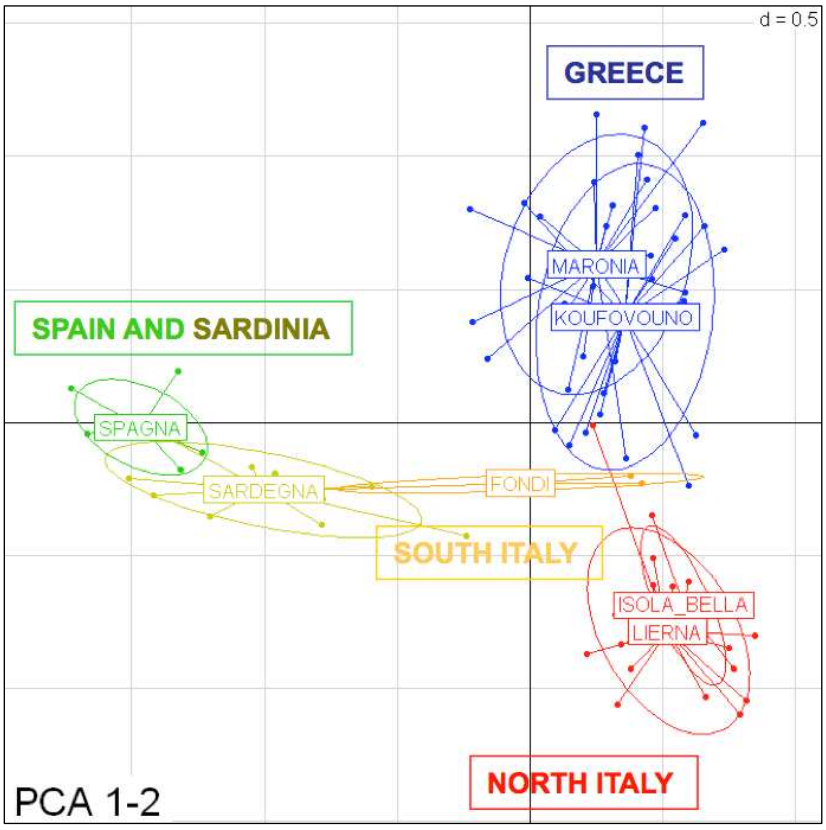


Figure 3.9: First and second components of a principal component analysis (PCA) of 7-locus micro satellite genotype from 89 *Myotis capaccinii* samples from Europe

Using again Adegenet package we performed an assignment test, in order to see if all samples could be plotted in the same colonies. All samples were regrouped in their own colonies, expect for one sample found in the colony of Lierna and another one in the colony of Isola Bella. Again this could be explained considering that this two colonies are close one to each other (Fig. 3.10).

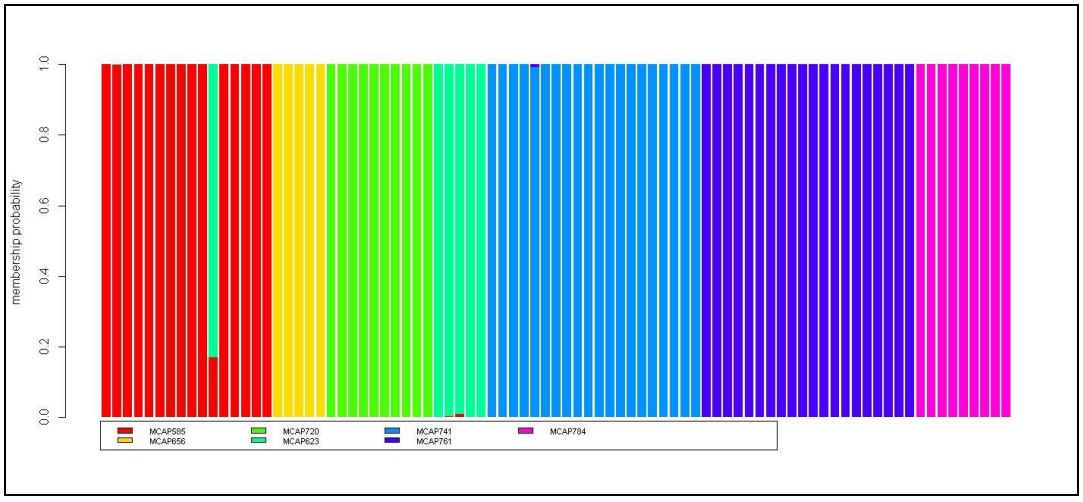


Figure 3.10: assignment test output, each sample was regrouped in its own colony. Red: Isola Bella, Yellow: Fondi, Green: Sardinia; White green: Isola Bella; Blue: Maronia; Violet: Koufovouno; Purple: Spain

We then run STRUCTURE to investigate the global genetic structure of *Myotis capaccinii*'s European population.

The programme was run with five independent simulations for each of K from 1 to 10, each one of 1 000 000 iterations, following a burn-in period of 100 000 iterations. In all simulations, no admixture ancestry model and independent allele frequency models (with l = 1) were used in the first steps. If the population structure is subtle, the model of correlated allele frequencies is often more effective to detect this structuring (Falush et al., 2003).

In the next step, we therefore analysed the data by using the correlated allele frequencies model. The likelihood of K [i.e. Ln Pr(X|K)], obtained using the online software Structure Harvester, was used to infer the number of real populations in the datasets. In this case the optimal K was 3 (Fig. 3.11; Table 3.17).

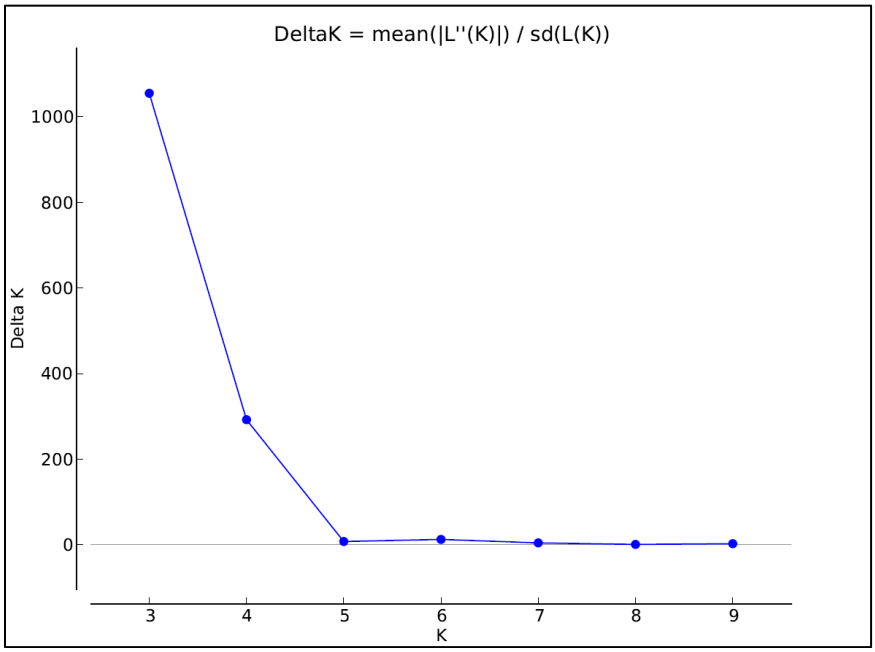


Figure 3.11: Estimate number of population (K) derived from the structure clustering analyses. The magnitude of Delta K was calculated using the method described by Evanno et al. (2005) and implemented in Structure Harvester

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln"(K)|	Delta K
2	5	-1453.960000	3.768023	-	-	-
3	5	-1339.080000	0.083666	114.880000	88.240000	1054.669725
4	5	-1312.440000	0.114018	26.640000	33.320000	292.235732
5	5	-1319.120000	0.192354	-6.680000	1.480000	7.694154
6	5	-1327.280000	0.327109	-8.160000	4.120000	12.595208
7	5	-1331.320000	0,334664	-4.040000	1.500000	4.482107
8	5	-1333.860000	0.364692	-2.540000	0.340000	0.932294
9	5	-1336.060000	0.296648	-2.200000	0.760000	2.561959
10	5	-1337.500000	0.387298	-1.440000	-	-

Table 3.17: Evanno Table, K = populations; Reps = replicates; Mean LnP(K) = log probability of the data; Stdev LnP(K) = standard deviation of log probability of the data; Ln'(K) = $LnP(K)_n - LnP(K)_{n-1}$; $|Ln''(K)| = Ln'(K)_n - Ln'(K)_{n-1}$; Delta K = [Ln''(K)]/Stdev LnP(K)

The plot showed a specific population genetic pattern, in which it is possible to identify three different groups characterizing the entire European population. One group is typically Italian, in fact here we found the two North_Italian colonies of Lierna and Isola Bella, the second includes samples collected in Sardinia and Spain. The latest is the one with the two Greek colonies of Maronia and Kovoufono. A particular situation was showed by the colony of Fondi, in fact here we can identify all of the three genetic components of the groups previous described, showing possible contact zone of different genetic lineages (Fig. 3.12).

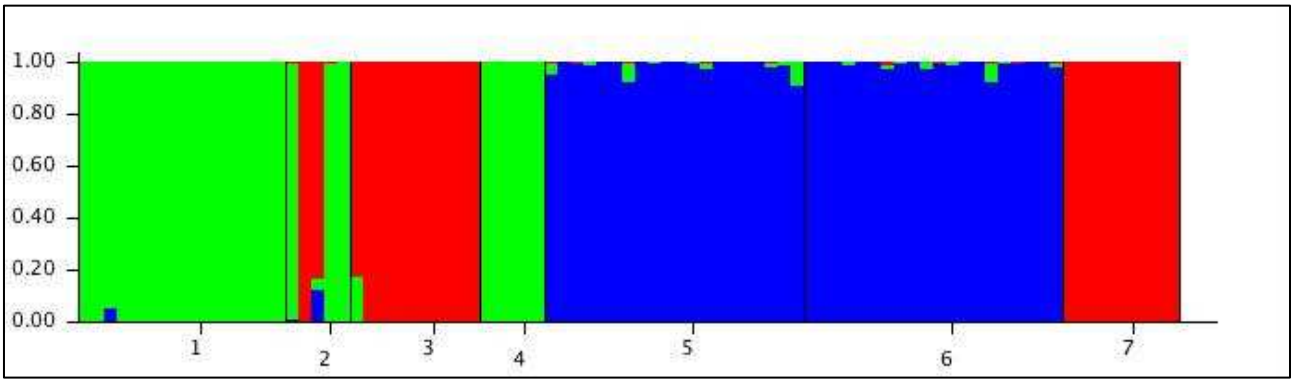


Figure 3.52: Bayesian clustering and individual assignment output from the program Structure show genotypic clustering according to geographic distribution of the colonies with some evidence of admixture within some cluster. Arrows Vertical axis is the probability of assignment (Q) to each of the clusters, and the horizontal axis represents the nursery colonies sampled. 1: Lierna; 2: Fondi; 3: Sardinia; 4: Isola Bella; 5: Maronia; 6: Koufovouno; 7: Spain.

3.1.2.2 Population genetics analysis and microsatellite variability

We used the 89 distinct *Myotis capaccinii* genotypes invasively and non-invasively detected in this study to estimate some of the chief parameters of Population Genetics using even with this species the programs GeneAlex v. 6.0 (Peakall & Smouse, 2005; 2006) and Genetix v.4.2 (Belkhir *et al.*, 2001).

All *loci* were polymorphic in the Europe bat populations, showing different values of heterozygosis (**HO** = 0,14-0,71; **HE** = 0,46-0,83) and a mean number of alleles per *locus* of

10 ranging from 4 (H29-G25) to 12 (D15) (Table 3.18). Some loci showed a low HO (F19, H29 and E24) others instead high HO (H19 and A13), however all these values are comparable to those found by Bilgin, HO = 0,10-0,75; HE = 0,27-0,82).

	A13	G25	D15	E24	H19	H29	F19	Mean Value
Na	8	4	12	6	11	4	11	10.87
Hobs	0.7160	0.4405	0.5119	0.3889	0.6905	0.3095	0.1429	0.4572
Hexp	0.7372	0.6407	0.8034	0.4540	0.7970	0.4646	0.8351	0.6760

Table 3.18: values of *Myotis capaccinii* Ho and He for each locus, Na = number of allele observed

Only colonies of Sardinia, Spain and Maronia were in Hardy-Weinberg equilibrium. In total we found 15 private alleles, most of them were in colony of Maronia, Greece (Table 3.19).

	Hexp	Hobs	Namean	Np	Fis	Fst
LIERNA	0.5230	0.5115	3,28	2	0.05548	0.25321
FONDI	0.5367	0.5071	2,71	0	0.19318	0.25296
SARDEGNA	0.5093	0.4571	2,85	0	0.17360*	0.25341
ISOLA BELLA	0.4914	0.5714	2,57	1	0.04192	0.25630
KOUFOVOUNO	0.6097	0.6484	5,28	4	-0.03625	0.24275
MARONIA	0.7001	0.5904	5,85	8	0.19421*	0.24395
SPAGNA	0.3166	0.5238	1,57	0	-0.61963*	0.26313

Table 3.19: genetic diversity in 7 *Myotis capaccinii* nursery colonies genotyped at 6 unlinked microsatellite *loci.* Ho = observed heterozygosity, He = expected heterozygosity, NAmean = mean number of alleles per *locus* (direct count), NP = number of private alleles. Departures from Hardy–Weinberg equilibrium were assessed for the population from average multilocus FIS values, FST = effect of subpopulations or individual diversity coefficient within the population

The global AMOVA (Table 3.20) indicated that, using all of the loci, the percent of variation attributable to population differences was 74,56%. The Fst values for each locus were quite high (Table 2), also indicating a good differentiation in microsatellites. These values also showed that, for each locus, within population differentiation comprised at least 87% of all the observed differentiation

	Fst	Within population variance	Among population variance
GLOBAL	0.25432	74,56%	25,44%
A13	0.23105	76,89%	23,11%
G25	0.38696	61,30%	38,70%
D15	0.28512	71,48%	28,52%
E24	0.12013	87,98%	12,02%
H19	0.12172	87,82%	12,18%
H29	0.21264	78,73%	21,27%
F19	0.35501	64,50%	35,50%

Table 3.20: the results of global and locus-by locus AMOVAs for microsatellites based on Fst , and the distribution of variance

ARLEQUIN was used also to calculate the overall level of nuclear genetic differentiation among European populations of *M. emarginatus*, which was relatively hight (FST= 0.24432), and significant ($P < 0.001$). All pairwise FST values between colonies were significant at the

5% level and ranged between -0.0076 (Isola Bella — Lierna) and 0.55485 (Spain — Isola Bella; Table). Samples from Spain showed a the highest Fst value with a significant level (P ≤ 5%) (Table 3.21).

The microsatellite data set also showed no evidence for isolation by distance; there was only a weak correlation of geographical and genetic distances (R2 = 0.0357, P = 0.85).

	LIERNA	FONDI	SARDINIA	ISOLA BELLA	KOUFOVOUNO	MARONIA	SPAIN
LIERNA	0.00000						
FONDI	***0.16445***	0.00000					
SARDINIA	***0.35196***	***0.27032***	0.00000				
ISOLA BELLA	-0.00766	***0.22658***	***0.38214***	0.00000			
KOUFOVOUNO	***0.15520***	***0.17948***	***0.28031***	***0.16213***	0.00000		
MARONIA	***0.21767***	***0.17692***	***0.28842***	***0.22712***	***0.02675***	0.00000	
SPAIN	***0.49723***	***0.42983***	***0.17416***	***0.55485***	***0.38218***	***0.36109***	0.00000

Table 3.21: estimates of population differentiation among the 7 nursery colonies of *Myotis capaccinii*

The FST linearized, FST/(1-FST) genetic distances evaluated with ARLEQUIN, were used to built a rootless NJ tree, which is also consistent with a major subdivision between Italian, Greece and Sardinian and Spanish samples (Fig. 3.13). In particular, all Italian colony samples that were found into the peninsula cluster in one group. These analyses of nuclear variation clearly indicate that bats from Italy and Greece are more closely related to each other than the populations found farther in Spain and Sardinia. In turn, all of those analyses suggest that there are three different genetic groups in the European continent.

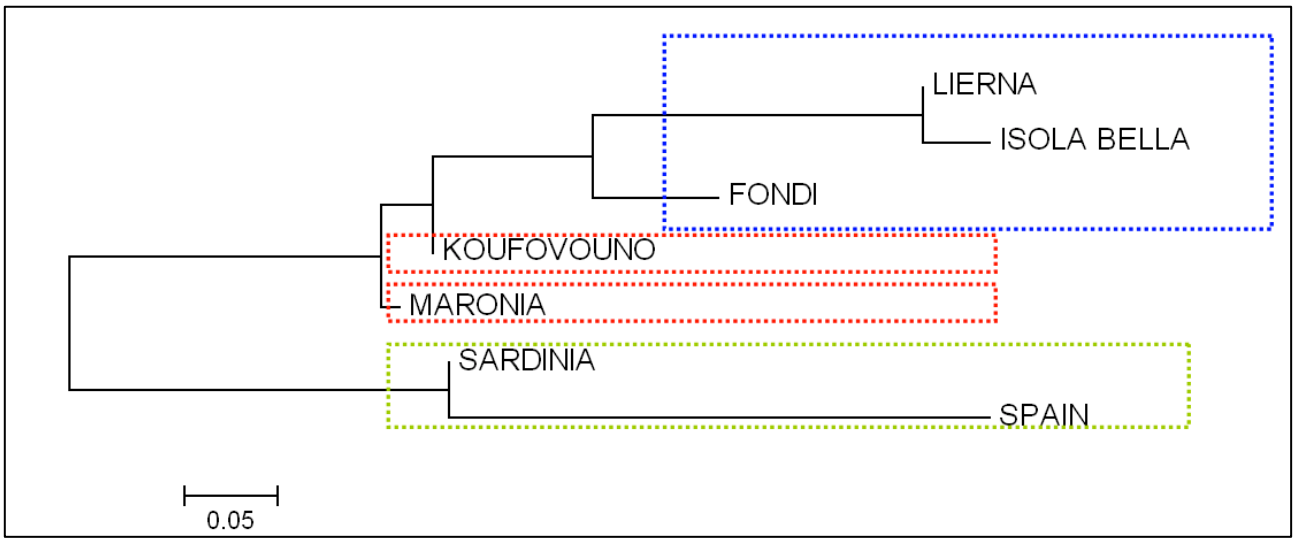


Figure 3.13: Unrooted NJ trees of linearized pairwise FST among nursery colonies derived from STR loci

Finally we used these genetic distance to make a PCA, even this confirm the result showed in the NJ tree (Fig. 3.14).

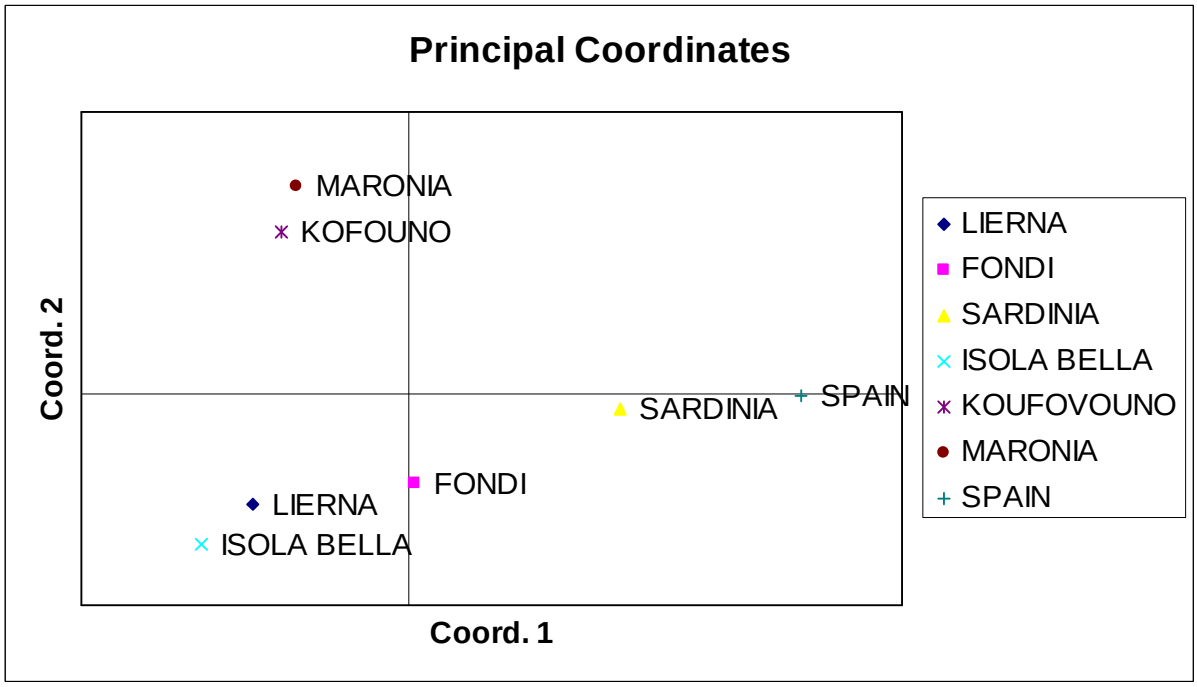


Figure 3.14: PCA graph of linearized pairwise FST among nursery colonies derived from STR loci

3.1.3 ASSIGNMENTE TEST

We performed an assignment test in Genalex using both tissues and faecal samples of both species. It showed that tissues and faeces of both species are unambiguously assigned without errors and doubts. Results are more clear considering the PCA graph obtained using the Adegenet package (Fig. 3.15).

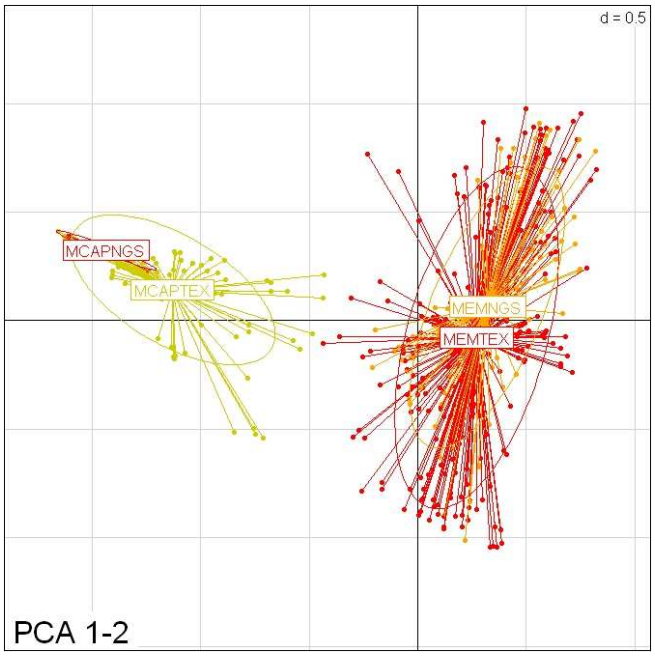


Figure 3.15: First and second components of a principal component analysis (PCA) of 5-locus micro satellite genotype from 89 *Myotis capaccinii* and 269 *Myotis emarginatus* samples from Europe

Structure shows the same result, in fact even in this case both samples, tissues and faeces, are assigned to two species with certainty (Fig. 3.16).

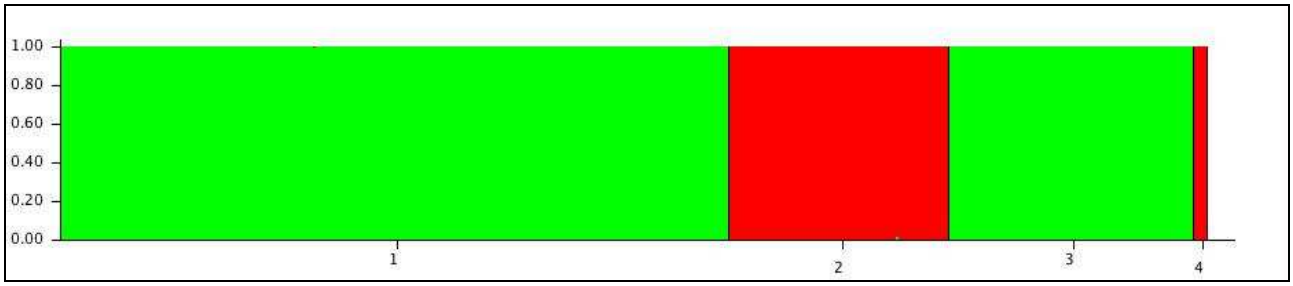


Figure 3.16: Genetic clusters obtained with two groups, whitch indicated the two species, *Myotis emarginatus* and *Myotis capaccinii*. The numbers were respectively: 1: *Myotis emarginatus* (inasive samples), 2: *Myotis capaccinii* (invasive samples); 3: *Myotis emarginatus* (NGS); 4: *Myotis capaccinii* (NGS)

3.2 SEQUENCES

mtDNA CONTROL REGION analyses

We sequenced only the first 345 bp and 374 bp of the CR region for *Myotis emarginatus* samples and *Myotis capaccinii* samples, respectively, because several bats are heteroplasmic for a variable number of 6 bp repeats as described also by Castella and Ruedi (Castella et al., 2001). These R2 repeats (Fumagalli et al., 1996), usually exceeding 50 copies, occur upstream from the replication origin of the H-strand. The R2 motif sequenced in this study is CGCATA in both species as describe for *Myotis myotis* (Castella et al., 2001).

This R2 motif has been also found in other species of *Myotis*, for example in *M. myotis* from Spain, Poland and Bulgaria as well as in *M. blythii* from Switzerland (Castella, unpublished observations; cit in Castella et al., 2001) and is identical to the R2 motif reported in the bat *N. noctula* (Petit et al., 1999).

3.2.1 *Myotis emarginatus*

The second hypervariable segment (HVII) of the control region was newly sequenced in 345 European bats. Because samples from Spain and Morocco were lower than others colonies we decided to consider them as two big colonies. Of the 345 aligned nucleotide positions, 31 were variable among the entire data set. Most inferred substitutions (22 out of 31) were transitions, with only nine transversions and no alignment gap, a typical pattern of substitution among closely related mtDNA sequences (Avise 2000). These variable sites defined 39 distinct haplotypes among the 31 European colonies (Table 3.22.).

	8	***32***	***35***	***43***	***44***	***73***	***150***	***170***	***178***	***183***	***188***	***196***	***215***	***222***	***243***	***244***	***245***	***246***	***248***	***251***	***253***	***263***	***271***	***272***	***274***	***277***	***279***	***282***	***285***	***288***	***303***
H1	C	A	A	C	T	C	G	A	C	A	A	A	C	C	G	G	T	T	C	T	G	G	C	C	G	G	A	G	G	G	C
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H8	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	.	.	.
H10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
H11	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T
H15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.
H16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
H19	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.

	8	32	35	43	44	73	150	170	178	183	188	196	215	222	243	244	245	246	248	251	253	263	271	272	274	277	279	282	285	288	303
H1	C	A	A	C	T	C	G	A	C	A	A	A	C	C	G	G	T	T	C	T	G	G	C	C	G	G	A	G	G	G	C
H20	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
H21	.	.	.	.	.	.	.	G	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H22	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
H24	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
H25	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
H28	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
H31	.	.	G	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.
H33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H34	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.
H35	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H36	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H37	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.
H38	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.
H39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.

Table 3.22: list of 39 *Myotis emarginatus* haplotypes with the 31 variable positions

All these haplotypes (H1 to H39) have never been described before in this species and 6 of them are located in the Italian peninsula, one in Sardinia, one in Spain and the remaining 19 in Greece The base frequencies for Adenine, Cytocine, Guanine and Thymine were 29%, 21%, 21% and 27%, respectively.

3.2.1.1 HAPLOTYPE ANALYSIS

Using the software NETWORK (Fluxus-engineering.com) we constructed a network of all different haplotypes, to investigate the possible relationship among them. The software run using the Median Joining algorithm.

As obtained with microsatellites, we don't have a clear genetic pattern with Dloop too. The network didn't show any evident cluster but one common haplotype from which all others developed. Haplotypes are coloured to match broad geographic regions where samples were taken. Figurexx shows that only few of them are founded in different localities.

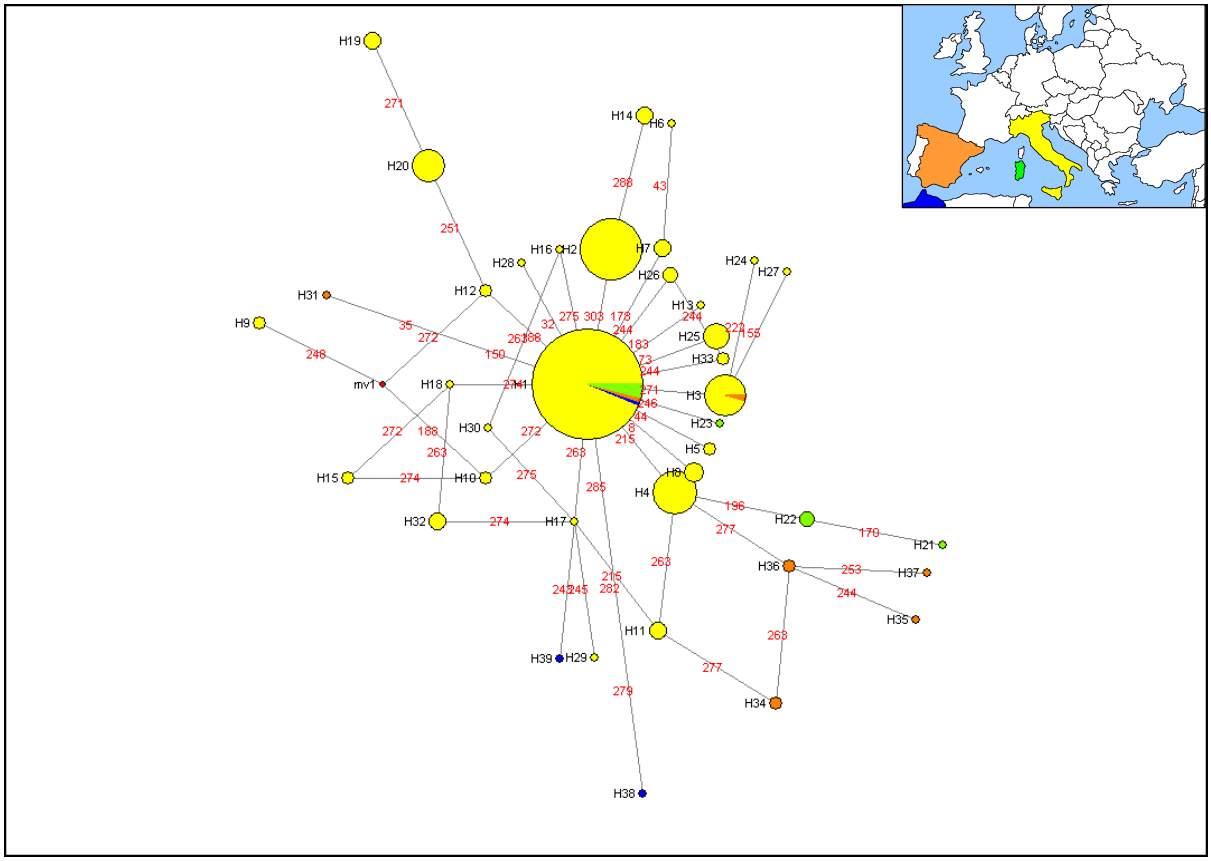


Figure 3.17: phylogenetic network of 39 *Myotis emarginatus* haplotypes. Nodes are color coded on the basis of location, and their size is proportional to the frequency of sequences. Branches are approximately sized to the number of mutations.

As obtained with microsatellites, we don't have a clear genetic pattern with Dloop too. The network didn't show any evident cluster but one common haplotype from which all others developed. Haplotypes are coloured to match broad geographic regions where samples were taken. Figurexx shows that only few of them are founded in different localities.

In order to have a more defined graphic representation of relationship among haplotypes, we made a phylogenetic analyses using GENEIOUS software (Biomatter TD) and its PAUP* plugin .The model used in building the neighbour-joining tree was TrN +G (Tamura e Nei, 1993) as revealed by MODELTEST.

This NJ tree showed that the 39 haplotypes are not very different among them, and the possible existence of haplogroups can not be considered due to the low level of bootstrap obtained for each nodes. We used then another analysis, we run a Bayesian analysis with MrBayes on Geneious (Fig. 3.18). A sequence of *Myotis myotis* Dloop was used as outgroup. Supports of nodes were assessed as posterior probabilities, with the initial 2000,000 generations considered as burn-in.

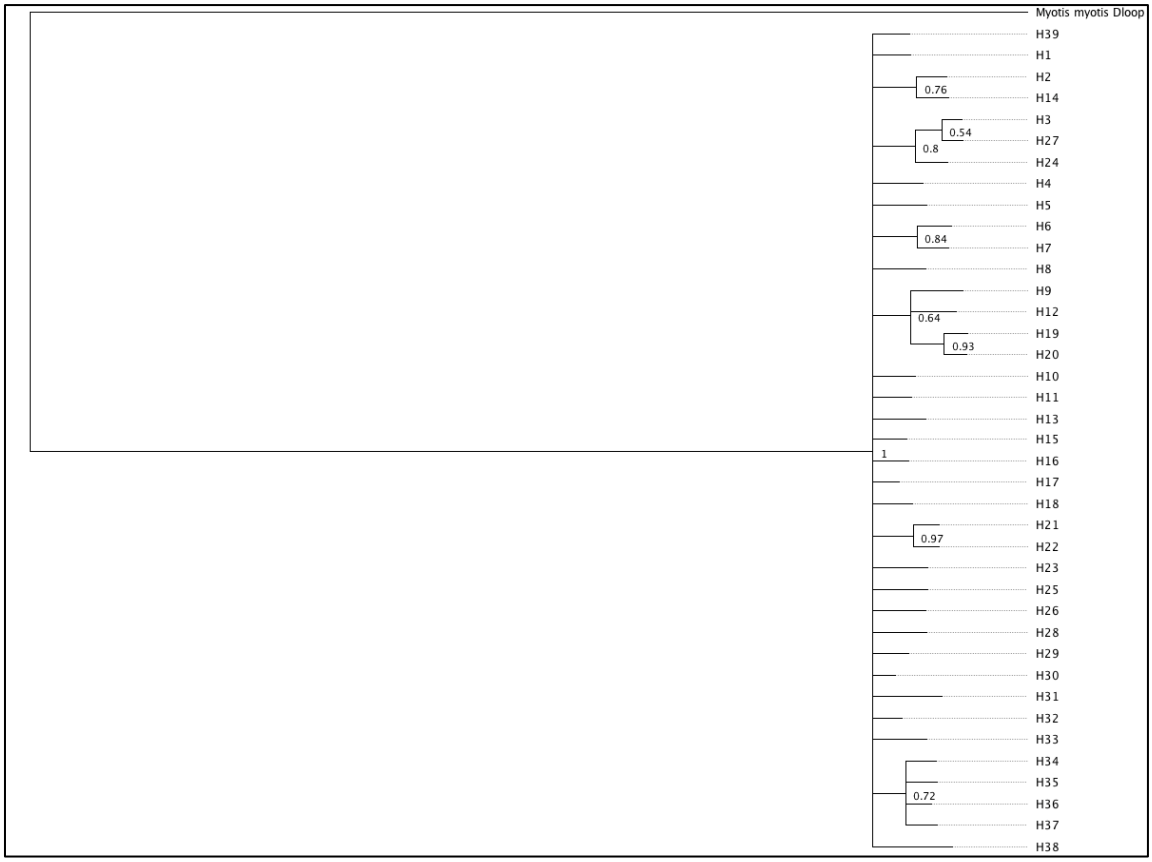


Figure 3.18; Bayesian tree showing phylogenetic relationships of the 39 distinct European haplotypes. Nodes supported by high (>0. 50) posterior probability are indicated.

Again the Bayesian tree confirms what we found on the Network, there is not a clear genetic pattern also using Dloop. We had only few clusters, which were high supported, but grouped sequences from different geographic locations, the only exceptions were haplotypes found in Spain (H34-H37), those in Sardinia (H21-H22) and in North Italy (H2, H14).

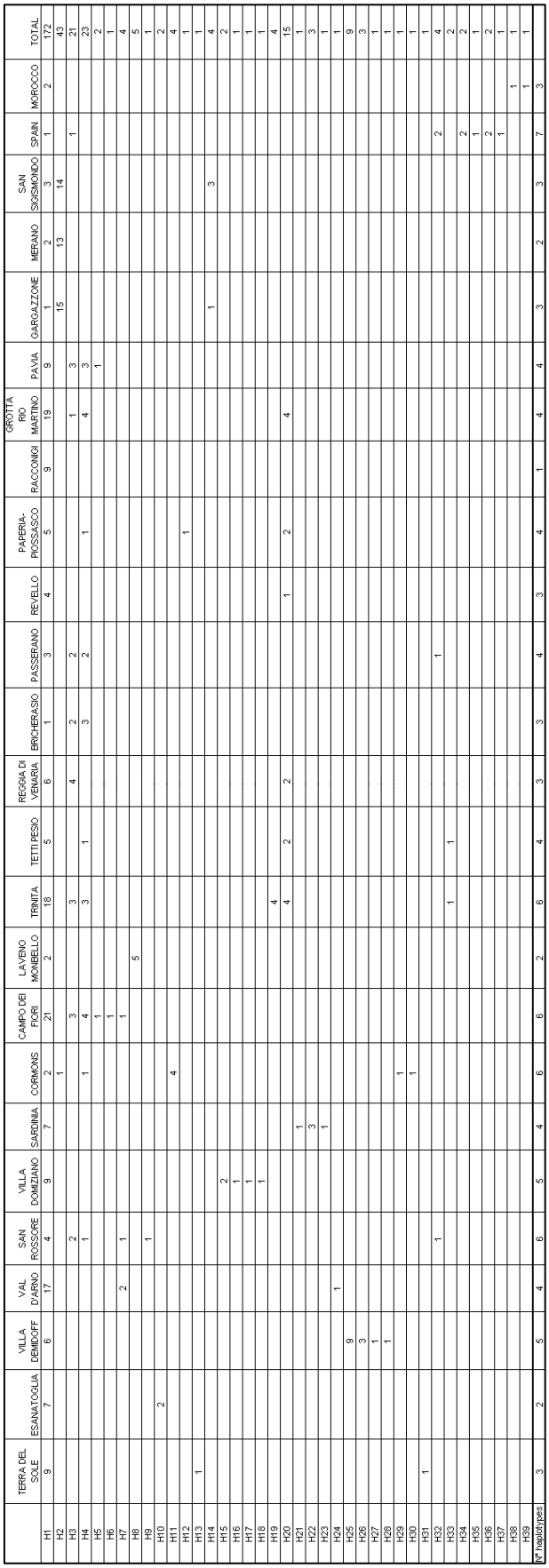
Specifically the tree showed the close relationship among all the haplotypes, indicated that these different sequences are the result of quite recent event of mutation.

The mean uncorrected genetic divergence of haplotypes is 0,9%, (range 0.4%–1.8%,). These values confirm the low variability noticed in *Myotis emarginatus*.

3.2.1.2 COLONIES

The most common haplotype in Italy and Europe is H1, characterizing 171 of 345 individuals (49,57% of bats) and found in all colonies. H2 is a Haplotype located principally in the three colonies of Trentino Alto Adige (San Sigismondo, Gargazzone and Merano).H11 was found only in the colony of Cormons, near the border with Slovinia. Whereas H20 is characteristic of most of the colonies in North –Western Italy (Piedmont). Finally haplotypes H34-H37 were only in Spain and H38-H39 only in Morocco. Globally we have haplotypes which are in common and some of them belongs to a single colony.

At the population level, each Italian colony contained a number of distinct haplotypes (Table 1), included from a minimum of 1 (Revello) to a maximum of 6 (Trinità, Campo dei Fiori and Cormons).

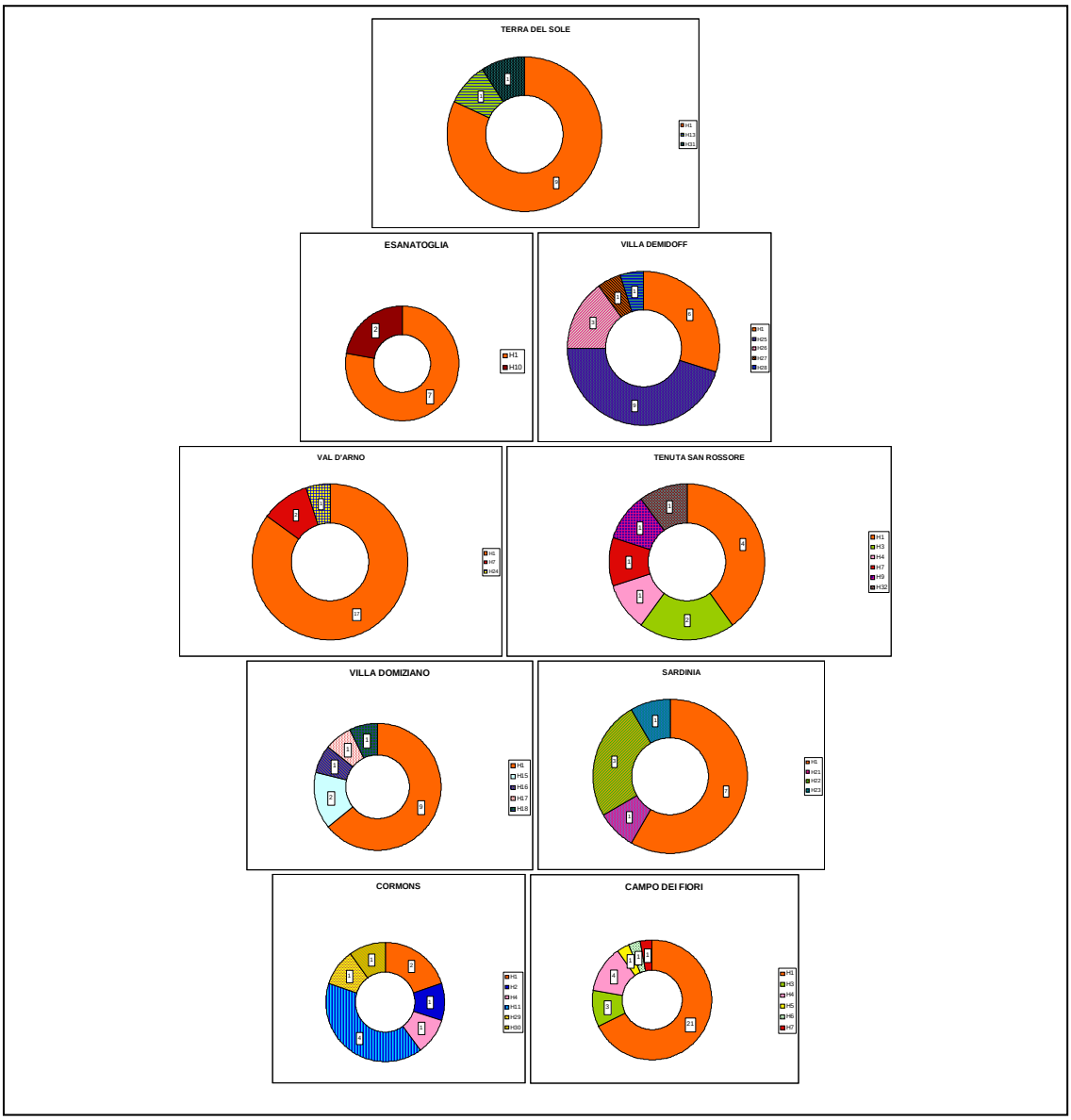


	TERRA DEL SOLE	ESANATOGLIA	VILLA DEMIDOFF	VAL D'ARNO	SAN ROSSORE	VILLA DOMIZIANO	SARDINIA	CORMONS	CAMPO DEI FIORI	LAVENO MOMBELLO	TRINITA	TETTI PESIO	REGGIA DI VENARIA	BRICHERASIO	PASSERANO	REVELLO	PAPERIA-PIOSSASCO	RACCONIGI	GROTTA RIO MARTINO	PAVIA	GARGAZZONE	MERANO	SAN SIGISMONDO	SPAIN	MOROCCO	TOTAL
H1	9	7	6	17	4	9	7	2	21	2	18	5	6	1	3	4	5	9	19	9	1	2	3	1	2	172
H2					2			1			3		4	2	2				1	3	15	13	14	1		43
H3					1			1	4		3	1		3	2		1		4	3				1		21
H4									1											1						23
H5									1																	2
H6				2	1				1	5											1		3			4
H7					1																			2		5
H8				1																						1
H9		2																								2
H10								4																		4
H11																	1									1
H12	1																									1
H13																										4
H14						2																	3			2
H15						1																				1
H16						1																				1
H17						1																				4
H18											4															15
H19							1				4	2	2			1	2		4							1
H20							3																			3
H21							1								1											1
H22				1																						1
H23			9																							9
H24			3																							3
H25			1																							1
H26								1																		1
H27																										1
H28								1																		1
H29																										1
H30	1														1								2			4
H31					1						1	1												2		4
H32																										2
H33																								2		2
H34																								1		1
H35																								2		2
H36																								1	1	2
H37																										1
H38																										1
H39																									1	1
N° haplotypes	3	2	5	4	6	5	4	6	6	2	6	4	3	3	4	3	4	1	4	4	3	2	3	7	3	

Table 3.23: distribution of 39 haplotypes in the European colonies

The mean values of haplotype is 3,65 which is similar to the haplotype diversity found in the rest of Europe (mean *A* = 5, range 3–7). These values are very similar to those identified by Ruedi et al. (2008) on *Myotis myotis*, a species of the same genus whose population genetic structure was studied using the same Dloop marker.

At the population level, Italian colonies contained 33 distinct haplotypes (Table 3,23)), the mean number for these colonies is 3,60 with a range 1-6, while this value is less higher in Spain and Morocco. This result is more clear with a graphic representation (Fig.3.19) of each colonies.



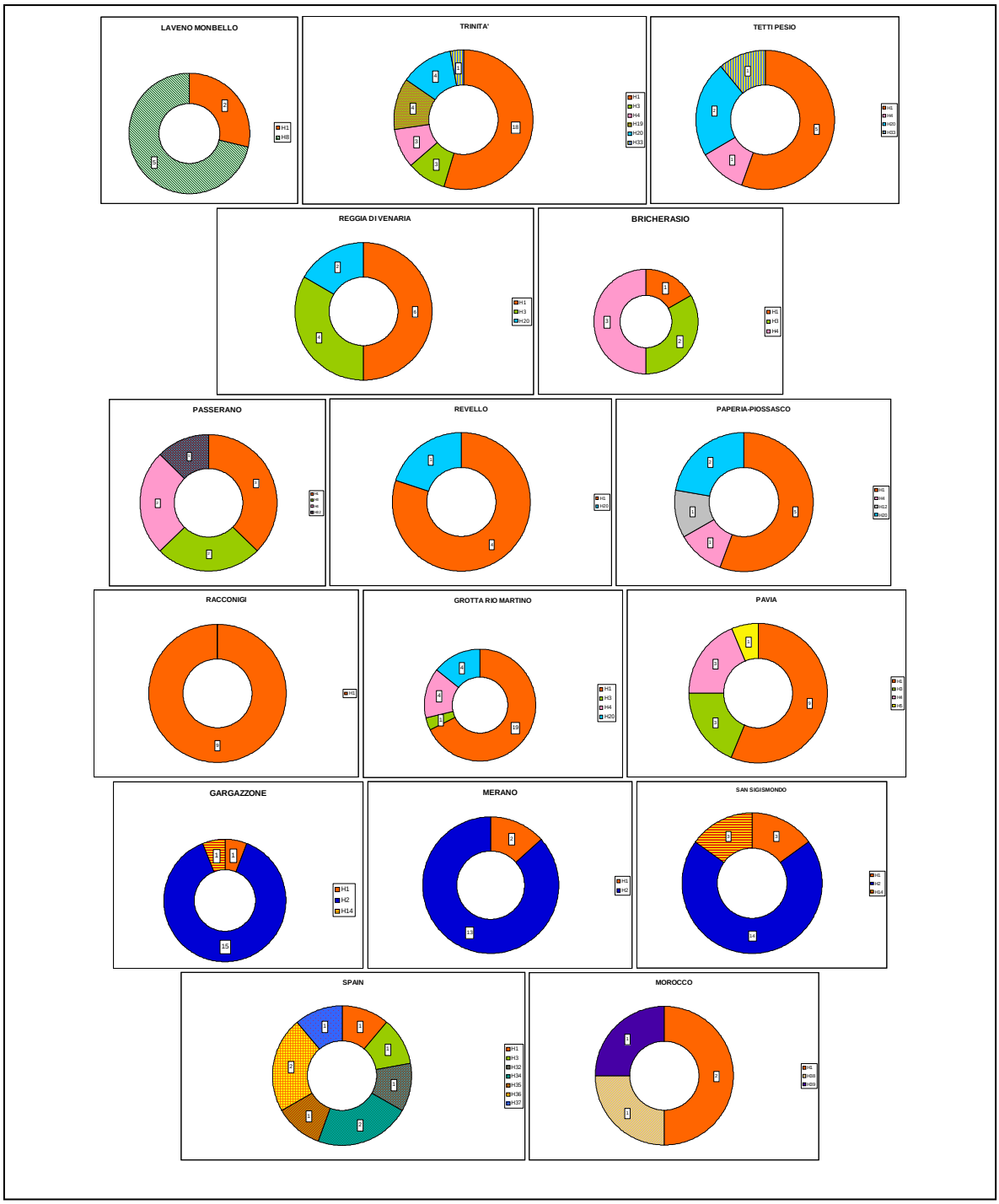


Figure 3.19: graphic rapresentation of distribution of 39 haplotypes in the 25 European colonies

Plotting this different graphs onto a map of Europe (Fig. 3.20), consider haplotypes, the most common are: H1 which spreads across entire Europe, and H2 located in the north of Italy; H4 and H20 were found in the piedmont colonies and both of them are showed in most of these colonies. Others are private but located only in Iberian Peninsula and in the Central and Eastern part of Italy.

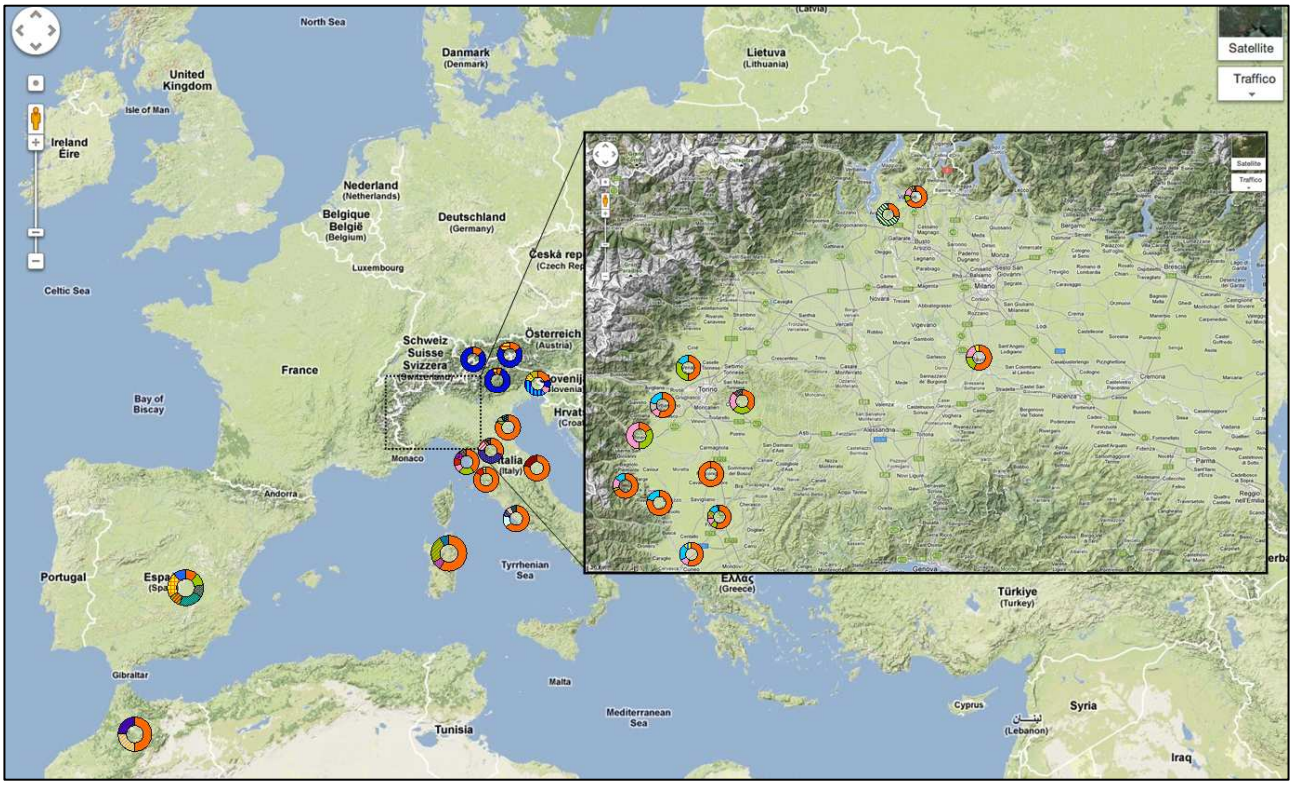


Figure 3.60: Map showing the location of 25 sampled *Myotis emarginatus* nursery colonies. In each colonies is showed the frequency of each haplotype

The most interesting thing is that the three colonies located on the North-Italy showed a haplotype pattern completely different from the others, in fact only few samples had haplotype 1, while the majority of them showed haplotype H2, found only in these three colonies. Also the colony of Cormons is quite interesting, in fact it is the only one showing haplotype H11; it could be possible that H11 is a common haplotype in the Balkans. Having sampled nine different colonies in Piedmont, the results showed the strict connection between them, in fact only in these colonies there were H20, again as in the colony of Cormons, this haplotype could be typical also in the French colonies, as per their location near the French border.

At nucleotide level, the variability of the different colonies is rather heterogeneous, with values of nucleotide diversity ranging from a minimum of $\pi = 0$ to a maximum of $\pi = 0.008$. The highest values ($\pi = 0.008$) were found in Spanish colonies, composed by a marked coexistence of haplotypes which are quite far away considering the NJ tree. Except for the Moroccan samples showing a π similar to Spain, 0,007, the remaining colonies are mostly characterized by a dominant haplogroup, therefore less diversified ($\pi \leq 0.005$).

Haplotype diversity rangs from a minimum of $h = 0$ to a maximum of $h = 0.93$, with a trend that reflects the results of nucleotide variability (Table 3.24).

COLONY	π	h
BRICHERASIO	0.003494	0.7333
CAMPO DEI FIORI	0.002047	0.5290
VILLA DI DOMIZIANO	0.002362	0.5934
CORMONS	0.004580	0.8444
ESANATOGLIA	0.001193	0.3889
GARGAZZONE	0.000722	0.2279
GROTTA RIO MARTINO	0.002559	0.5159
LAVENO MONBELLO	0.001461	0.4762
MERANO	0.000760	0.2476
PAPERIA PIOSSASCO	0.003411	0.6944
VILLA DEMIDOFF	0.003040	0.7158
PASSERANO	0.004179	0.8214
PAVIA	0.002380	0.6500
RACCONIGI	0.000000	0.0000
REGGIA DI VENARIA	0.003352	0.6667
REVELLO	0.002454	0.4000
SAN SIGISMONDO	0.001647	0.4895
TENUTA DI SAN ROSSORE	0.005406	0.8444
TERRA DEL SOLE	0.001678	0.3435
TETTI PESIO	0.003751	0.6944
TRINITA	0.004102	0.6761
VAL D'ARNO	0.001503	0.3632
SARDINIA	0.004004	0.6364
SPAIN	0.008455	0.9333
MOROCCO	0.007701	0.8333

Table 3.24: haplotype diversity (h), nucleotide diversity (π) for the 25 European colonies

For each pair of populations we calculated in ARLEQUIN ΦST values as described by table 3.25 reporting the matrix of values obtained from the analysis of nucleotide sequences.

The overall low fixation index (ΦST = 0.2988, P < 0.001) confirms that there is a week genetic structure among the European colonies at the mitochondrial level. The higher values are comparing the three North Italian colonies located in Trentino-Alto Adige with the all others. However, the values ΦST are not generally so high among Europe. Colony of Cormons showed high values of pair-wise differences too.

AMOVA showed that the global Φst was 0.2988, if we considered all colonies in one group, called in this case Europe. The Φst, after geographically grouping the colonies (Italy, Italy-Sardinia, Spain and Morocco), was 0.0771 (P < 0.005). The percent of differentiation attributable among group differences was 7,71% when the four groups were grouped separately. In both case the genetic variability can be explained by differences across individuals within populations/groups.

	BRICHERASIO	CAMPO DEI FIORI	VILLA DI DOMIZIANO	CORMONS	ESANATOGLIA	GARGAZZONE	GROTTA RIO MARTINO	LAVENO MONBELLO	MERANO	PAPERIA PIOSSASCO	VILLA DEMIDOFF	PASSERANO	PAVIA	RACCONIGI	REGGIA DI VENARIA	REVELLO	SAN SIGISMONDO	TENUTA DI SAN ROSSORE	TERRA DEL SOLE	TETTI PESO	TRINITA	VAL D'ARNO	SARDINIA	SPAIN	MOROCCO
BRICHERASIO	0.00000																							
CAMPO DEI FIORI	***0.14666***	0.00000																						
VILLA DI DOMIZIANO	***0.27329***	***0.08813***	0.00000																					
CORMONS	***0.18483***	***0.35722***	***0.33042***	0.00000																				
ESANATOGLIA	***0.31862***	0.05948	0.00010	***0.37226***	0.00000																			
GARGAZZONE	***0.72840***	***0.63552***	***0.66242***	***0.65096***	***0.76198***	0.00000																		
GROTTA RIO MARTINO	***0.75970***	0.03345	***0.10051***	***0.32600***	0.07415	***0.60252***	0.00000																	
LAVENO MONBELLO	***0.49327***	***0.43506***	***0.42981***	***0.48272***	***0.54349***	***0.82074***	***0.39717***	0.00000																
MERANO	***0.68804***	***0.58748***	***0.61278***	***0.61056***	***0.72198***	-0.02602	***0.55309***	***0.79633***	0.00000															
PAPERIA PIOSSASCO	0.19244	***0.73851***	***0.75367***	***0.29746***	0.15675	***0.65947***	-0.02394	***0.40833***	***0.60965***	0.00000														
VILLA DEMIDOFF	***0.30568***	***0.21810***	***0.21573***	***0.40310***	***0.20923***	***0.62816***	***0.21899***	***0.43250***	***0.58370***	***0.23900***	0.00000													
PASSERANO	-0.07129	0.01610	***0.11965***	***0.20824***	***0.11258***	***0.63322***	0.05477	***0.36288***	***0.57949***	0.09594	***0.19754***	0.00000												
PAVIA	0.02590	-0.02252	***0.10836***	***0.29271***	0.09658	***0.65370***	0.03568	***0.42815***	***0.60440***	***0.12585***	***0.20888***	-0.05142	0.00000											
RACCONIGI	***0.40502***	-0.01136	0.05484	***0.40816***	0.12500	***0.84875***	0.02257	***0.70353***	***0.82387***	0.16610	***0.19915***	***0.11252***	0.05556	0.00000										
REGGIA DI VENARIA	0.13198	***0.10301***	***0.15454***	***0.34950***	***0.14897***	***0.63572***	0.05856	***0.39943***	***0.58591***	0.04177	***0.22478***	0.02672	0.05214	0.14174	0.00000									
REVELLO	0.20830	0.04660	0.05689	***0.28892***	0.08388	***0.72693***	-0.09143	***0.44633***	***0.67918***	-0.14313	0.16680	0.04096	0.06271	0.12621	-0.03071	0.00000								
SAN SIGISMONDO	***0.61734***	***0.55042***	***0.55271***	***0.57635***	***0.60568***	-0.01291	***0.52379***	***0.69902***	-0.00336	***0.55018***	***0.55238***	***0.52544***	***0.55066***	***0.65421***	***0.53948***	***0.56442***	0.00000							
TENUTA DI SAN ROSSORE	0.02104	0.00408	0.04263	***0.22816***	-0.00290	***0.54557***	0.02657	***0.27456***	***0.49639***	0.02760	***0.15623***	-0.07582	-0.01900	0.01067	-0.01237	-0.04981	***0.45923***	0.00000						
TERRA DEL SOLE	***0.27914***	0.02682	0.05895	***0.36017***	0.05192	***0.77432***	0.04957	***0.47689***	***0.66733***	0.12847	***0.18962***	0.04300	0.06321	-0.01975	***0.12483***	0.02410	***0.57549***	-0.00884	0.00000					
TETTI PESO	0.14914	0.08321	***0.10430***	***0.26828***	0.09421	***0.63195***	-0.04596	***0.36637***	***0.57799***	-0.09886	***0.19305***	0.05415	0.07519	0.08251	0.02639	-0.15199	***0.52347***	0.00562	0.06910	0.00000				
TRINITA	0.11638	***0.09689***	***0.13625***	***0.37355***	***0.10970***	***0.57380***	0.01640	***0.32679***	***0.46683***	-0.02867	***0.21496***	0.05230	0.06412	0.08225	-0.03026	-0.07520	***0.45905***	0.02614	***0.09987***	-0.03120	0.00000			
VAL D'ARNO	***0.30726***	0.00437	***0.07974***	***0.41530***	0.06175	***0.68078***	***0.06369***	***0.49797***	***0.63071***	***0.16836***	***0.21788***	***0.10577***	0.05883	-0.03128	***0.12723***	0.05215	***0.56009***	0.02137	0.01226	***0.10656***	***0.11121***	0.00000		
SARDINIA	0.07893	***0.13834***	***0.18542***	***0.22728***	***0.17667***	***0.67743***	***0.13755***	***0.38567***	***0.56829***	***0.15752***	***0.26352***	0.06329	***0.10173***	0.17708	***0.20318***	0.11518	***0.53241***	0.08676	0.15997	0.11441	***0.17765***	***0.20389***	0.00000	
SPAIN	0.11101	***0.36144***	***0.33022***	***0.17542***	***0.37213***	***0.59450***	***0.33609***	***0.38613***	***0.55351***	***0.26231***	***0.37872***	0.13535	***0.26835***	***0.32826***	***0.30410***	***0.22605***	***0.55014***	***0.18640***	***0.29584***	***0.23853***	***0.32247***	***0.39786***	***0.21363***	0.00000
MOROCCO	0.16596	***0.21407***	0.13693	0.16149	0.16236	***0.65179***	0.18884	***0.34114***	***0.59823***	0.12779	***0.23362***	0.08143	***0.17773***	0.21605	0.15088	0.03130	***0.53837***	0.04130	0.14428	0.08927	***0.16584***	***0.21977***	0.15751	0.18662

Table 3.25: estimates of population differentiation among the 24 nursery colonies of *Myotis emarginatus*

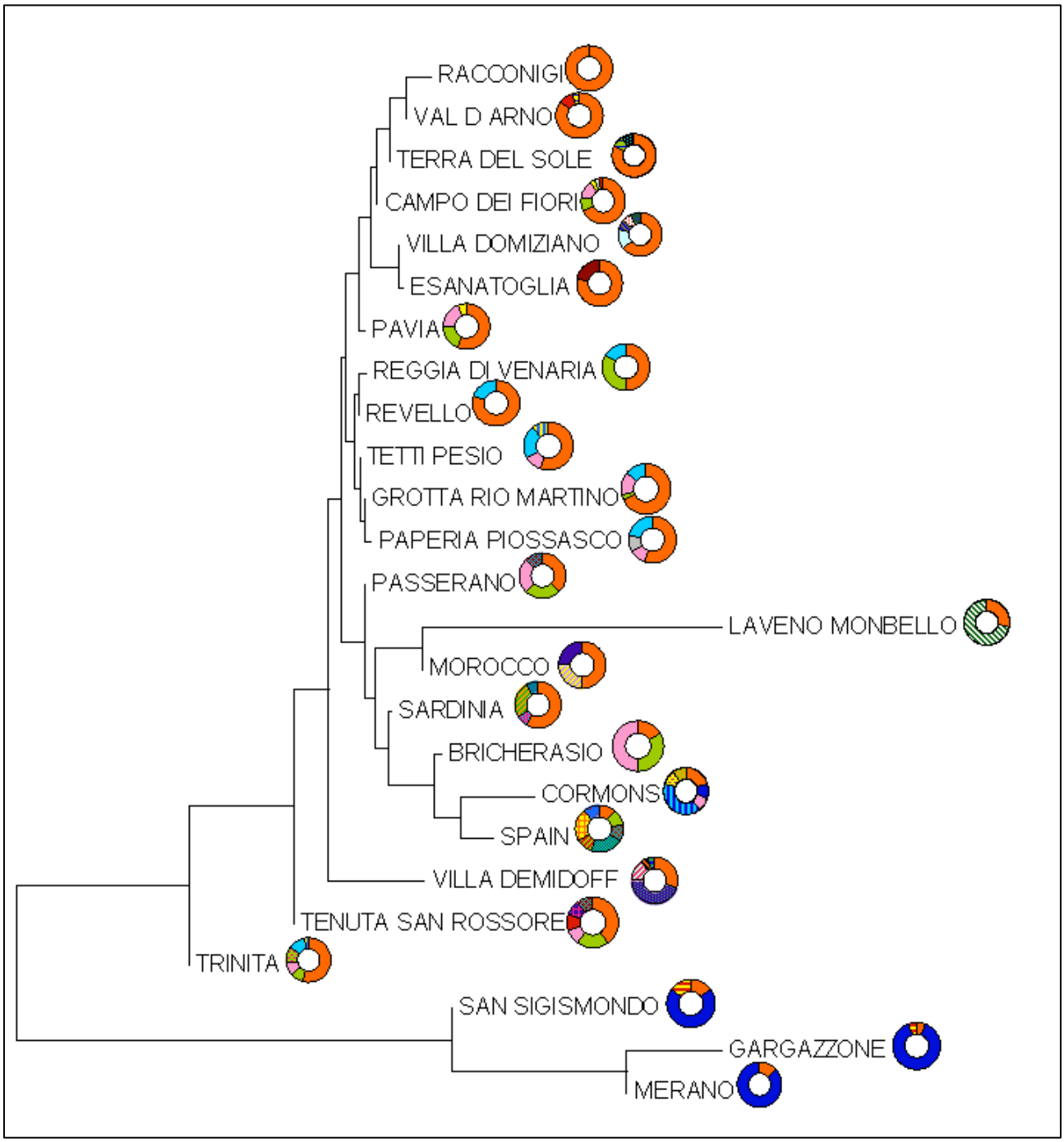


Figure 7.21: unrooted NJ trees of linearized pairwise ΦST among nursery colonies derived from mtDNA

The genetic relationships between colonies were visualized on an NJ tree based on a matrix of pair-wise ΦST values linearized as ΦST/(1 – ΦST) (Slatkin 1995), derived from the mtDNA data set. The NJ is presented in Fig. 3.21.

This NJ tree highlights the close relationship among the three different colonies of North Italy, San Sigismondo, Gargazzone and Merano, located near the border with Austria. In could be possible to identify inside the main group of this tree that most of the Colonies of Piedmont are regrouping all together

The tree supports also the heterogeneity among the Italian and European populations. The colonies of Spain and Morocco are also intermingled with the Italian samples, further suggesting that they share closer relationships than with other European colonies characterized by the widespread haplotype 1 (Fig. 3.21).

The values of mismatch analysis (Fig 3.22), Tajma D Test and FU test (Table 3.26) indicated that the global sample and all colonies fitted the expectation of a constant size population model. Looking at the graph in Figure 3.22, which represents the result of the analysis of Mismatch carried out for the whole sample, it is possible to note that the bars are well adapted to the curve expected, producing a typical distribution of the model in constant population size. Values in the table, always in reference to the entire sample, are significant for testing the SSD, then confirm the goodness of fit and allow you to accept the hypothesis of constant size population. Also the measured values for the D-test Tajima and the Fs-test are indicative of a stable population over time as though both pf them were negative but not highly significant.

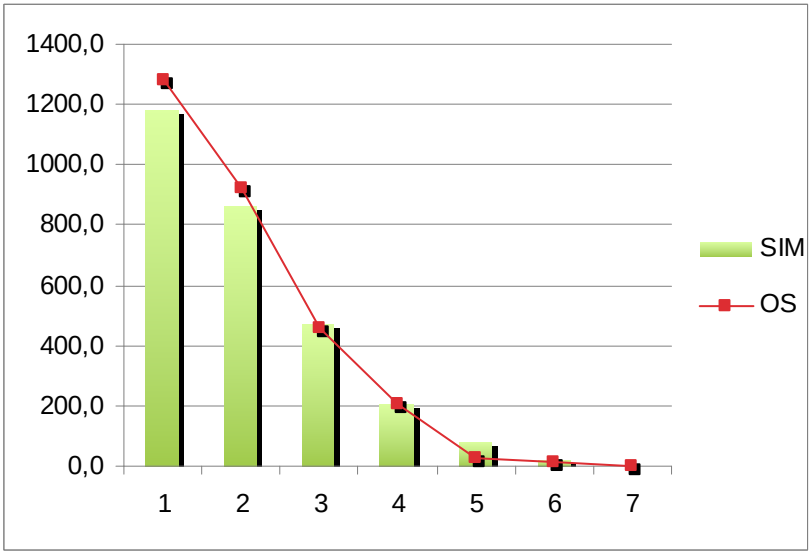


Figure 3.22: the observed and expected mismatch distributions in the European population of *Myotis emarginatus*

Statistics	BRICHERASIO	CAMPO DEI FIORI	VILLA DOMIZIANO	CORMONS	ESANATOGLIA	GARGAZZONE	GROTTA RIO MARTINO	LAVENO MOMBELLO	MERANO	PAPERIA PIOSSASCO	VILLA DEMIDOFF	PASSERANO	PAVIA	RACCONIGI	REGGIA DI VENARIA	REVELLO	SAN SIGISMONDO	TENUTA SAN ROSSORE	TERRA DEL SOLE	TETTI PESO	TRINITA	VAL D'ARNO	SARDINIA	SPAIN	MOROCCO	GLOBAL SAMPLE
TAJIMA D TEST	1.39259	-1.26434	-0.56505	0.20350	0.15647	-1.50358	-0.49523	0.55902	-0.39883	0.02527	-0.35434	-0.52474	-0.41395	0.00000	0.32192	-0.97256	-0.11187	-1.63600	-1.59996	-0.68914	0.21911	-1.63814	-0.05716	-0.14425	-0.79684	-0.41152
Tajima's D p-value	0.93800	0.10400	0.28800	0.65200	0.76100	0.06300	0.35400	0.82600	0.28500	0.53500	0.42400	0.30500	0.35200	1.00000	0.88900	0.18900	0.37700	0.05700	0.05000	0.32700	0.62900	0.02300	0.50500	0.48700	0.16900	0.41556
FU FS TEST	0.02028	-2.86744*	-1.86969*	-2.29007*	0.47744	-1.68032*	-0.09930	0.58867	0.13336	-0.82233	-0.89563	-0.62111	-0.82161	0.00000	0.86516	1.04042	-0.07875	-2.18321*	-0.53700	-0.62734	-0.67210	-1.61348*	-0.05015	-2.31399	0.46110	-0.65908
FS p-value	0.43400	0.01100	0.03100	0.03000	0.41300	0.01500	0.47000	0.46000	0.28200	0.15000	0.20500	0.20000	0.16500	N.A.	0.66300	0.63300	0.35500	0.03700	0.13500	0.22000	0.36300	0.03900	0.42000	0.05100	0.50800	N.A.
Tau	1.53125	0.74609	1.26562	2.14844	0.54297	3.00000	1.86914	0.71680	2.92969	1.31836	1.21680	1.51172	0.98047	0.00000	0.74414	0.00000	0.67188	1.72070	3.00000	1.73438	2.25000	1.00391	2.44727	2.77930	2.96875	1.56391
Theta0	0.00176	0.00000	0.00703	0.00000	0.00352	0.00000	0.00000	0.00000	0.90000	0.00000	0.00000	0.00000	0.00000	0.00000	0.44824	0.00000	0.00000	0.00000	0.00000	0.01230	0.00156	0.00000	0.00000	0.58008	0.00352	0.07832
Theta1	99999	99999	2.80547	99999	99999	0.31162	1.22505	99999	3.60000	20.95000	99999	99999	99999	0.00000	99999	99999	99999	99999	0.54268	4.52695	2.56499	0.66074	2.40249	22.71484	29.68750	48003.19969
SSD	0.02399	0.00408	0.00357	0.05529	0.00629	0.00292	0.00504	0.01718	0.28138	0.00892	0.00339	0.01108	0.02840	0.00000	0.00201	0.32000	0.01440	0.00825	0.01124	0.00796	0.00572	0.00016	0.03227	0.00925	0.09119	0.03816
(SSD) p-value	0.53900	0.53400	0.76800	0.09900	0.43700	0.46500	0.72900	0.26100	0.13200	0.66800	0.62900	0.64200	0.10300	0.00000	0.91300	0.00100	0.22500	0.71600	0.47300	0.78900	0.70100	0.73600	0.40400	0.62400	0.44800	0.48384

Table 3.26: results of mismatch distribution, SSD, Tau, Theta0 e Theta1 and neutrality test Tajima's D-test and Fu's Fs-Test

Finally the mitochondrial data set showed no evidence for isolation by distance; as revealed by Mantel Test (R^2 = 0.045, P = 0,250).

3.2.1.3 GLOBAL DLOOP RESUME

Considering global sampling, we found on a total number of 39 haplotypes, 30 haplotypes which are private and 9 shared among colonies (Fig. 3.23). We must consider that in the shared haplotypes there is H1, which is the most common across Europe.

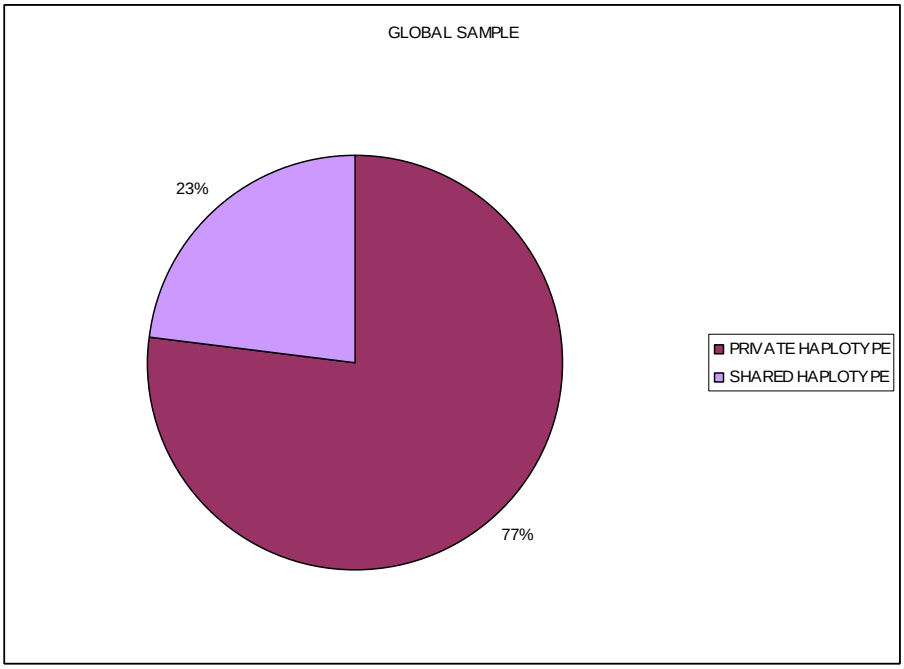


Figure 3.23: mtDNA, percentage of private and shared haplotypes on whole sampling

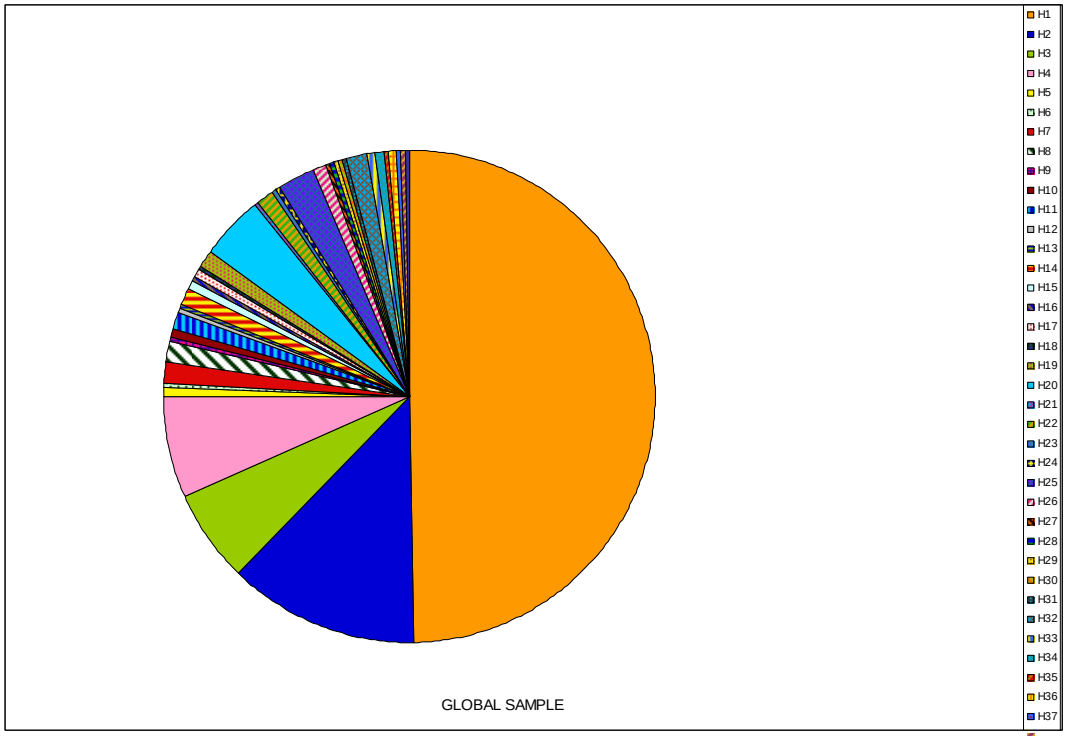


Figure 3.24: frequency of 39 haplotypes on global sample

3.2.2 *Myotis capaccinii*

The second hypervariable segment (HVII) of the control region was newly sequenced in 97 European bats, most of the samples coming from the Italian peninsula. Because samples from Spain and Sardinia were lower than other colonies we decided to consider them as two big colonies. Of the 374 aligned nucleotide positions, 57 were variable among the entire data set. Most inferred substitutions (49 out of 57) were transitions, with only eight transversions and no alignment gap, a typical pattern of substitution among closely related mtDNA sequences (Avise 2000). These variable sites defined 27 distinct haplotypes among the seven European colonies (Table 3.27.).

HAPLOTYPE	38	78	114	140	143	149	152	164	165	170	184	196	198	200	202	203	204	208	209	227	228	231	242	244	248	252	262	264	265	266	267	268	269	274	275	276	277	278	282	283	284	287	292	294	299	300	311	312	314	322	327	330	332	333	345	358	359
H1	T	C	G	G	A	A	T	C	A	T	A	G	G	G	C	A	G	A	A	T	G	A	A	A	A	T	C	T	A	A	G	G	A	A	A	A	G	G	A	C	T	A	A	T	A	T	A	C	G	C	T	G	G	C
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	.	.	A	G	G	C	.	G	.	.	.	.	.	.	.	G	.	.	.	.	G	.	C	T	.	.	.	.	.	G	.	A	.	.	.	.	.	.	A	.	.	G	C	.	T	.	T	.	.	.	T
H8	.	.	.	A	G	G	C	.	G	.	.	.	.	.	.	.	G	.	.	.	.	G	.	C	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	C	.	T	.	T	.	A	.	T
H9	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	G	.	G	.	.	.	A	.	.
H10	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	G	.	G	.	.	.	A	.	.
H11	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	G	.	G	.	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	G	.	G	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	G	.	G	.	.	.	.	.	.	.
H14	.	T	A	.	G	.	C	G	G	C	.	A	A	.	T	T	A	G	G	C	A	G	.	.	.	C	.	C	G	G	A	A	T	.	.	G	.	T	G	T	.	.	.	C	.	C	.	G	.	G	.	A	.	C	A	.	A
H15	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	.	G	.	.	.	.	.	.	.
H16	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	G	.	G	.	G	.	.	.	.	.	.	.
H17	.	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	.	G	.	.	.	.	.	.	.
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	.	G	.	.	.	.	.	.	
H19	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	C	.	.	T	G	.	G	.	.	.	.	.	.	.
H20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	.	G	.	.	.	.	.	.	.
H21	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	.	G	.	.	.	.	.	.	.
H22	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	T	G	.	G	.	.	.	.	.	.	.
H23	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	.	G	.	.	.	.	.	.	.
H24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	T	G	.	G	.	.	.	.	.	.	.
H25	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	A	.	.	G	.	G	.	.	.	.	.	.	.
H26	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	.	G	.	.	.	.	A	.	
H27	.	.	.	A	G	G	C	.	G	.	.	.	.	.	.	.	G	.	.	.	.	G	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	C	.	T	.	T	.	A	.	T

Table 3.27: : list of 27 *Myotiscapaccinii* haplotypes with the 57 variable positions

All of these haplotypes (H1 to H27) have never been described in previous studies for this species. The base frequencies for Adenine, Cytocine, Guanine and Thymine were 29%, 21%, 21% and 27%, respectively, and rate variation was calculated as to be equal among-sites. Among these, 6 are located in the Italian peninsula, one in Sardinia, one in Spain and the remaining 19 in Greece.

3.2.2.1 HAPLOTYPE ANALYSIS

Using the software NETWORK (Fluxus-engineering.com), it has been developed a network of all haplotypes identified across Europe (Fig. 3.25). We used the *median-joining* or *MJ* network algorithm.

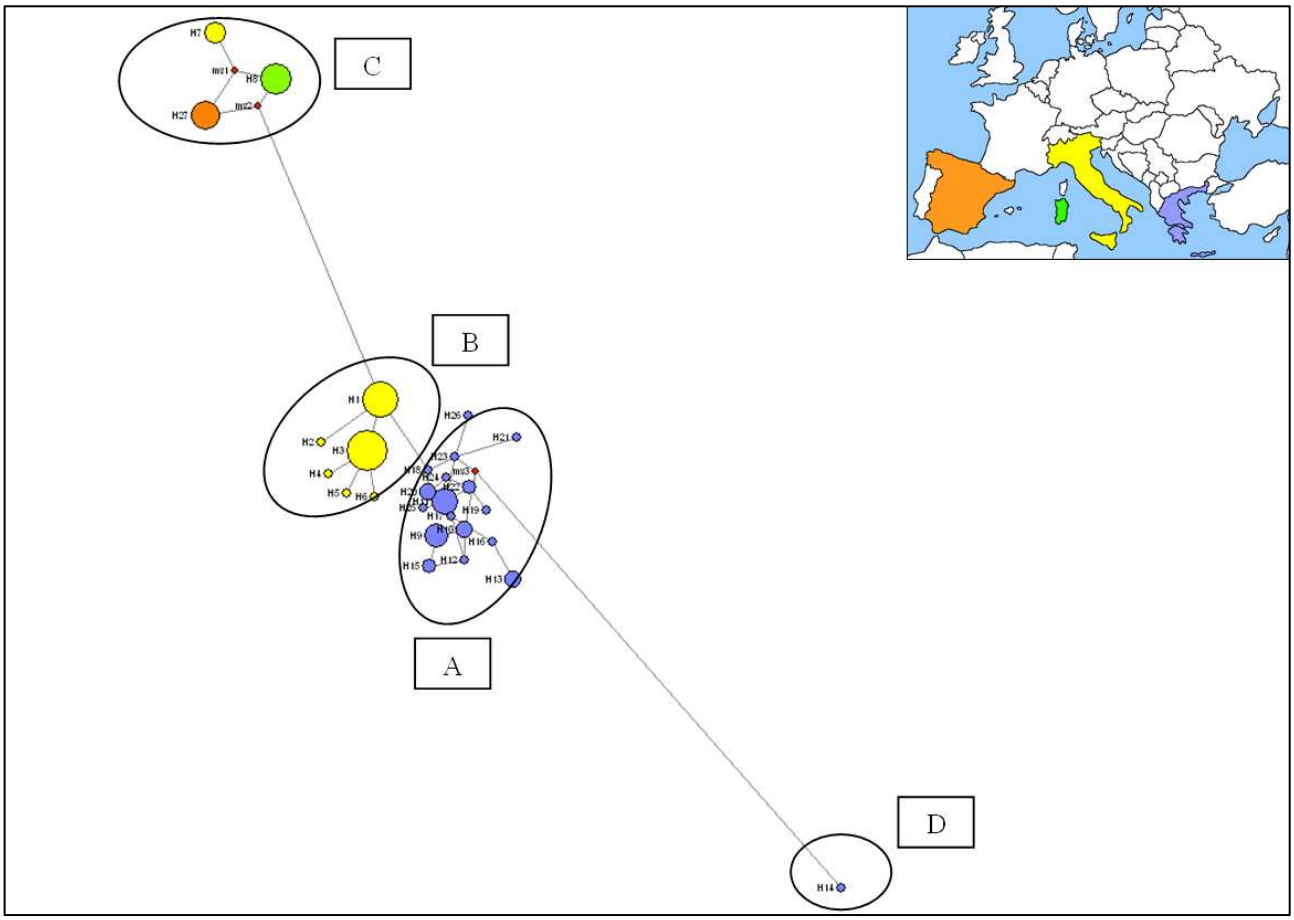


Figure 3.25: phylogenetic network of 27 *Myotis capaccinii* haplotypes. Nodes are color coded on the basis of location, and their size is proportional to the frequency of sequences. Branches are approximately sized to the number of mutations.

Specifically, the results show the distribution of haplotypes in four different haplogroups. Each haplotype is represented by a circle whose size is proportional to its frequency in the sample. Each mutational event is represented by connecting lines among haplotypes, while the nodes correspond to haplotypes that the software considers intermediate between two mutational steps, but that are not present in the sample under study.

The two haplotypes identified in the northern Italy (Isola Bella and Lierna) cluster into haplogroup B. Haplogroup C regroups haplotypes identified in Italy, specifically in the Fondi's colony but also in Sardinian and Spanish colonies. These three haplotypes make a haplogroup which spreads from Spanish to Italian peninsula. Finally, D and A haplogroups contain haplotypes found in Greece. Of particular interest is the haplotype H14, the only inside haplogroup D, in fact in the network it is graphically represented far from the others, indicating that the variation among the others sequences is very high.

Phylogenetic analyses of *M. capaccinii* were made using GENEIOUS software (Biomatter LTD) and its PAUP* and MyBayes plugins .The model used in building the neighbour-joining tree was TrN +G (Tamura e Nei, 1993) as revealed by MODELTEST.

Figure 3.26: Neighbour-joining tree showing phylogenetic relationships of the 27 distinct European haplotypes of Myotis emarginatus and 2 haplotypes of *Myotis punicus* and *Myotis myotis* (outgroup). The Bayesian tree (not shown) was almost identical. Nodes supported by high (> 50%) bootstrap (in bold) or posterior probability (in italics) are indicated.

NJ tree (Fig.3.26) shows the relationship among the 27 different haplotypes related to *Myotis capaccinii*, *Myotis myotis* was set as an outgroup. As anticipated by the analysis made with the software Network, haplotypes were regrouping 4 major clades (indicated in the figure by the letters A, B, C, D, and marked with different colors). Clades D is clearly separated from others, supporting also a high bootstrap value, both with NJ and MrBayes. Note that the composition of the haplotype clades confirms and reflects the distribution of the different haplotype distribution within haplogroups, proposed in the network on Fig.3.25.

The phylogenetic reconstructions support the existence of four major clades within Europe, one of which is present both in Italy and Sardinia and Spain. One divergent haplotype found in the colony of Kovoufovuno (H14) clusters making a specific haplogroup D, which is very far away from the other (Fig.3.26). This unexpected relationship was highly supported by bootstrap (100%) and posterior (1,00) value. Haplogroup B is also highly supported in these phylogenetic reconstructions and is found exclusively in northern Italy. The other haplogroup is distributed in the Peloponnese region of Greece (haplogroup A). The mean uncorrected genetic divergence of haplotypes ranges from 0.6% (within haplogroup B) to 0.8% (within

haplogroup A), while each haplogroup differs from the others by a mean distance of 2.9% (range 2.3–11.3%, Table 3.28).

HAPLOGROUPS	B	C	A	D
B	0,000			
C	0,050	0,000		
A	0,023	0,060	0,000	
D	0,113	0,118	0,097	0,000

Table 3.28: genetic divergence between haplogroups

3.2.2.2 COLONIES

We don't have a very common haplotype spread among different European colonies, some of them cluster in the same haplogroup as showed by previous analysis. We obtained this result because all haplotypes belongs to a single colony. In the northern Italian colonies, the most common haplotyes are H1 and H3, while for Spain is H27 and for Sardinia is H7. The highest variability was found within the two Greek colonies, Maronia and Koufovouno, in fact in this case we described 9 and 10 different haplotypes respectively.

A single bat sampled in the colony of Koufovouno Greece bears the haplotype H11, which is otherwise found in the other Greek colony of Maronia. In the same colony there is the haplotype H14 which is the only one included into haplogroup D (Table 3.29).

HAPLOTYPE	HAPLOGROUP	LIERNA	ISOLA BELLA	FONDI	SARDINIA	KOUFOVOUNO	MARONIA	SPAIN	TOTAL
H1	B	14							14
H2	B	1							1
H3	B		19						19
H4	B		1						1
H5	B		1						1
H6	B		1						1
H7	C			5					5
H8	C				10				10
H9	A					6			6
H10	A					3			3
H11	A					1	6		7
H12	A					1			1
H13	A					3			3
H14	D					1			1
H15	A					2			2
H16	A					1			1
H17	A					1			1
H18	A					1			1
H19	A						1		1
H20	A						3		3
H21	A						1		1
H22	A						2		2
H23	A						1		1
H24	A						1		1
H25	A						1		1
H26	A						1		1
H27	C							8	8

Table 3.29: distribution of 27 haplotypes in the European colonies

At the population level, Italian colonies contained seven distinct haplotypes (Table 3.29), the mean number for these colonies is 2 with a range 1-4, while this value is higher in the Greeks colony and less in Spain and Sardinia. This result is more clear with a graphic representation (Fig.3.27) of each colonies.

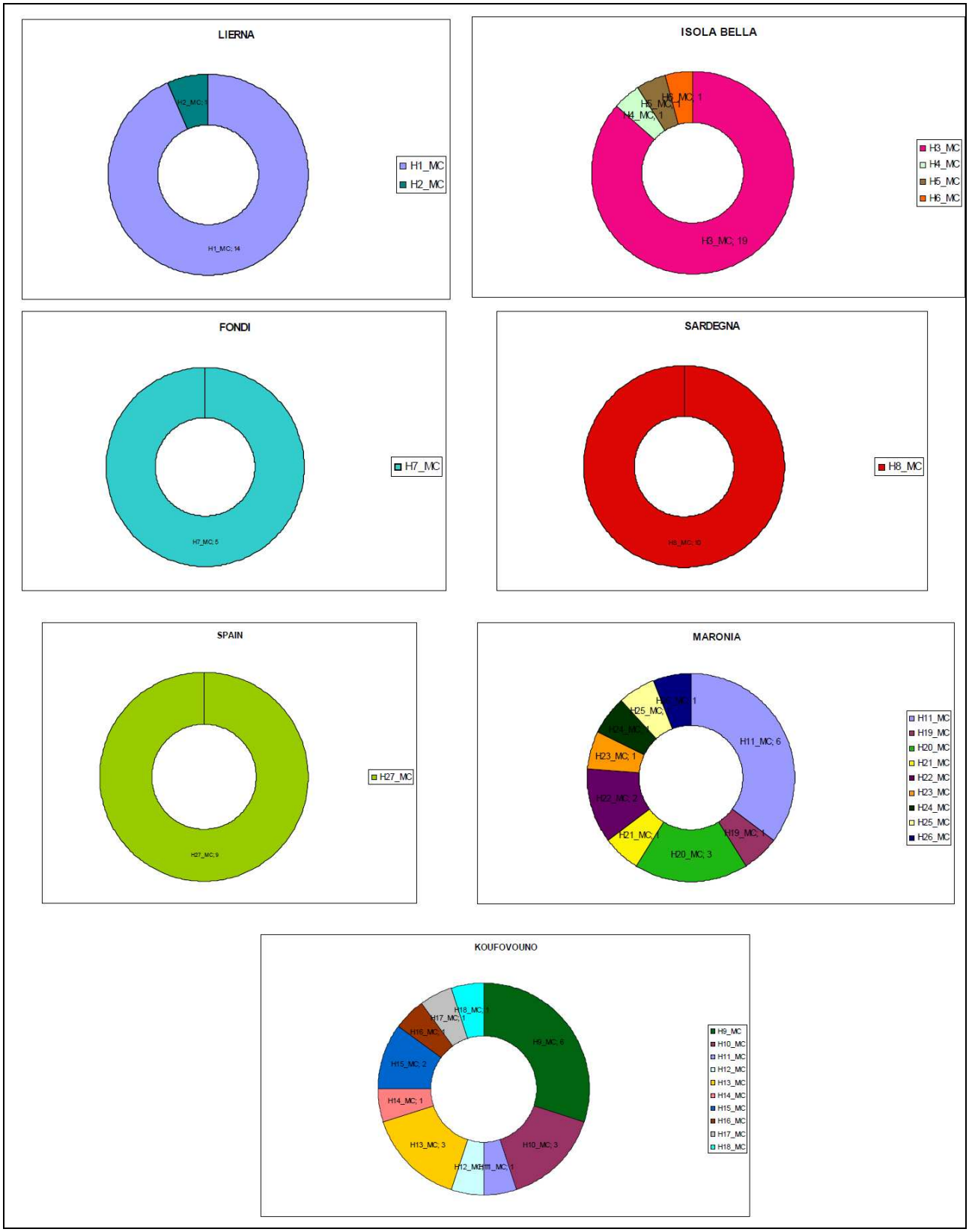


Figure 3.27: graphic rapresentation of distribution of 27 haplotypes in the 7 European colonies

These different graphs have been plotted onto a map (Fig. 3.29) showing that each colony has its own haplotypes.

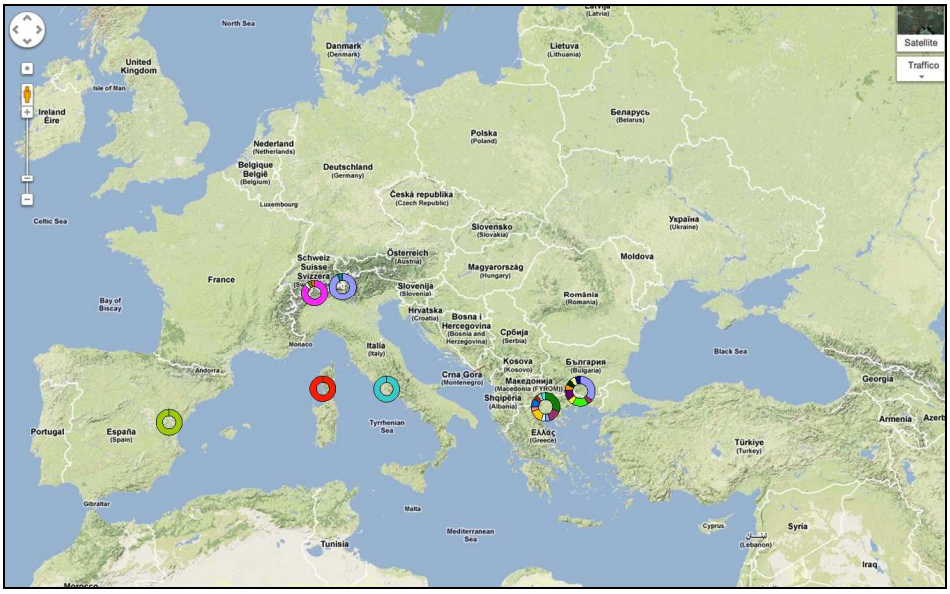


Figure 3.29: Map showing the location of 25 sampled *Myotis emarginatus* nursery colonies. In each colonies is showed the frequency of each haplotype

Looking at haplogroups, the colonies are composed of bats from a single haplogroup, only the one of Maronia, from Greece, is admixed with two haplogroups (Fig. 3.30). This suggests that these colonies were founded by very different matrilineages, and/or that divergent matrilineages persisted over considerable evolutionary periods.

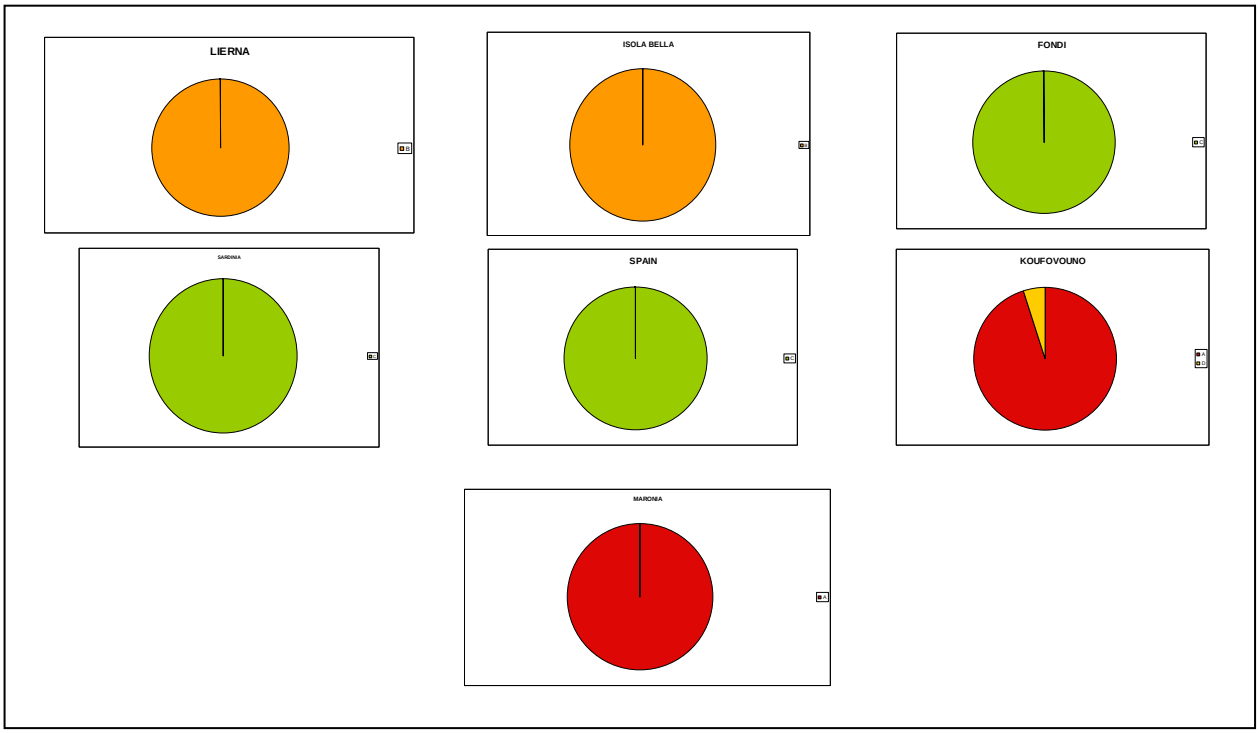


Figure 3.30: graphic rapresentation of distribution of 4 haplogroups in the 7 European colonies

Plotting these different graphs onto a map of Europe, it is clear that across Europe there are three different haplogroups. One is located in North Italy, both in colonies of Lierna and Isola Bella, second one in Greece and the lateste across Spain and South-Italy, Sardinia included. Finally the presence of haplogroup B in the colony of Koufovouno could indicate the presence of another different haplotype in Turkey and more specifically in Middle-Asia.

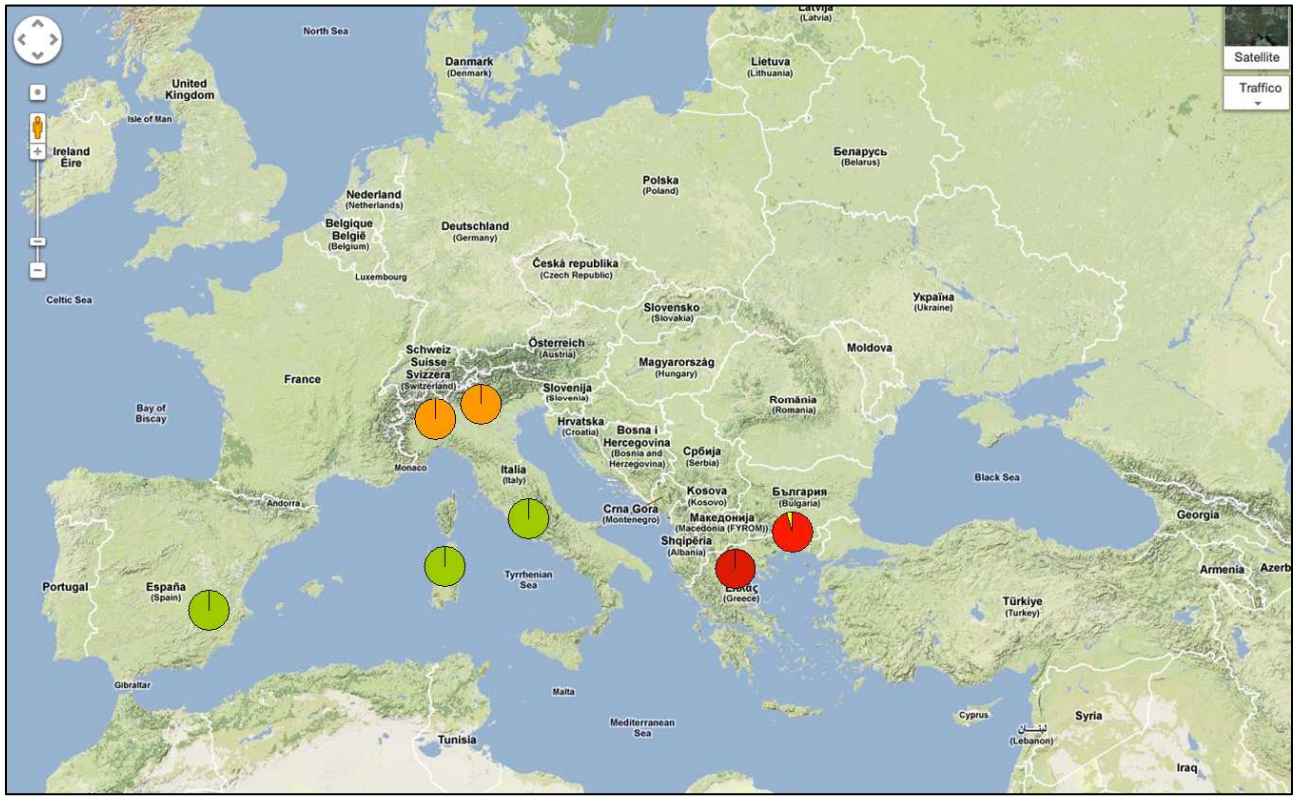


Figure 3.31: Map showing the distribution of 4 haplogroups of *Myotis capaccinii* in nursery colonies. Mitochondrial DNA haplogroups correspond to those presented on the phylogenetic reconstruction in Fig. 3.26 and pie charts show their frequencies in each colony

The mitochondrial variability within colonies was evaluated in terms of haplotype diversity (h) and nucleotide (π), using the software ARLEQUIN 2.0 (Schneider et al., 2000), as shown in Table 3.30

	π	h
LIERNA	0.000357	0,1333
FONDI	0.000000	0.0000
SARDINIA	0.000000	0.0000
ISOLA BELLA	0.000972	0,2597
KOUFOVOUNO	0.013862	0,8842
MARONIA	0.004421	0,8693
SPAIN	0.000000	0.0000

Table 3.30: : haplotype diversity (h), nucleotide diversity (π) for the 7 European colonies

At nucleotide level, the variability of the different colonies is rather heterogeneous, with a range of values of nucleotide diversity that ranges from a minimum of $\pi = 0$ to a maximum of $\pi = 0.013$. The highest values ($\pi = 0.013$) were found in colonies of Koufovouno, colonies

which are in fact composed by a marked coexistence of haplotypes belonging to different clades (A and D). The remaining colonies are mostly characterized by a dominant haplogroup, therefore less diversified ($\pi \leq 0.088$).

Regarding haplotype diversity goes from a minimum of h = 0 to a maximum of h = 0.88, with a trend that reflects the results of nucleotide variability.

For each pair of populations was calculated using ARLEQUIN ΦST value. Below there is a table with the matrix of values obtained from the analysis of nucleotide sequences (Table 3.31).

	LIERNA	ISOLA BELLA	FONDI	SARDEGNA	KOUFOVOUNO	MARONIA	SPAGNA
LIERNA	0.00000						
ISOLA BELLA	***0.78604***	0.00000					
FONDI	***0.99393***	***0.98329***	0.00000				
SARDINIA	***0.99495***	***0.98524***	***1.00000***	0.00000			
KOUFOVOUNO	***0.67492***	***0.73514***	***0.81764***	***0.84699***	0.00000		
MARONIA	***0.84370***	***0.86891***	***0.93725***	***0.95121***	***0.30612***	0.00000	
SPAIN	***0.99472***	***0.98474***	***1.00000***	***1.00000***	***0.82703***	***0.94462***	0.00000

Table 3.31: estimates of population differentiation among the 7 nursery colonies of *Myotis capaccinii*

The overall high fixation index (ΦST = 0.8767, P < 0.001) suggests a very strong genetic structure among the European colonies at the mitochondrial level. The lower values are between the two Greek colonies, and it can be explained with the high variability of these colonies and with the lower level of variation of their haplotypes. Surprisingly, the colonies of Isola Bella and Lierna are separated by only 60 km straight distance, yet they show a remarkable high level of genetic differentiation (ΦST = 0.78, P < 0.001).

AMOVA showed that the global Φst was 0.867, if we considered all colonies in one group, called in this case Europe. The Φst after grouping the colonies as clades A, B and C was 0.897. The percent of differentiation attributable among group differences was 78,62% when clade A, B and C were grouped separately. This was not done for clade D as it was monomorphic. In both case the genetic variability can be explained by differences across individuals among populations/groups.

The genetic relationships between colonies were visualized on an NJ tree based on a matrix of pairwise ΦST values linearized as ΦST/(1 – ΦST) (Slatkin 1995), derived from the mtDNA data set. The NJ are presented in Fig. 3.32. This NJ tree supports the existence of three different groups among Europe, and highlights the close relationships between the central-south Italian colony of Fondi and those of Sardinia and Spain, located across the Tyrrhenian Sea. Greek colonies are also intermingled with the Italian samples, suggesting further that they share closer relationships among them.

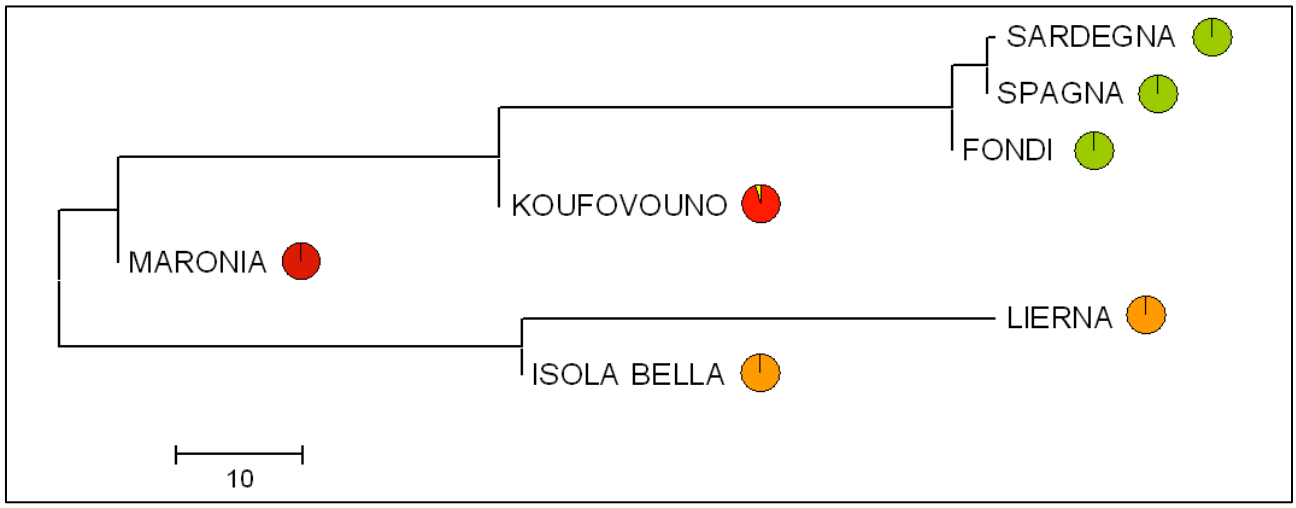


Figure 3.32: unrooted NJ trees of linearized pairwise ΦST among nursery colonies derived from mtDNA

The values of mismatch analysis (Fig 3.33), Tajma D Test and FU test (Table 3.32) indicated that the global sample and all colonies fitted the expectation of a constant size population model.

Looking at the graph in Figure 3.33, which represents the result of the analysis of Mismatch carried out for the whole sample, it is possible to note that the bars are well adapted to the curve expected, producing a typical distribution of the model in constant population size. Values in the table, always in reference to the entire sample, are significant for testing the SSD, then confirm the goodness of fit and allow you to accept the hypothesis of constant size population. Also the measured values for the D-test Tajima and the Fs-test are indicative of a stable population over time as though both pf them were negative but not highly significant.

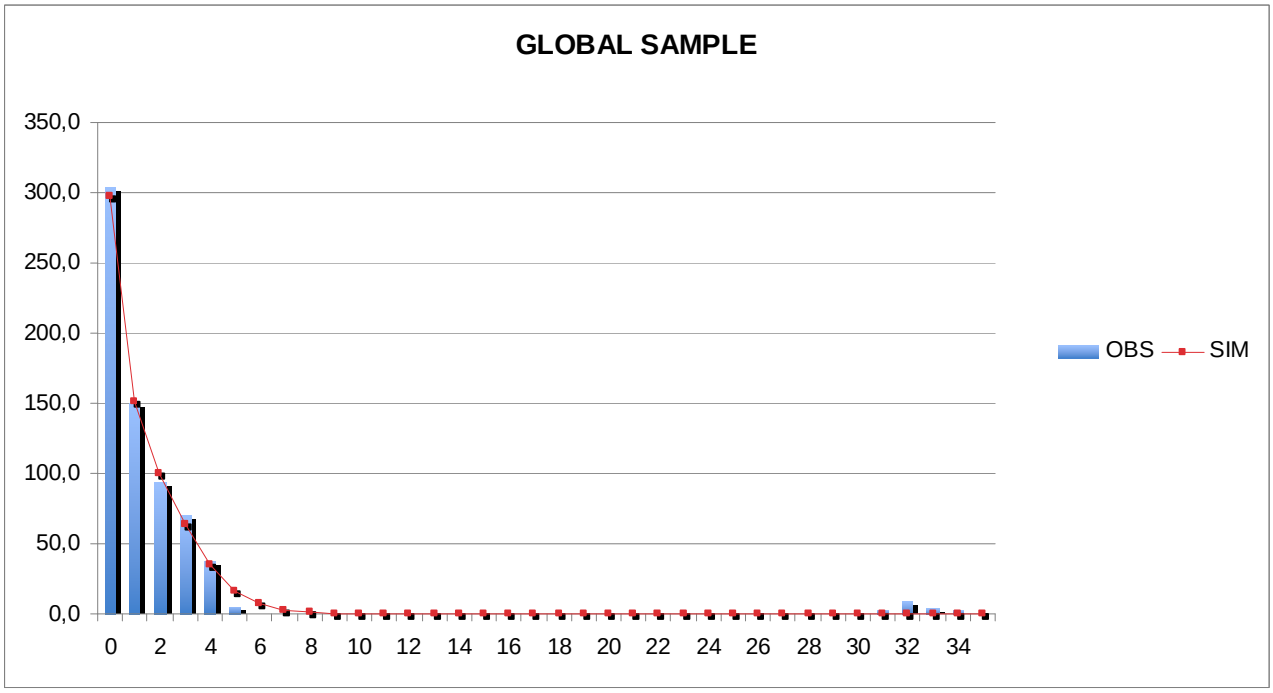


Figure 3.33: the observed and expected mismatch distributions in the European population of *Myotis capaccinii*

	GLOBAL SAMPLE	LIERNA	ISOLA BELLA	FONDI	SARDEGNA	KOUFOVOUNO	MARONIA	SPAGNA
TAJIMA D TEST	-0,83639 P=0,47943	-1,15945 P=0,16000	-1,87763*	0,0000 P=1,0000	0,0000 P=1,0000	-1,81305*	-1,00458 P=0,17700	0,0000 P=1,0000
FU FS TEST	1,68753 P=N.A.	-0,64899 P=0,11000	-2,20561 *	0,0000 P=N.A.	0,0000 P=N.A.	0,74365 P=0,35400	-4,55443*	0,0000 P=N.A.
TAU	1,49079	2,98242	3,0000	0,0000	0,0000	2,75000	1,70312	0,0000
THETA0	0,01808	0,11250	0,0000	0,0000	0,0000	0,01055	0,00352	0,0000
THETA1	14.287,31493	0,45000	0,34824	0,0000	0,0000	11,40625	99999	0,0000
SSD	0,00897 P=0,20257	0,03785 P=0,10100	0,00213 P=0,49700	0,0000 P=0,0000	0,0000 P=0,0000	0,01548 P=0,31600	0,00733 P=0,50400	0,0000 P=0,0000

Table 3.32: results of mismatch distribution, SSD, Tau, Theta0 e Theta1 and neutrality test Tajima's D-test and Fu's Fs-Test

Finally the mitochondrial data set showed no evidence for isolation by distance; as showed by Mantel Test (R^2 = 0,104, P = 0,152).

3.2.2.3 GLOBAL DLOOP RESUME

Considering global sampling, we found on the total number of 27 haplotypes, 23 haplotypes which are private and only 4 that are shared among colonies.

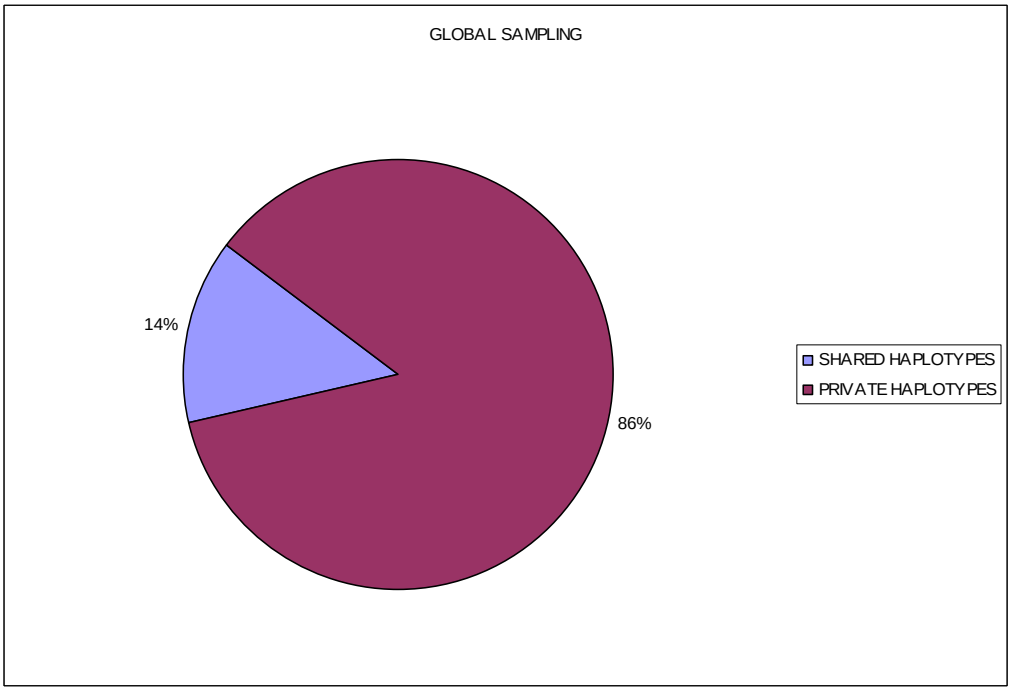


Figure 3.34: : mtDNA, percentage of private and shared haplotypes on whole sampling

In conclusion the most common haplotypes findings among Europe are H1, H3 and H8, which represent the 44% of the total.

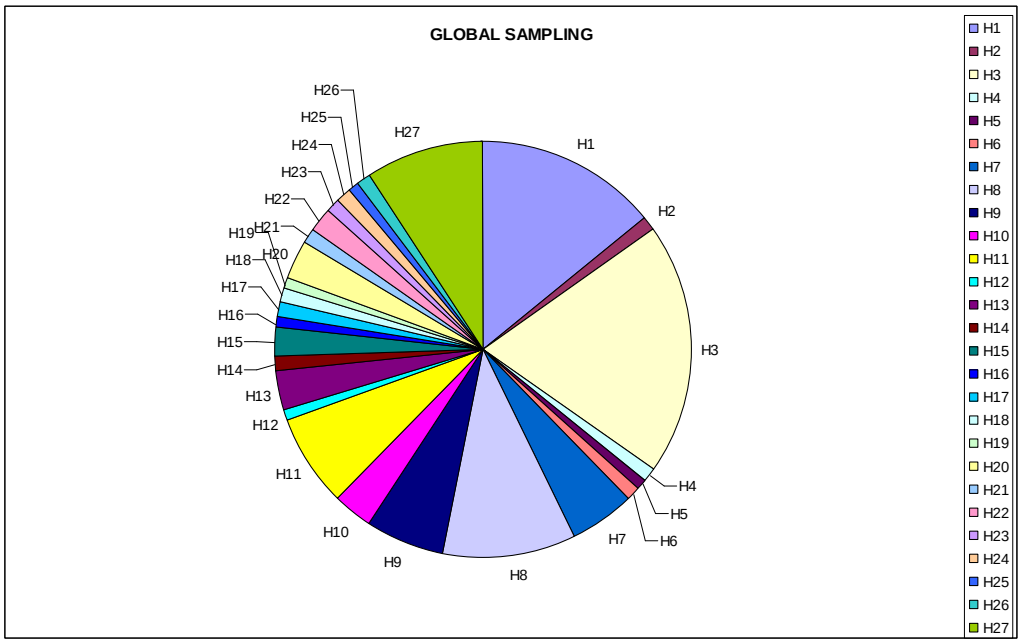


Figure 3.35: frequency of 27 haplotypes on global sample

CYTOCHROME B

3.2.3 *Myotis emarginatus*

We sequenced 1140 bp of CytB, we used for these analysis the second 772 in order to make a comparison using also those in GeneBank, with accession numbers AF376849, DQ120902-DQ120905 and EU360637- EU360639.

There was a total of 23 haplotypes, 8 of these haplotypes (H01-H02, and H15 to H20) were already described in previous work (Ibanez et al., 2006), but the other 15 haplotypes were not been described yet in other European samples. There were 26 sites that were polymorphic, 4 of which were parsimony informative (Table 3.33).

Global nuclear composition is 32,5% T, 24,6% C, 30,0% A and 12,8% G.

	6	13	14	15	18	19	69	81	105	108	110	123	159	165	179	254	443	459	471	473	570	618	638	658	710	759
H1	C	C	G	A	A	T	C	C	T	T	T	A	A	C	C	A	A	C	T	C	T	T	C	C	C	T
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.
H4	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C
H6	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.	T	.	.
H7	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	.	.
H8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.
H9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.
H10	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.
H11	.	G	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C
H12	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H13	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H14	.	A	A	C	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H15	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C
H16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.
H17	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H18	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
H19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.
H20	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	T	.
H21	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	.	T	.	.	.	.	.	T	.	.
H22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.
H23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	T	.

Table 3.33: list of 23 *Myotis emarginatus* CytB haplotypes with the 26 variable positions

3.2.3.1 HAPLOTYPES ANALYSIS

Using the software NETWORK (Fluxus-engineering.com), it was developed a network of all haplotypes identified across Europe (Fig. 3.36). We used the *median-joining* or *MJ* network algorithm.

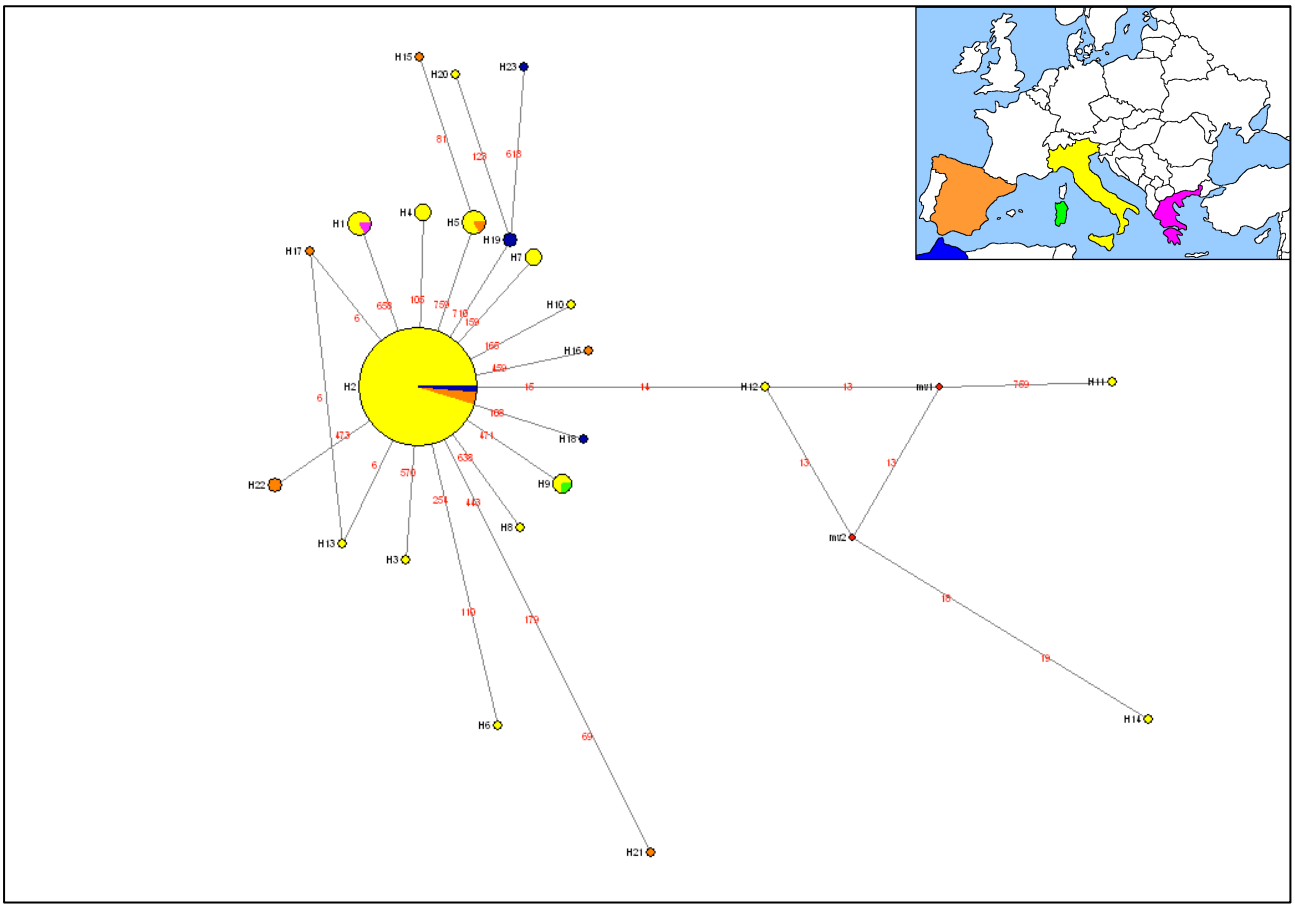


Figure 3.36: phylogenetic network of 23 *Myotis emarginatus* CytB haplotypes

As obtained with microsatellites and Dloop, we don't have a clear genetic pattern with CytB too. The network didn't show any evident cluster. There is one common haplotype from which all others developed. Haplotypes are coloured to match broad geographic regions where samples were acquired from. As we can see, only few of them are founded in different localities, in fact some are only in Spain and Morocco.

In order to have a more defined graphic representation of relationship among haplotypes and investigate which could be, we made a phylogenetic analyses using GENEIOUS software (Biomatter TD) and its PAUP* and MyBayes plugin .The model used in building the neighbour-joining tree was TrN +G (Tamura e Nei, 1993), as revealed by MODELTEST. The NJ (not showed) had also in this case low value of bootstrap, then we performed a Bayesian analysis and a Maximun Parsimony analysis. We used a sequence of CytB from *Myotis tricolor* as an outgroup (Fig. 3.37).

Figure 3.37: Bayesian tree showing phylogenetic relationships of the 23 distinct European haplotypes.The bootstrap support values for the nodes, for Bayesian analysis and MP analyses are represented in the same order. *M. tricolor* was used as outgroups.

The Bayesian tree showed that there isn't a clear relationship among different haplotypes, as already obtained with Microsatelittes and Dloop. We had few clusters, but without any relationship with the geographic distribution. Only one of these had haplotypes found in Morocco (H19-H20, H23).

Considering colonies we had a very common haplotype spread across the colonies from Spain to Cormon, in the North-East of Italy. Only few haplotypes were private.

	BRICHERASIO	CAMPO DEI FIORI	VILLA DOMIZIANO	CORMONS	ESANATOGLIA	GARGAZZONE	GROTTA RIO MARTINO	LAVENO MOMBELLO	MERANO	PAPERIA PIOSSASCO	VILLA DEMIDOFF	PASSERANO	PAVIA	RACCONIGI	REGGIA DI VENARIA	REVELLO	SAN SIGISMONDO	TENUTA SAN ROSSORE	TERRA DEL SOLE	TETTI PESIO	TRINITA	VAL D'ARNO	SARDINIA	SPAIN	MOROCCO	GREECE	GLOBAL SAMPLE
H1																										1	1
H2	4	34	8	3	6	12	11	5	10	5	10	5	5	6	7	4	14	6	6	5	5	17	3	5	1		197
H3								1																			1
H4					3																						3
H5					1	2			1								1							1			6
H6																			1								1
H7																			2			1					3
H8																											1
H9							3		1														1				4
H10																			1								1
H11						1																1					1
H12																						1					1
H13																						1					1
H14											1																1
H15																							1				1
H16																							1				1
H17																							1				1
H18																								1			1
H19																								2			2
H20																								1			1
H21																							1				1
H22																								2			2
H23																									1		1

Table 3.34: distribution of 23 haplotypes in the European colonies

3.2.4 *Myotis capaccinii*

The laboratory protocols yielded a total of 657 base pairs of cytochrome-b for the samples of *M. capaccinii*. For furthers analysis we used also the sequence already discovered by Bilgin et al.(2008), which were deposited in GenBank with accession numbers EU475878-EU475885. There was a total of fifteen haplotypes, six of these haplotypes (H01, and H11 to H15) were already described in Bilgin *et al*. (2008), but the other nine haplotypes had not been described yet in other European samples. There were 47 sites that were polymorphic, 41 of which were parsimony informative (Table 3.35).

The base frequencies for Adenine, Cytocine, Guanine and Thymine were 29,5%, 25,5%, 12,1% and 32,9%, respectively, all these values are comparable to ones of the previous study (Bilgin et al., 2008) .

	7	38	44	57	59	62	77	122	126	131	149	167	174	191	197	219	224	230	242	251	254	256	257	275	278	281	314	326	350	365	374	422	431	452	453	488	530	531	554	563	575	587	591	614	632	640	641
H1	C	T	T	T	G	T	T	T	T	T	T	T	A	T	C	A	C	A	T	A	G	T	G	T	T	A	C	T	A	A	C	C	C	G	T	C	G	C	C	T	T	A	C	C	A	T	T
H2	.	C	C	C	A	C	.	C	C	.	C	.	G	C	T	G	T	G	.	.	A	C	A	C	C	.	T	.	G	G	T	.	T	A	C	T	A	.	.	C	C	G	T	T	.	C	C
H3	.	C	C	C	A	C	.	C	C	.	C	.	G	C	T	G	T	G	.	.	A	C	A	C	C	.	T	.	G	G	T	A	T	A	C	T	A	.	.	C	C	G	T	T	.	C	C
H4	.	C	C	C	A	C	.	C	C	.	C	.	G	C	T	G	T	G	.	T	A	C	A	C	C	.	T	.	G	G	T	.	T	A	C	T	A	.	.	C	C	G	T	T	G	C	C
H5	.	C	C	C	A	C	.	C	C	.	.	.	G	C	T	.	T	G	.	.	A	C	A	.	C	.	T	.	G	.	T	.	T	A	C	T	A	T	T	.	C	G	T	.	G	C	C
H6	.	C	C	C	A	A	.	C	C	.	.	.	G	C	T	.	T	G	.	.	A	C	A	.	C	.	T	.	G	.	T	.	T	A	C	T	A	T	T	.	C	G	T	.	G	C	C
H7	.	C	C	C	A	A	.	C	C	.	.	.	G	C	T	G	T	G	.	.	A	C	A	C	C	.	T	.	G	.	T	.	T	A	C	T	A	.	.	C	C	G	T	T	G	C	C
H8	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	C	C	C	A	A	.	C	C	.	.	.	C	C	T	G	T	G	.	.	A	C	A	C	C	.	T	.	G	.	T	.	T	A	C	T	A	.	.	C	C	G	T	T	G	C	C
H10	A	C	C	C	A	C	.	C	C	.	.	C	G	C	T	G	T	G	.	.	A	C	A	C	C	.	T	.	G	.	T	.	T	A	C	T	A	.	.	C	C	G	T	T	G	C	C
H11	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 3.35: list of 15 *Myotiscapaccinii* CytB haplotypes with the 47 variable positions

3.2.4.1 HAPLOTYPE ANALYSIS

Using the software NETWORK (Fluxus-engineering.com), it was developed a network of all haplotypes identified across Europe (Figure 3.38). We used even in this case the *median-joining* or *MJ* network algorithm.

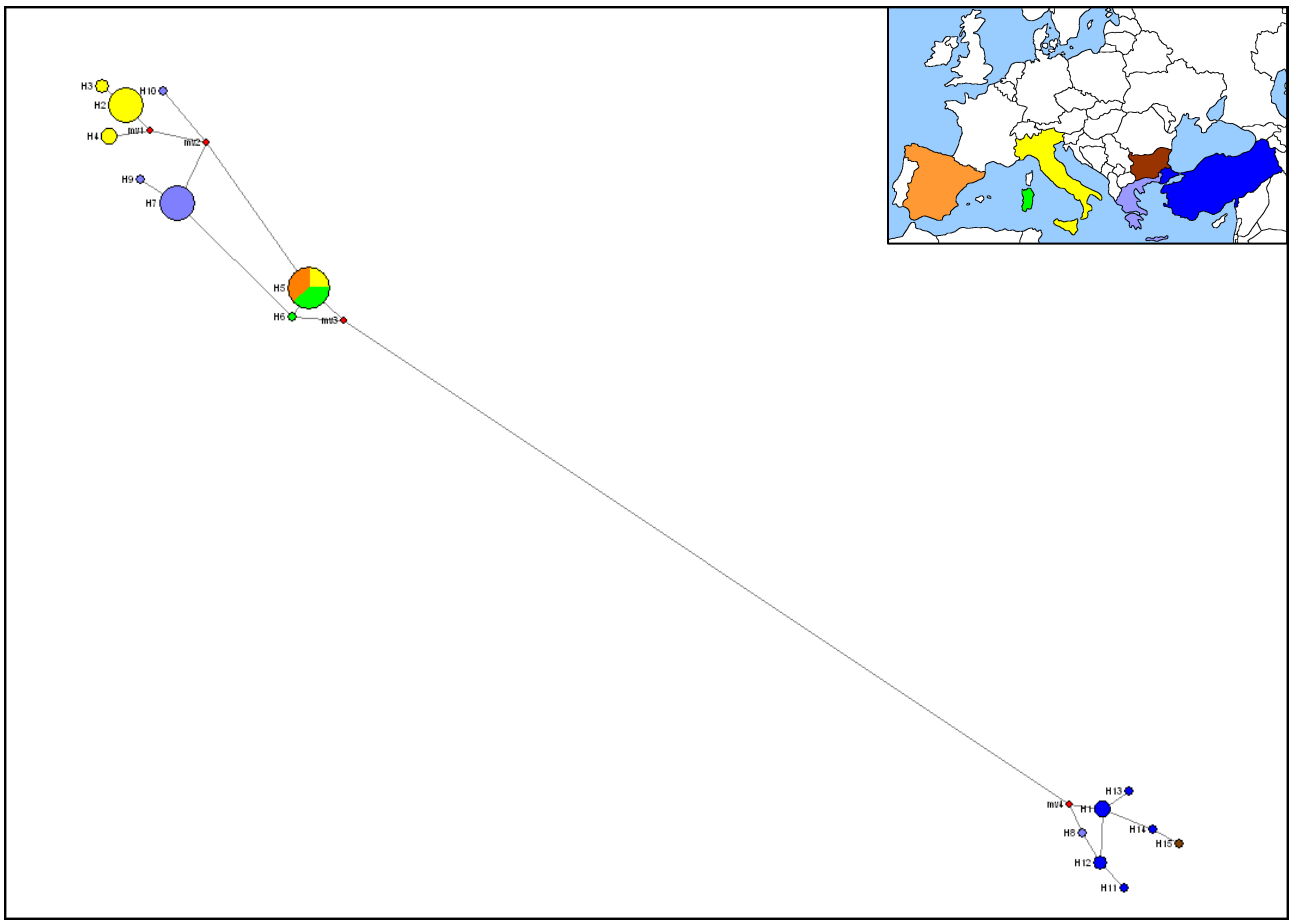


Figure 3.38: phylogenetic network of 15 *Myotis capaccinii* CytB haplotypes

Specifically, the result shows a similar distribution of haplotypes found with Dloop, in these case we have three different haplogroups. One regrouped all samples found in Turkey and Bulgaria by Bilgin plus one found by us in the colony of Kovofouno. All other Italian and Greek samples clustered into the same haplogroup, while as we already saw for Spanish, Sardinian and Southern Italian samples, they moved into another haplogroup.

Phylogenetic analyses of *M. capaccinii*, using neighbour-joining, maximum parsimony and Bayesian analysis (Fig. 3.39) showed the presence of three main clades, with high bootstrap support, confirming the same topology that we found using NETWORK.

The distribution of these three clades indicated a west–east split and a west split. These clades will be referred to as clades B C and A, the previous two clades were already described by Bilgin, here we identified another one, clade A, which is spread among the south Mediterranean area, from Spain to south Italy. The model used in building the neighbour-joining tree was GTR+G (Tavaré 1986) as revealed by MODELTEST. However, the boundary was not absolute. One individual with a clade B haplotype was found, along with clade C haplotypes, in a cave in Greece (Koufovouno), this was described also by Bilgin, who found clade C in Bulgaria, Greece and Turkish Thrace.

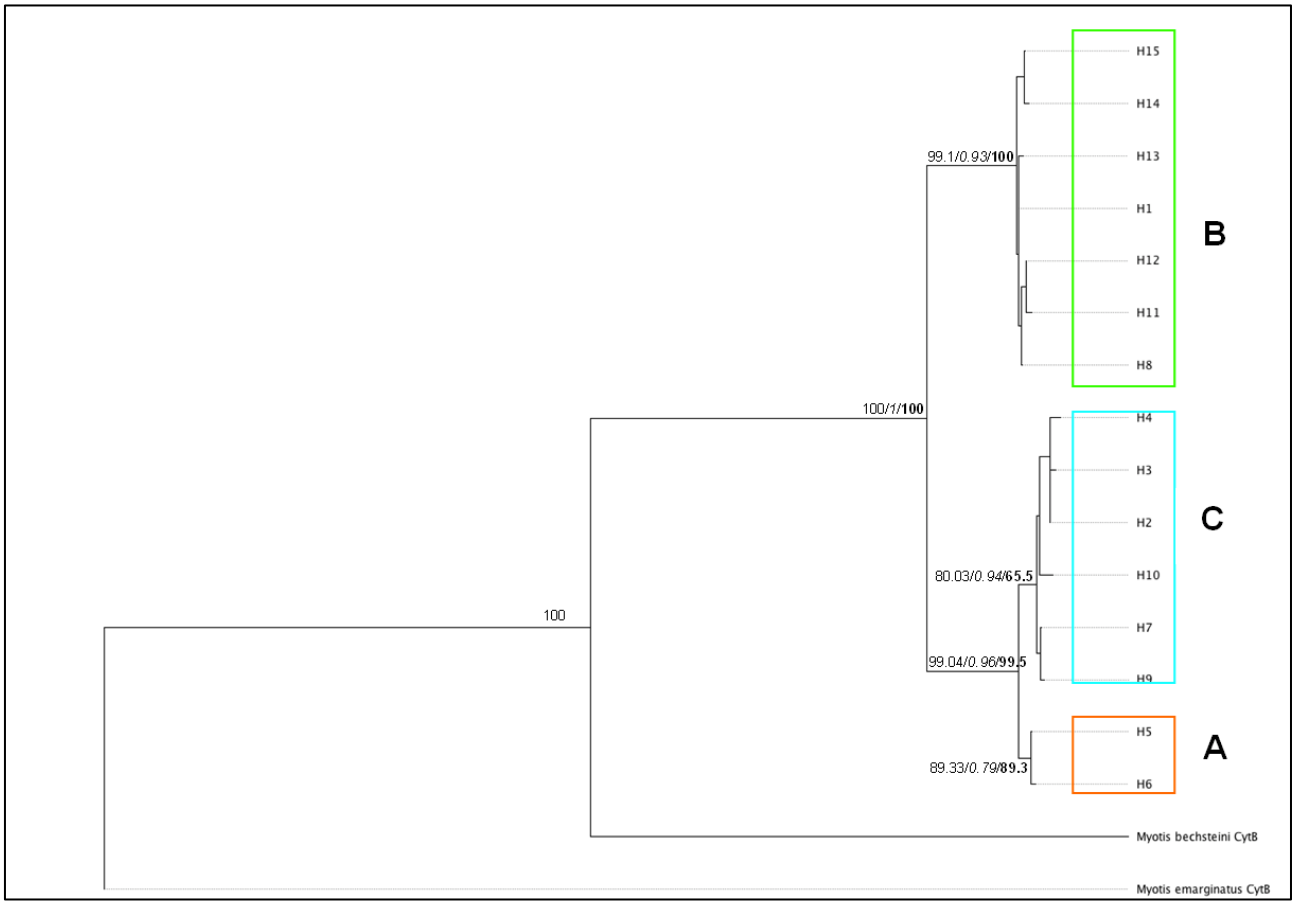


Figure 3.39: The neighbor-joining tree for *M. capaccinii*. The bootstrap support values for the nodes, differentiating the clades C and B, and C and A for NJ/Bayesian/MP analyses are represented in the same order. *M. bechsteinii* and *M. emarginatus* were used as outgroups.

The divergence between clades B and C, expressed as Dxy (the number of changes per site), was 5.90%, and between B and A was 1,30%; the value of difference between B and C is similar to one detected by Bilgin, 5,10% (Table 3.36). The percent difference within clades B, C and A were 0.61%, 0,41% and 0,13%, respectively.

	C	B	A
C			
B	5,90%		
A	5,30%	1,30%	

Table 3.36: genetic divergence between haplogroups

Tajima's molecular clock test showed that a hypothesis of a molecular clock could not be rejected (P = 0.72).Based on a molecular clock of 4.8% divergence per million years [for cytochrome-b in *M. myotis* (Ruedi & Mayer 2001)], the time of divergence for clades B and C was calculated to be approximately around 580.000 years ago, which is similar to one identified by Bilgin (around 500.000). The time of divergence between clades A and B was 130.000 years.

At colony level we could see that each colony had his own haplotype, some of Greek haplotypes are in the same haplogroup of Italian ones (Table 3.37). This could demonstrate that between Italian colonies and the Greek ones there is a strictly genetic pattern.

HAPLOTYPE	HAPLOGROUP	LIERNA	ISOLA BELLA	FONDI	SARDINIA	KOUFOVOUNO	MARONIA	GREECE	SPAIN	BULGARIA-GREECE	TURKEY	TOTAL
H1	B										1	1
H2	C	14										14
H3	C	2										2
H4	C		3									3
H5	A			5	8				8			21
H6	A				1							1
H7	C					7	8					15
H8	B					1						1
H9	C						1					1
H10	C							1				1
H11	B										1	1
H12	B										1	1
H13	B										1	1
H14	B										1	1
H15	B									1		1

Table 3.37: distribution of 15 haplotypes in the European colonies

On the map the three different haplogoups followed a typical geographic distribution, it showed also the close relation between Italian and Greek colonies over the other haplogroup including the colonies from Spain to South Italy (Fig. 3.40).

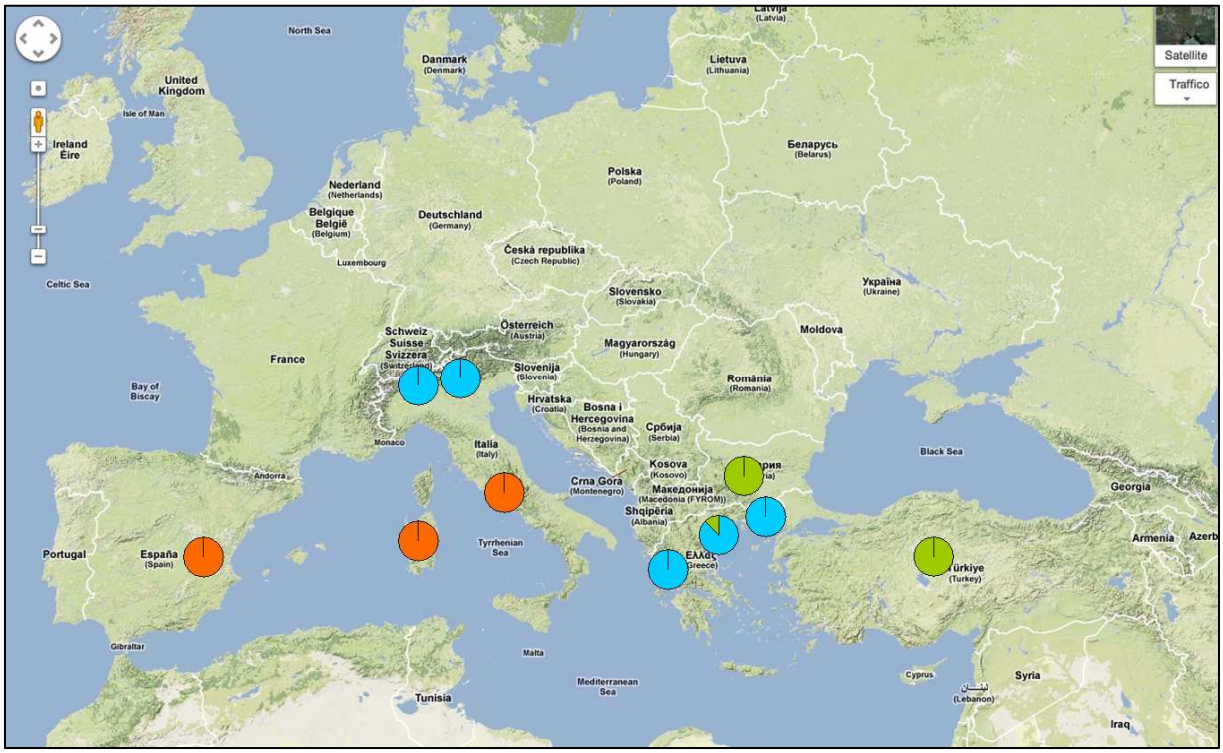


Figure 3.40: Map showing the distribution of 3 haplogroups of *Myotis capaccinii* in nursery colonies. Mitochondrial DNA haplogroups correspond to those presented on the phylogenetic reconstruction in Fig. 3.39 and pie charts show their frequencies in each colony

3.3 FUTURE PRESPECTIVE

Here a brief description about internship done at UCD (Univeristy College Dublin) under the supervision of Prof. Emma Teeling.

3.3.1 *Myotis emarginatus* AND *Myotis capaccinii* NUCLEAR INTRONS

Of the 13 *Myotis emarginatus'* samples and 12 *Myotis capaccinii* samples used in the nuclear intron study (Table 3.38 and 3.39); we used BGN for both species, while other six introns only on *Myotis capaccinii* (ABDH11, ACOX2, ACPT, COPS7A and ROGDI). We decided to follow this way considering the previous result obtained with microsatellites and mtDNA.

Considering BGN we found only one haplotype on *Myotis emarginatus*, indicated that the species is the same across Europe, so that no taxonomic implications are possible.

For *Myotis capaccinii* there were 4 haplotypes. Due to the X-linked nature of this gene all females will carry two copies and therefore have the potential to be heterozygous which will equate to possessing two haplotypes. Nuclear introns do not evolve as quickly as many regions of mtDNA or nuclear microsatellites do. At the end, considering the results we got, we expect to obtain a low value of genetic diversity using this molecular marker.

Myotis emarginatus	
COUNTRY	N° SAMPLES
ITALY	4
ITALY (SARDINIA)	2
SPAIN	2
MOROCCO	3
TUNISIA	2

Myotis capaccinii	
COUNTRY	N° SAMPLES
ITALY	4
ITALY (SARDINIA)	2
SPAIN	2
GREECE	2
IRAN	3

Table 3.38 and 3.39: samples of *Myotis emarginatus* and *Myotis capaccinii* analyzed for BGN

As in many cases countries had several sequences which were all of the same haplotype, indices of genetic variability could not be calculated.

Myotis emarginatus					
COLONY	**N°SAMPLES**	**N°HAPLOTYPES**	**HAPLOTYPES**	**π**	**h**
CAMPO DEI FIORI	2	1	H1	-	-
VILLA DOMIZIANO	2	1	H1	-	-
SARDINIA	2	1	H1	-	-
SPAIN	2	1	H1	-	-
MOROCCO	2	1	H1	-	-
TUNISIA	2	1	H1	-	-

Myotis capaccinii					
COLONY	**N°SAMPLES**	**N°HAPLOTYPES**	**HAPLOTYPES**	**π**	**h**
LIERNA	2	1	H1	-	-
FONDI	2	1	H2	-	-
SARDINIA	2	1	H2	-	-
SPAIN	2	1	H2	-	-
KOUFOVOUNO	2	1	H2	-	-
IRAN	3	2	H3, H4	0.002564	0.6667

Table 3.40: results of *M. emarginatus* and *M. capaccinii* BGN analisys. (h), nucleotide diversity (π), allelic richness

Within the phylogenetic network (Fig. 3.41) constructed for *Myotis capaccinii* for the BGN intron it can first be noted that the greatest distance between the two more genetically distant samples is very low. Haplotypes are coloured to match broad geographic regions where samples were acquired from. There are two haplotypes from Iran which form a diverse clade, and other two haplotypes which encompass all other samples used in this study from the west of the species range, Spain/Sardinia and also some samples from the east Fig. 3.41 shows that the majority of samples studied share one of two haplotypes, with the remainder of haplotypes having low frequencies. For this marker the east forms the most diverse clade.

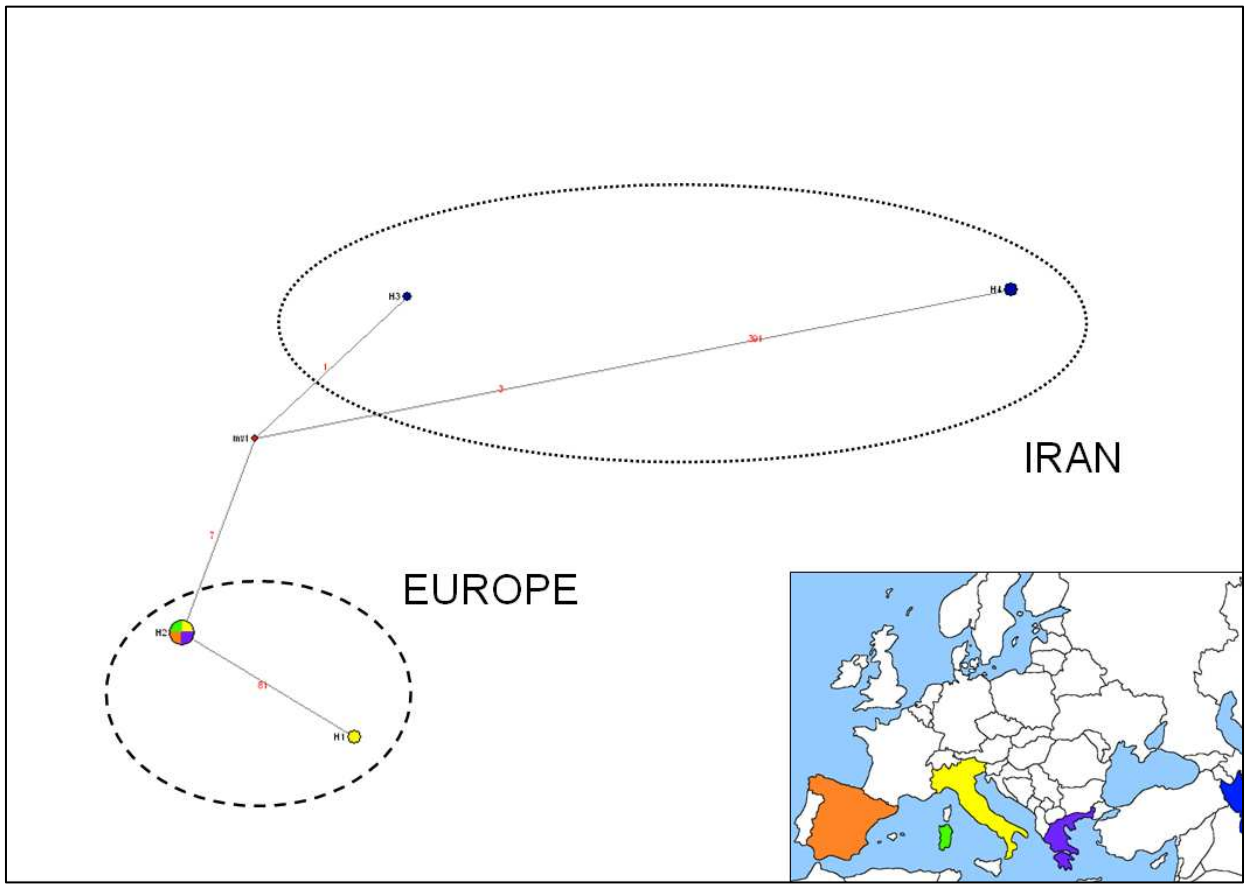


Figure 3.41: phylogenetic network of 4 *Myotis capaccinii* BGN haplotypes

3.3.2 Comparison of BGN between *Myotis emargiantus* and *Myotis capaccinii*

We aligned the two BGN sequences of *Myotis emarginatus* and *Myotis capaccinii*, in order to evidence differences between two species. We used haplotype H1 for *Myotis emarginatus* and haplotype H2 for *Myotis capaccinii*, which is the most common among our samples.

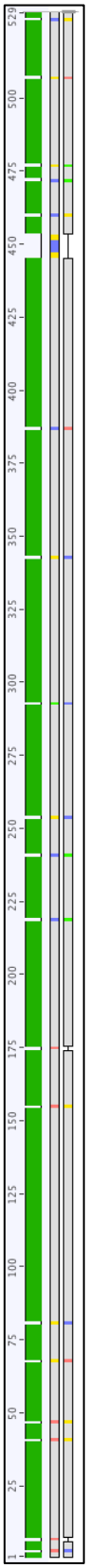


Figure 3.42: *M. emarginatus* and *M. capaccinii* BGN sequence aligned

On 529 bp, the percent of identity is 94,9%. The principal difference between the two sequences was represented by an insertion of 8 bp at position 443 on *Myotis emarginatus*' BGN sequence. In total there are 18 variable positions and two more insertions of one base (positions 7 and 175) between *Myotis emargiantus* and *Myotis capaccinii*.

3.3.3 VARIATION AMONG SPECIES

We used the sequence obtained with other six nuclear introns to investigate the amount of difference between species. We made a comparison among *Myotis capaccinii* and three other species, *Myotis schaubi*, *Myotis nattereri* and *Myotis eschalerai*; for all of these species there are sequences in NCBI genebank. The comparison was made considering nucleotide diversity (π) and haplotype diversity (h) (Table 3.41).

	ABDH11			
	π	h	N°sequences	N°haplotypes
Myotis capaccinii	-	-	9	1
Myotis escalerai	0.001782	0.5385	13	5
Myotis nattereri	0.007538	0.9095	21	10
Myotis schaubi	-	-	2	1
	ACOX2			
	π	h	N°sequences	N°haplotypes
Myotis capaccinii	0.002901	0.8222	10	5
Myotis escalerai	0.000668	0.7821	13	6
Myotis nattereri	0.009107	0.9649	19	16
Myotis schaubi	-	-	2	1
	ACPT			
	π	h	N°sequences	N°haplotypes
Myotis capaccinii	0.007335	1.000	2	2
Myotis escalerai	0.012852	0.6813	14	6
Myotis nattereri	0.043298	0.9825	19	16
Myotis schaubi	0.008475	1.000	2	2
	COPS7A			
	π	h	N°sequences	N°haplotypes
Myotis capaccinii	0.001095	0.6667	7	3
Myotis escalerai	0.002060	0.5934	14	5
Myotis nattereri	0.005511	0.9825	19	16
Myotis schaubi	-	-	2	1
	ROGDI			
	π	h	N°sequences	N°haplotypes
Myotis capaccinii	0.000881	0.3778	10	3
Myotis escalerai	0.000438	0.2949	13	3
Myotis nattereri	0.003259	0.5956	17	7
Myotis schaubi	-	.	2	1

Table 3.41: nucleotide diversity (π) and haplotype diversity (h) of *Myotis capaccinii* and three other species of Myotis using ABDH11, ACOX2, ACPT, COPS7A and ROGDI

Considering all primers, the highest values of both π and h was obtained using ACPT. In global the two species *Myotis escalerai* and *Myotis natterei* showed higher values than the other two species considering all nuclear introns markers. However only one primer, ABDH11, showed one haplotype from *Myotis capaccinii*.

CHAPTER 4

DISCUSSION

The work described here has led, for the first time, to describe the population structure of the two bats species of the genus *Myotis*, *Myotis emarginatus* and *Myotis capaccinii*. These species, like all bats are protected under the Habitats Directive, thus these results are of considerable importance because they allow to assess not only the differences identified between different colonies but also the status of "genetic health" of the same.

Last but not least also the fact that being *Myotis capacccinii* critically endangered, according to the IUCN, the results obtained can strengthen conservation activities for this species.

We investigated these two species at two types of genetic markers, mtDNA and nuclear DNA, to compare the results, strengthening them and checking if these results could be due to aspects of behaviour, influenced by the gender of the analysed bats; in fact the females of some species (eg *Myotis myotis*, *Myotis blythii*) are faithful to their colony of origin, while males can make even long journeys (Burland & Worthington Wilmer 2001, Kerth et al. 2002), as the typical situation described in mammals and in birds (Greenwood 1980).

Considering also that bats are protected species, we developed a non-invasive sampling method, which could eventually makes it easier and less harmful genetic research on these animals.

4.1 NGS

The aims of this sampling scheme of the research is to describe a method for the analysis of non-invasive bat samples (mainly faeces). From the collected data we can observe that the yield of recovered samples in the different colonies is satisfactory, a percentage of 51% is quite good considering that some of samples were recovered in mixed colonies in which there are not only the two species under investigation. Specifically considering the samples, which have passed the initial quality screening (109), the percentage of genotyped samples is equal to 90%, very high if compared to that obtained (91.1%) in the study of Puechmaille et al. (2007). This efficiency in the rendering is very important when considering studies to be made on a larger scale and with a larger sampling. Also the percentage of samples, which are positively amplified at mtDNA is very high, similar to that obtained for microsatellites (94.39% in total).

In our study we observed that the amplification success is much higher in colonies where there is the dominance of *Myotis emarginatus* or *Myotis capaccinii*, in fact most of the colonies, which are monospecific for those species, are those with the best yield for *Myotis emarginatus*. While colonies of Terra del Sole (Emilia-Romagna) and Trinità (Piedmont) are

those that show a lower yield, because they are polyspecific. In the only colony of *Myotis capaccinii* sampled with non-invasive method, Isola Bella (Piedmont), the yield is satisfactory but lower compared with that of the colonies, where there is the predominance of *Myotis emarginatus*, probably because in this colony there is not only *Myotis capaccinii*. The fact that 97 unique genotypes have been identified out of a total of 98 samples, it indicates that the technique of sampling can give a good result because it reduces significantly the chance of sampling the same individual more than once.

Using STRUCTURE, Genetix and GENEALEX, we have observed how the two species separate themselves without showing uncertain situations, in particular we have seen that for the non-invasive samples, which are exactly assigned to their respective species. This indicates that the set of microsatellites, used on the two species, allows the discrimination without ambiguity and confirms that the non-invasive samples belong to one of the two species of this study.

The genotyping error values (mean ADO 31% and mean FA 0.0 %) indicate that our Multitube protocol is efficient and gives reliable results.

The following work can demonstrate that it is possible to extract a good quality DNA from bat fecal samples, it is possible to identify individuals and assign them to one of the two species under study. With this type of protocol catches are no longer necessary, so the sampling can be performed in a single time without giving troubles to the colony. It is necessary to consider also that this method of sampling is easy to perform and very cheap.

But it is important to remember that a good performance, especially in case when you select one or two species, is possible when you work on unique and not mixed colonies. In fact in this case it could be possible that only some of the collected samples could give good results, as it is possible that the others belong to different species, which live in the colony. But the method is useful if you want to identify all species present in a mixed colony. Finally, this method is very useful if you are doing studies on species endangered or protected by law, as in our case the bats.

4.3 *Myotis emarginatus*

This is the first work by far about Italian and European *Myotis emarginatus* .populations. In this case the work took a little more time because no one had tested microsatellite markers for this species before, so part of the work involved the development of a STR. Once again CR, haplotypes we found are the first for the species, while those of CytB are in addition to other previously identified.

4.3.1 Analysis of the population structure

These results show *Myotis emarginatus* is a species with a not well-defined genetic structure. The fact that the specimens of Morocco at microsatellite level separate, albeit weakly, from all others and using STRUCTURE a genetic component is common throughout Europe but the only one that was identified in Moroccan samples, may support the hypothesis of African origin of the species. This result also may indicate that its spread in Europe had followed two lines of development: from the Straits of Gibraltar, and from Egypt, then moved to the Balkans. This hypothesis needs to be confirmed by the analysis of samples from Greece and Balkans.

The CR shows that even with the mtDNA marker it is very difficult to determine and describe a possible genetic structure in Europe. In fact, throughout Europe it is presented in many analyzed colonies with a high percentage the haplotype H1, some colonies show private haplotypes but in the three colonies in North Italy (Gargazzone, St. Sigismondo and Merano) we identified a haplotype (H2) which is present in the absolute majority, while the H1 haplotype identified only in a few individuals.

It is also interesting the situation of the colonies located in Piedmont near the French border, they have two haplotypes identified only in these colonies, H4 and H20. These haplotypes might be characteristic of colonies of France *Myotis emargiantus* but in this case a further sampling would needed. Similar situation was found in the colony of Cormons, located in Friuli-Venezia Giulia region, 2 km from the Slovenian border. The presence, only in this colony, of haplotype H11 with a good percentage (4 samples on total) would suggest the arrival of this haplotype from Balkan colonies.

Haplotype H2 was found only in colonies of Trentino-Alto Adige, suggesting that females of *M. emarginatus* are very philopatric, and did not emigrate out of the border near Austria.

In other Italian colonies private haplotypes were identified, this could be indicative of relatively recent mutation events, given the presence in all the colonies of haplotype H1 and the low value of genetic variability among all identified haplotypes (mean 0,9%) and values of overall ΦST, 0.2988, and their parwise values between colonies. This result also emerges from the network of haplotypes, this shows an ancestral haplotype (H1) from which drift the other 38. Furthermore, both the network and the tree obtained by MrBayes show how it is difficult to find at least a distribution of haplogroups or haplotypes, which follow a geographical pattern.

This phylogeographical study shows that *Myotis emarginatus* from the Apennine peninsula bears mitochondrial lineages that are identical or very similar to those found elsewhere in Europe, suggesting that this region did not serve as a major refuge for current *Myotis*

emarginatus mitochondrial diversity or that those lineages expanded post glacially to many other areas in Europe. Same consideration could be done for haplotypes found in Spain, in this case in fact most of the haplotypes are private for Spain, but are very similar to those found in Italy or Morocco.

Values of haplotype and nucleotide variability reflected the composition of the single colonies. In all the colony, with the exception of that of Racconigi, we found at least two haplotypes, excluding this colony the range of haplotypes of all the analysed colonies varies from a minimum of 2 to a maximum of 7, with an average value of 3,60.

The values of parwise ΦST then highlighted even more as the colonies of Northern Italy are the most distant to the genetic level from all others. This situation is very well represented in the NJ tree obtained with these same linearized distances.

The results of the Mismatch Distribution and its tests associated (SSD) and tests of neutrality Tajima D and Fu-Fs-test, all carried out using the mitochondrial marker, indicate that the global population of *Myotis emarginatus* and their colonies remained demographically stable over time.

The analysis conducted with the CytB largely confirms what was observed with the previous markers. Also in this case the network showing the presence of an ancestral and common haplotype for the majority of individuals sampled (H2); the few haplotypes discovered and their poor diffusion spread across the colonies, and their location within the network and the impossibility to determine distinct haplogroups with PAUP and Mrbayes analysis, confirmed the probable recent mutational events that have affected this species.

In conclusion, what happened on this species, is probably an event of recent differentiation which is still in progress. It could then lead to a greater level of variability in the future if the genetic factors that could cause isolation will increase, especially regarding the Northern Italy colonies. But in agreement with results of the latter studies, we did not detect any pattern that maps to the geography of the European colonies when we used any of the three markers. In the case of the mitochondrial marker, we obtained deeply divergent clades, but those were not geographically partitioned. The absence of population structure could be due to a high effective population size and a high gene flow or a combination of both.

The lack of phylogeographic structure in the *Myotis emarginatus* is not unexpected because considering his ecological traits and tropic niches (Flaquer et al., 2008) it is clear that this species can be found in most part of Europe and the dispersal in this species is not constrained by open areas, rivers and mountains, which represent barriers to gene flow for other bats as *Myotis myotis* (Ruedi et al., 2008).

Considering the phylogeny of this species and in particular the work of Stadelmann (2004) on the phylogeny. of African *Myotis*, it emerges, as a node of interest, the split between the lineages leading to *M. tricolor* and to *M. emarginatus*. This divergence is dated at about 5.98 ± 1.18 MYA.

His phylogenetic position in this Ethiopian clade (Stadelmann et al., 2004) suggests that it is derived from one African ancestor that secondarily colonized Eurasia (Fig. 4.1) .

Figure 4.1: hypothetical scenario of colonization of Europe by the species *M. emarginatus*, according to the phylogenetic results (Stadelmann et al. 2008) who indicated an African origin.

This situation would seem confirmed by the results of the microsatellite, where a genetic component typical for Moroccan samples was identified also in Europe and even the CytB and dloop data showed that, in fact in samples from Morocco was found the ancestral haplotype of the two markers that turns out to be also those spread throughout Europe.

Moreover considering, the fossil record shows that the presence of *Myotis emarginatus* in Italy and Europe is relatively recent, the oldest fossils are dated to the late Pleistocene (Tata, 2005; Sevilla, 2011) and there is no trace of the oldest presences. This would indicate that the colonization of Europe by this species is fairly recent.

Mitochondrial markers are thus more likely to conserve a trace of the early colonization history or of the foundation of a colony than nuclear STR markers, which would be more suited to measure current levels of population gene flow (see Heckel *et al*. 2005). Although *M. emarginatus* populations in Italy are indeed about 10 times less structured at nuclear vs. mitochondrial markers (*F*ST = 0.024 vs. ΦST = 0.29), both classes of markers support a weak differentiation among colonies. This suggests that both males and females are vagile and that

the phylogeographical patterns presented here certainly reflect the history of the whole populations, not just the movements of the females or males.

4.2 *Myotis capaccinii*

Excluding Bilgin's work (2008), these are the first results that take into account the majority of the distribution area of this species, for the first time the colonies have been analyzed not only at the level of nuclear DNA but also using the CR of mtDNA, thus identifying the first haplotypes available for this species.

4.2.1 Colony structure and phylogeographic relationship

Since this is an endangered species, living in a specific habitat, like lakes and rivers with a height of vegetation not exceeding 50m, few colonies are known in Italy as in Europe. The sampling allowed to cover a good part of the Italian peninsula, with two colonies in North Italy, very close together, the colony of Isola Bella on Maggiore Lake and that of Lierna on Como Lake. Then we sampled two colonies in Sardinia and Lazio near the city of Fondi. It is know that there are other colonies, one in Puglia and a second in Sicily, respectively. Future goal will be to collect some samples from these colonies, especially considering the results obtained using Fondi's samples.

In fact the results, considering both molecular markers, showed a clear separation between the colonies of North Italy with those of Fondi and Sardinia. Of particular interest is the fact that the colonies of Fondi seems to be closer to those of Sardinia. The fact that there is this difference between Fondi and Northern Italy might be due to the presence of the Apennines, which act as a barrier to the movement of individuals of this species, in addition to the used specific habitat.

When we considered together the Italian colonies and the European ones, the overall situation is very interesting. The choice of expanding the sampling was due, in particular, to the comparison made between sequences of CytB. We analysed together the Italian sequences with those in the NCBI database. Accordingly to these results and those Bilgin described in his work (2008), it immediately appeared clear the presence of individuals with a high genetic variability. The survey then moved from one "national" level to an "European" one.

Microsatellites results, as described by PCAs obtained respectively with Genalex and the package Adegenet, show the sampled colonies are genetically different one from each other, the only exception being the two colonies of North Italy, but this could be due to the short distance between them, approximately 50 km, in which there are no particular obstacles for the flight. But other interesting details emerged, including the close relationship between samples in Sardinia and ones in Spain, in addition to a possible role of connection between

the colony of Fondi, in South Italy, with the previous samples. This situation is shown in the graph obtained using Adegnet, which confirms the reports described above, but in addition it shows as the colonies of Greece, Maronia and Koufovouno, are close to the colonies of Northern Italy.

The same situation is then confirmed by STRUCTURE results, the best value of *K* is 3, meaning that we had three main genetic groups in Europe, three different genetic components. These groupings show that the colonies of North Italy are closely interlinked, as well as those of Greece and those of Sardinia with those of Spain, the colony of Fondi in this case confirms its possible connection role as graph shows, in fact it presents the three identified genetic components in Europe. A mixing of different genetic lines could have caused this admixture.

A microsatellite variability estimation carried out on the detected unique genotypes (Table 3.5; Table 3.6) showed that all the 7 used microsatellite loci were polymorphic in the European bat population presenting from 4 to 12 alleles per locus, and even a total of 15 private alleles.

Considering the values of He and those of Ho they are very similar to those identified by Bilgin (2008). The studied bat population resulted to be not completely in Hardy-Weinberg equilibrium due to deficit of heterozygotes (the probability to obtain by chance a value of FIS greater than the observed was P = 0.010).

In terms of microsatellites, single and combined analyses of the loci in all of the species and global AMOVAs suggested that at least 90% of the variation was distributed within populations. This value was also described by Bilgin (2008).

The values of Fst parwise are high and significant, which are reflected on the total value of fst, (0.24432, high and significant), indicating that there is a strong genetic structuring. The lowest value was found between the two colonies of northern Italy, thus confirming the existence of gene flow. The relationships between the colonies were analyzed using the linearized parwise FST distances, they were used to elaborate a NJ tree. This tree shows that the colonies of North Italy were genetically close to the Greek colonies, suggesting that individuals in the past may have moved from Greece to northern Italy from the Balkans or from southern Italy crossing the Adriatic coast. The lowering of the Mediterranean sea level during glacial maxima indeed reduced the Adriatic Sea to a fraction of its current size (Shackleton et al. 1984), thereby facilitating exchanges of the terrestrial fauna between the Balkans and the Apennine peninsula. No data are available for *M. capaccinii* sampled along the east Adriatic coast, thus this potential route of colonization can only be hypothesized. However, such trans-Adriatic relationships of lineages have been evidenced in a variety of other Italian organisms, including brown bear (Taberlet & Bouvet 1994), rock partridge

(Lucchini & Randi 1998), pond turtle (Fritz et al. 2005), brown trout (Splendiani et al. 2006), or white oak (Petit et al. 2002).

The presence of Fondi in the group of Italian colonies, confirms its role as a connection between the different genetic lines.

If we consider the results obtained with the mtDNA CR, the situation identified with microsatellites is further confirmed.

On a total of 27 Dloop haplotypes, only one of these, H11, is present in two colonies, those of Maronia and Kofouvouno, in Greece. All the others are not shared between the colonies; they are therefore private haplotypes that allow us to differentiate the colonies one from each other. The fact that these haplotypes are unique to individual colonies, suggests that females of *M. capaccinii* are very philopatric, and did not emigrate out of the colonies ranges.

The haplotypes were then analyzed by building a network and a NJ tree, that both confirmed the result of the existence of four common haplogroups for Europe. The NJ tree showed again the strict connection between North Italian and Greek colonies.

Again even with the CR, it emerges as Sardinian and Spanish samples are closely related and as the colony of Fondi could carry out a contact zone and expansion of this haplogroup, since all these haplotypes are grouped in the same haplogroup as shown in Figure 3.25. Whereas it emerges as the Italian haplogroup (B) is genetically close to that of the Greek haplotypes (A), this is clear both in the network and in the NJ tree. This result confirms the close relationship existing between northern Italy and Greece, identified by NJ tree of distances related to microsatellites' FSt.

It is interesting also the presence of a haplotype, H14, identified in Greece, which is completely different from the other, in fact, the difference, obtained comparing this sequence with other haplotypes is on an average of 11%. This haplotype made the haplogroup D, its presence in the Greek colony of Koufovouno may indicate the existence of other haplotype lines present between Bulgaria and Turkey.

From the framework presented so far it follows that, in the European context, mitochondrial variability appears to reflect, in a satisfactory manner, the geographical distribution, as also shown on Figure 3.31.

Haplotypic and nucletidic diversity reflected the composition of the different colonies, in fact the highest values were found in Greece, while the lowest values in Sardinia, Spain and Fondi. In this case all individuals showed the same haplotype, indicating that there is a situation of genetic isolation.

The ΦST value of 0.8767, high and significant, confirmed the existence of a strong structuring at the level of mtDNA, it is surprising that the value of ΦST between the two

colonies of northern Italy is very high, 0.78, indicating how there is no gene flow mediated by females. Because, whatever is the geographical distance that separates them, all the colonies are so divergent (average between pairs ΦST = 0.8767), a model "isolation by distance" is not adequate to explain the differentiation of the population compared to the mitochondrial DNA marker (Ruedi et al., 2003). As also it was confirmed by the subsequent Mantel test.

Using the linearized distances ΦST a NJ tree was constructed. This NJ tree supports the existence of three different groups among Europe, and highlights the close relationships between the central-southern Italian colony of Fondi and those of Sardinia and Spain, located across the Tyrrhenian Sea. Greek colonies are also intermingled with the Italian samples, further suggesting that they share closer relationships among them

Finally, also the results obtained using the CytB confirm and reinforce the hypothesis of the existence of three distinct groups across Europe, the NJ tree is strongly supported by all three analyzes (NJ, MrBayes, MP). They also confirm the results obtained in 2008 by Bilgin, who had previously identified haplogroups B and C, in this case it is added the haplogroup A grouping Sardinian, Spanish and South Italian (Fondi) haplotypes . Even in this case the relationship between the Greek colonies and those of northern Italy is represented by the grouping of identified haplotypes in a single haplogroup (C) (Fig. 4.2).

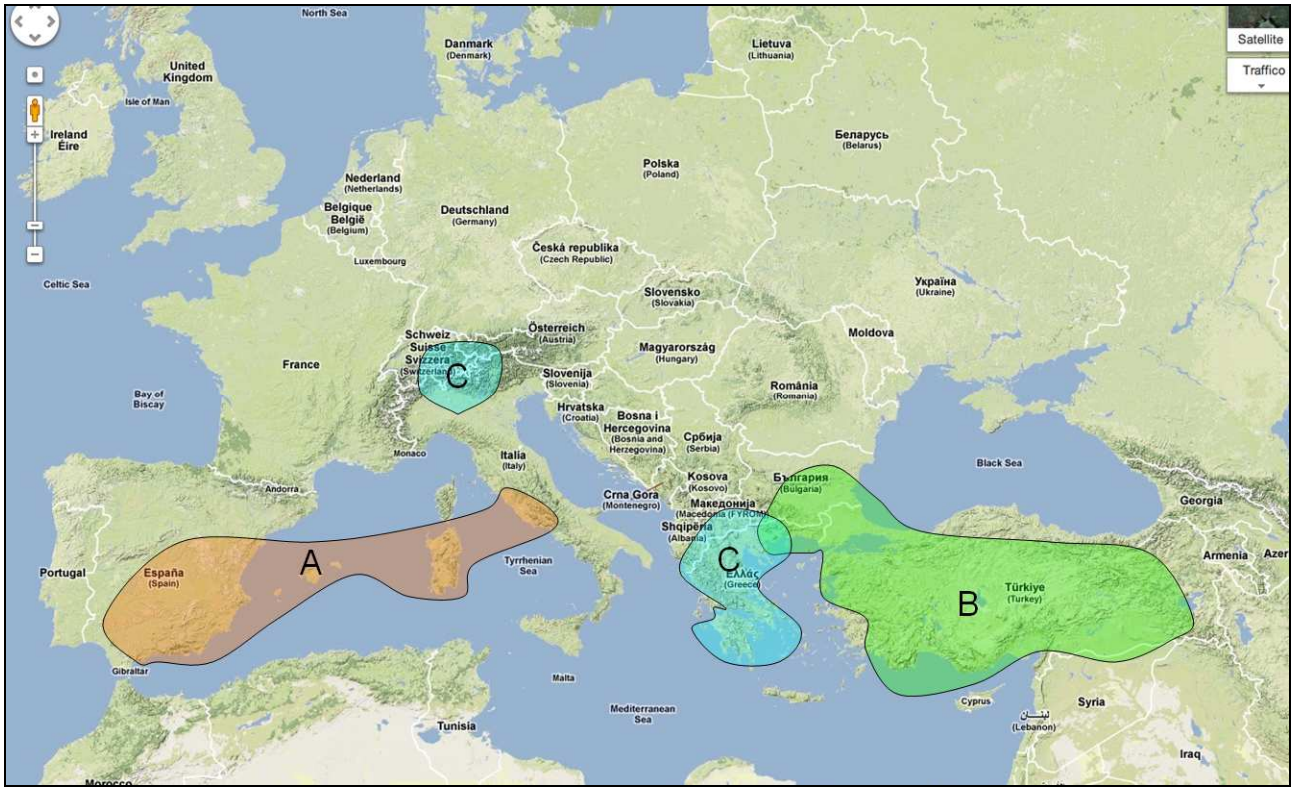


Figure 4.2: distribution of CytB haplogroups of *M. capaccinii* in Europe

The date of the split of the two CytB mitochondrial clades, B and C, was calculated to be around 580 000 years ago, within the Pleistocene. Isolation and differentiation in glacial refugia during Pleistocene have been recorded in other species in the region and in Europe. These include two bat species (Ruedi et al., 2008) , the brown bear (Taberlet & Bouvet 1994) and the meadow grasshopper (Syzmura et al. 1996). Hence the dating of the mitochondrial split for *M. capacinnii* to the Pleistocene is also corroborated by data from other species, and it is another example of the effect of Pleistocene glacial refugia in promoting genetic differentiation.

On the contrary the differentiation between haplogroups C and A would have occurred around 130.000 years ago, which it would correspond to an Early or Middle Pleistocene origin. The lowering of the Mediterranean sea level during glacial maxima indeed reduced the Tyrrhenian Sea to a fraction of its current size (Shackleton *et al*. 1984), thereby facilitating exchanges of the terrestrial fauna between the Spain and Balearic Island, between North Africa and Sardinia and the Apennine peninsula, as showed by the results obtained in the colony of Fondi. An important role could have been played by the Balearic Islands; therefore, in order to test this hypothesis, we collected additional samples of *Myotis capaccinii* from Spain and Majorca that will be analyzed in the future. The high similarity found in samples from Spain, Sardinia and Fondi greatly suggests a common origin for these individuals. The most sparing solution would be that the species originated in the east and subsequently colonized Europe via Turkey (Anatolia or Asia Minor).

Colonization of the Mediterranean islands in the past may have been facilitated by lower sea levels (Fig. 4.3). Biollaz *el al.* (2010) found that the most likely scenario for the Maghrebi bat (*Myoih punicus*) was the colonization from North Africa northwards to Sardinia, and further from Sardinia northwards to Corsica. After, higher sea levels isolated these island populations.

Fossil record for *M. capaccinii* showed that this species was in Italy at the beginning of the Pleistocene (2,58 MYA), other records were dated at Late Pleistocene and recent period (Tata, 2005).

In conclusion, the presence of three distinct groups, identified with three different markers can be explained by a first differentiation occurred about 580,000 years ago at the suture zone defined around the Turkish/Greek and Bulgarian border. The work also confirms the Bilgin's (2008) hypothesis that there should be another suture zone in the Balkans. In fact this is what we have shown, this idea is also supported with the results from *M. myotis*, where a suture zone was found in the Balkans, with refugial populations being found in western Europe (Ruedi & Castella 2003).

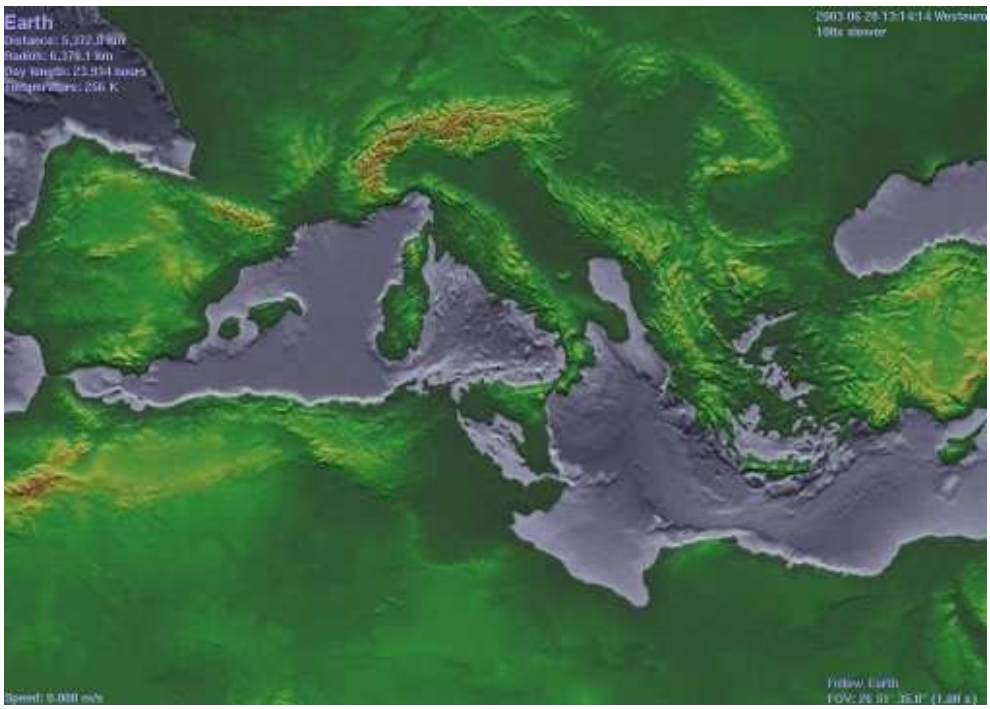


Figure 4.3: the mediterranean sea during the last ice age (Weichsel-Würm) in Europe

The presence of Haplogroup A can be explained by the effect of the ice age that caused the lowering of the Mediterranean Sea, allowing a movement of individuals from Spain to Sardinia and from Algeria and Morocco to Sardinia and southern Italy. We could assume this scenario because the species is known for North Africa but people were unable to find it during field work (Mudarra, 2009). We could also hypothesize the role of glacial refuge of Spain (Fig. 4.4).

Figure 4.4: : hypothetical scenario of colonization of Europe by the species *Myotis capaccinii*, according to our results and Bilgin's work (2008). The red lines indicated possible geographic barriers and Spain here is showed as a probable glacial refuge

Finally, the presence of the colony of Fondi in the NJ tree, obtained using Dloop, with the Italian colonies could be explained hypothesizing a phenomenon of mitochondrial introgression led by individuals from North Africa coming from Spain, which was used by them as glacial refuge. These individuals substitute the mitochondrial DNA, which was probably of individuals belonging to haplogroup B. The final consideration so is that individuals of Clade A are the invaders and suggest that the previous mtDNA genome was replaced by the local lineages of Clade A during its expansion into Europe, until the original genes was finally lost.

Both classes of markers support a strong differentiation among colonies and a trans-Tyrrhenian relationship of some populations (i.e.Sardinia, Spain and Fondi colonies in Fig. 3). This suggests that both males and females are less vagile than a flying mammal, and that the phylogeographical patterns presented here certainly reflect the history of the whole populations, not just the movements of the females.

4.2.2 Taxonomic implications

In the Eurasian transition, for *M. capaccinii*, there were two reciprocally monophyletic clades that could be treated as phylogenetic species [sensu Cracraft (1983)]. There was also some evidence for sympatry. Namely one individual belonging to clade B (potentially to subspecies *M. c. bureschi*) was found in a cave in Greece with clade C individuals, situation already described by Bilgin (2008). However, it is very likely that these represent single migrants and does not provide evidence for coexistence and differentiation of the two clades in sympatry, or for their reproductive isolation. The nuclear data regarding this sample, with variability at mtDNA level, showed that it is grouped with the other Greek samples. This suggested that there were no barriers to gene flow, hence from a biological species concept perspective; the two mtDNA clades do not qualify as separate species. However, it is important to note that, geographically, the cladistic bifurcation roughly coincides with the subspecies divide proposed for *M. c. bureschi* (Heinrich 1936) and *M. c. capaccinii*, supporting the idea of the existence of two subspecies (DeBlase 1980; Koopman 1994; Kumerloeve 1975) instead of a single subspecies proposed by Corbet (1978) and Spitzenberger & Helversen (2001) in the region. The percent variation between clades B and C, is 5.90% and between B and A, is 5,30%. These were comparable to that seen in other subspecies in the genus Myotis (e.g. 5.5% in cytochrome-b between *M. blythii blythii* and *M. blythii oxygnathus*) (Ruedi & Mayer 2001), another line of evidence suggesting that clades B and C might represent separate subspecies.

The results partially support the view presented by Albayrak & Aşan (2002) regarding the differences of the European and the Middle Eastern populations, in that, clade C haplotype

individuals were found exclusively in Europe. As for clade B, in fact it doesn't extend into Europe, and it is present only in Turkey and between the border of Bulgaria and Greece.
Considering these results, and bibliography regarding *M. capaccinii* (Heinrich 1936, DeBlase 1980; Koopman 1994; Kumerloeve 1975, Bilgin, 2008) we could identify samples from western Europe as *Myotis capaccinii capaccinii* and samples from Turkey and Bulgaria as *Myotis capaccinii bureschi*.
Because the values obtained using CytB and CR were remarkable high, we decided to see if these variations could identify different species. The possible existence of different species was investigated at the UCD during six moths internship. We used molecular intron markers, basically the obtained results showed that the differentiation among different samples collected across Europe and Asia were weak for saying that we are in presence of two different species. What we have found is that there is a differentiation between Asian samples, in this case from Iran, and European samples. The mutations are few but enough to identify a Western pattern and an Eastern one.

4.4 Future prospects

The work done at UCD in Dublin led to use for the first time on these two species different kinds of nuclear markers, specifically primers of nuclear intron genes. If for *Myotis emarginatus* it is not surprising the presence of a single haplotype diffused from Morocco to Italy, regarding *Myotis capaccinii* we confirmed the presence of two distinct geographical groups, with a variability not such as to justify the presence of two new species but at least to corroborate even more the hypothesis of the presence of two subspecies that constitute a single large complex.
The importance of these primers is then revealed by comparing together the two sequences of BGN of *Myotis emargiantus* and *Myotis capaccinii*, this primer in fact presents a high potential to perform phylogenetic studies between different species of bats, and also considering genetically related species belonging to the same family. A recent study of *Myotis nattererei* (Salicini et al, 2011) has shown that these primers may in fact help to discriminate easily unclear or doubtful phylogenetic relationships. In view of this possible development a particularly project relating to *Myotis emarginatus*, connected on its location within the African clade, could be the use of this primer set to confirm or refute the hypothesis of its origin. In fact the phylogenetic tree was constructed using only the CytB. Regarding *Myotis capaccinii* however, the variability was low, but an interesting result is represented by two individuals of the colony of Lierna. Because both have the H1 haplotype, found only in this colony, different from haplotype H2, the most common , only for a mutation. We would like to analyze other samples in order to understand whether this mutation is confined to the

colony or even to that of Isola Bella, and if so we could differentiate the two colonies of Northern Italy from the other on the basis of this single mutation.

CONCLUSIONS

The work described here has led for the first time to describe the population structure of the two bat species of the genus Myotis, *Myotis emarginatus* and *Myotis capaccinii*. Considering the fact that these species, like all bats, are protected by the Habitats Directive these results are very importance since they allow to allow to assess not only the differences identified between different colonies but even their "genetic health". Not unimportant also the fact that *Myotis capacccinii* being critically endangered, according to the IUCN, the results obtained can be implemented to strengthen conservation activities against this species.

This study showed that to use of non invasive genetic sampling (NGS) represents a powerful tool to study endangered species when its data are efficiently supported by additional ecological and field information, confirming that noninvasive genetic sampling methods can provide several issues that could not be addressed in any other way. In addition with the increasing attention paid to biodiversity and conservation, new methodologies are necessary to study species, particularly elusive and endangered ones. Non-invasive protocol showed her can readily be adapted to a broad range of species within chiropterans and beyond, and should become a useful tool in wildlife management and conservation.

Considering *Myotis emarginatus*, we discovered that this species haven't a clear genetic structure among Europe, probably due to a recent event of colonization started during the last glacial period, furthermore seem that it could be explained considering this species native from Africa as other work before showing (Stadelmann, 2006). We discovered also that the colonies of North Italy are more different than those Europeans, this could be an effect of the recent colonization of Europe, for this reason even the process of variation are recent and just started.

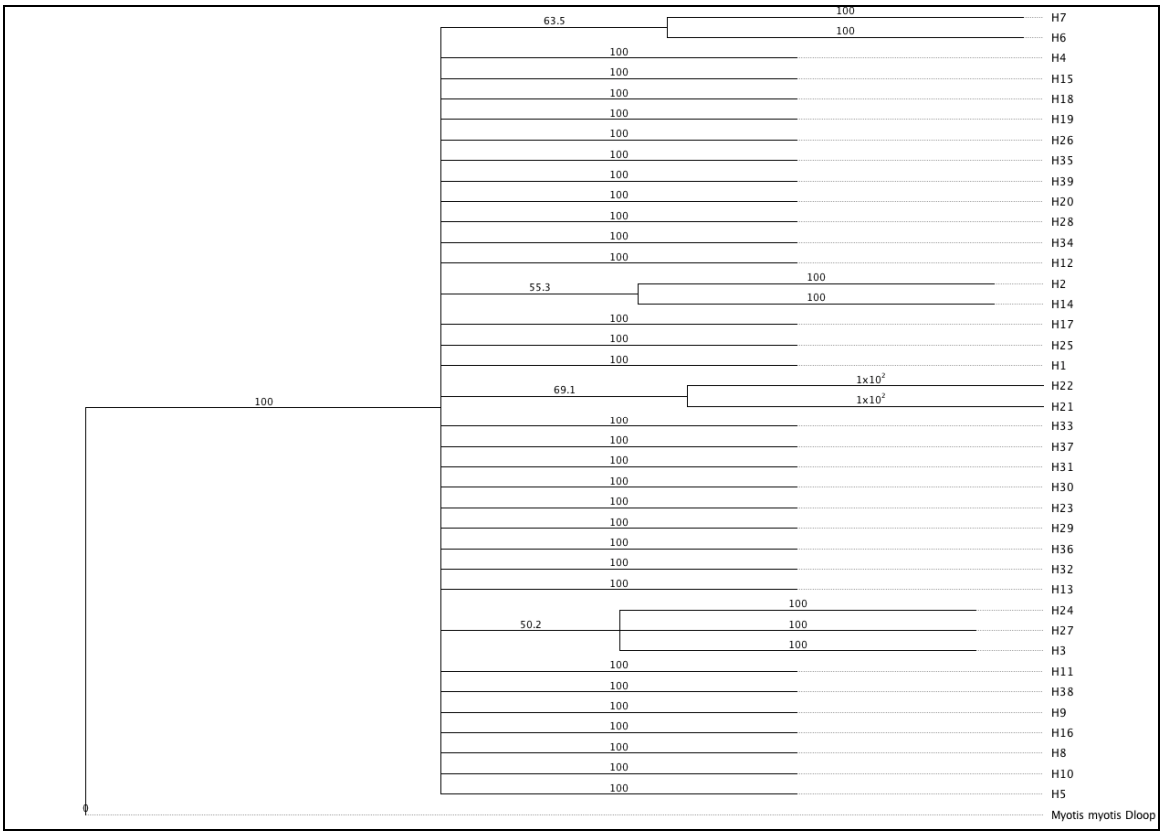
Our results on *Myotis capaccinii* showed, without any doubt, that in Europe we have three different big group. This was confirmed by results obtained with the two different marker, nuclear DNA and mtDNA. We confirm, moreover, that for this species there was an east–west divide in mtDNA as discovered by Bilgin (2008). We also detected another lineage, which started from Spain, crossed Sardinia and arrived to South Italy.

For the future we will analyzed more samples from other area, so that the whole areal of the two species will be covered; furthermore considering also the results obtained during the internship at UCD, we will use nuclear intron markers for investigating the relationships among species phylogentically related and for finding every possible mutations across populations of these two species, which could be useful to investigate their evolutionary history.

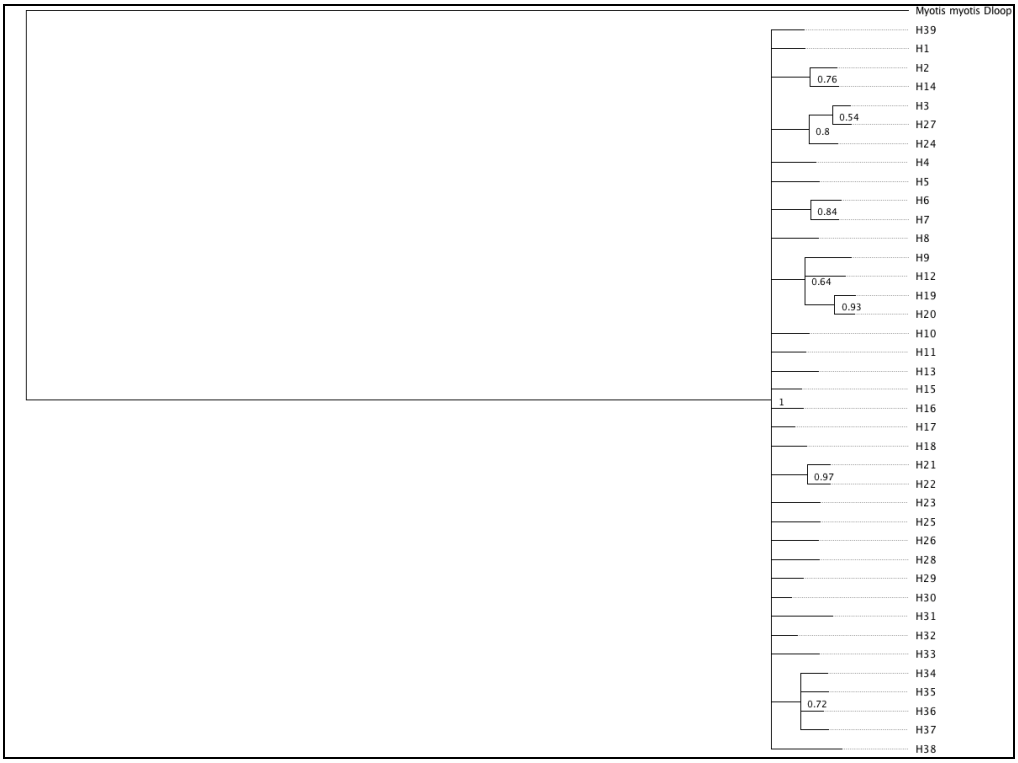
APPENDIX: TREES

DLOOP

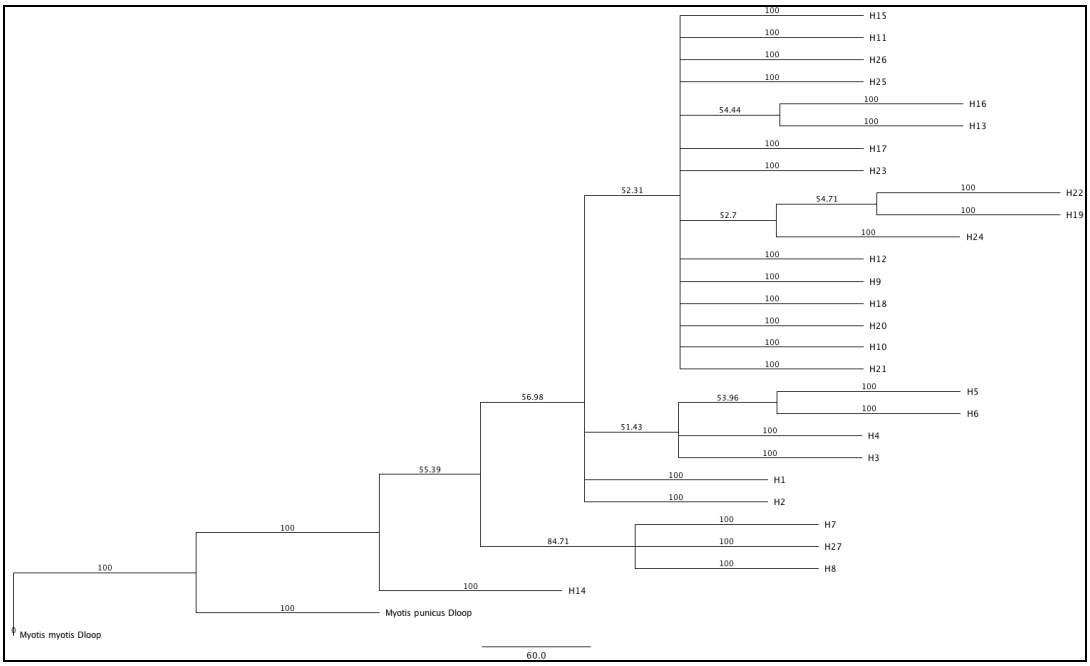
Myotis emarginatus, consensus NJ



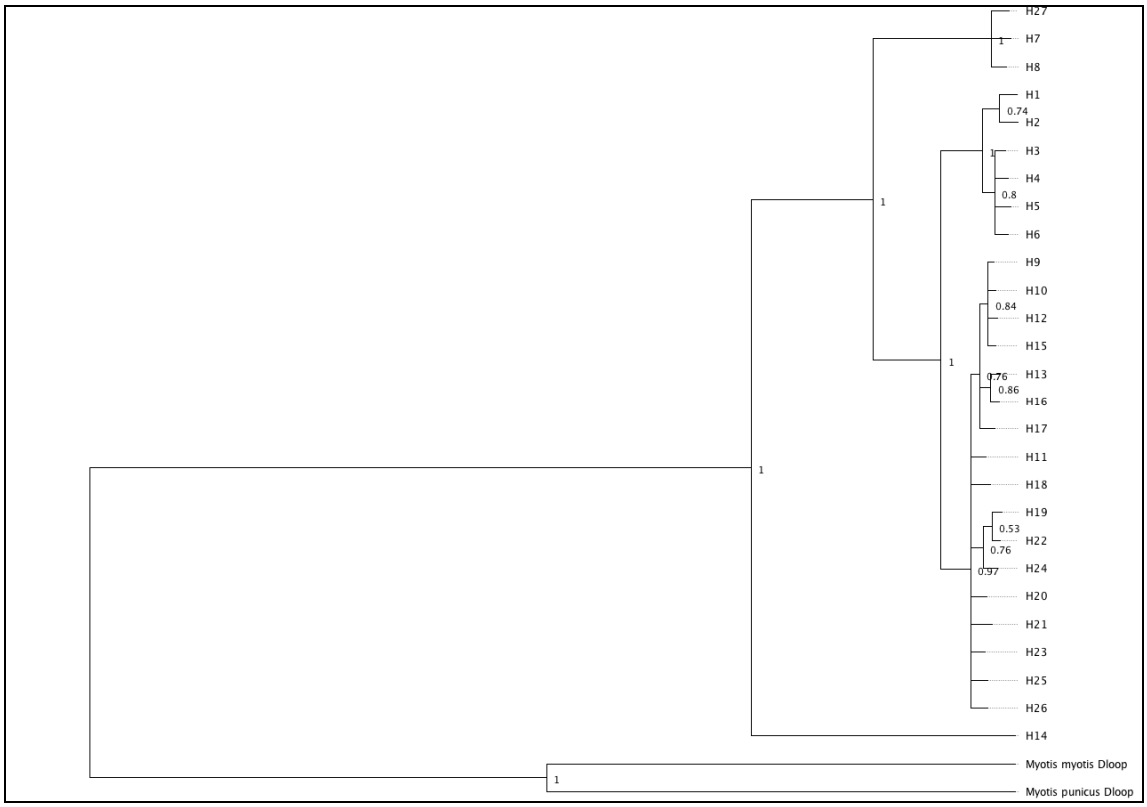
Myotis emarginatus, MyBayes tree



Myotis capaccinii, consensus NJ Tree

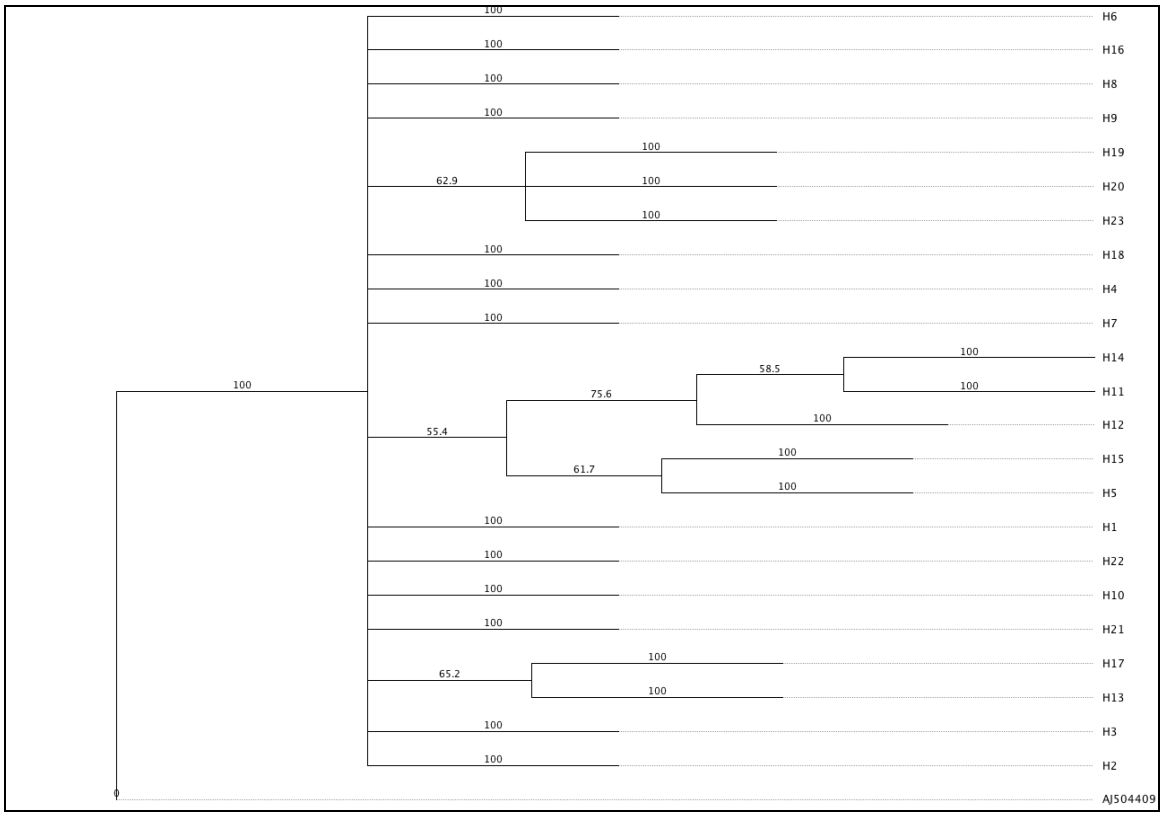


Myotis capaccinii, Mrbayes Tree

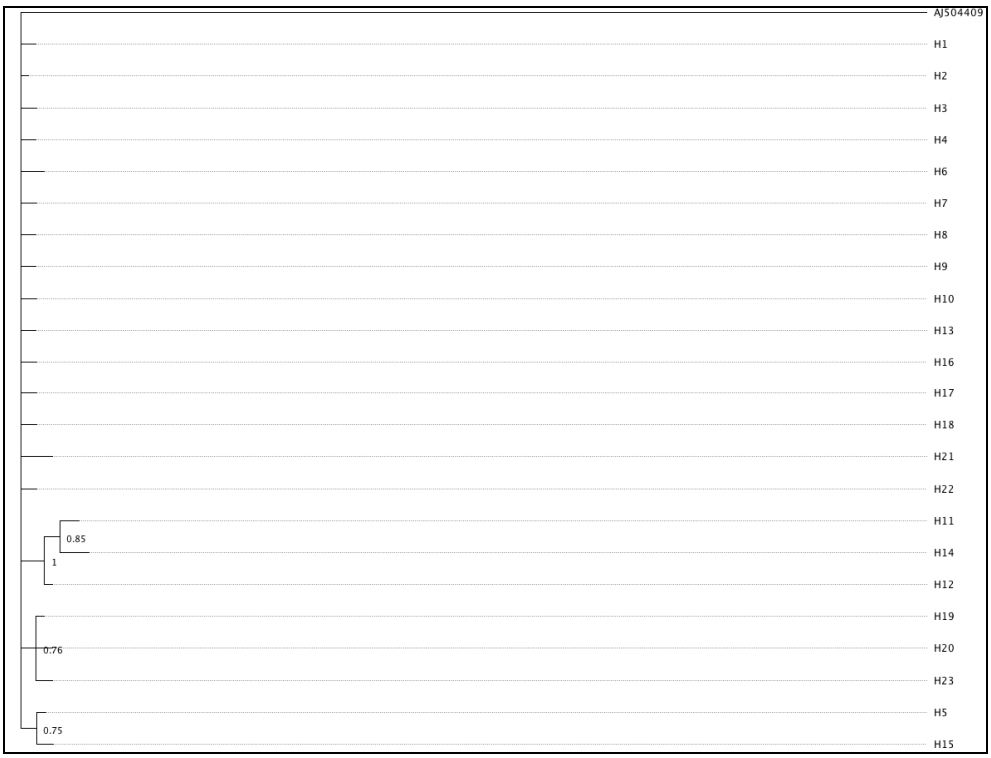


CytB

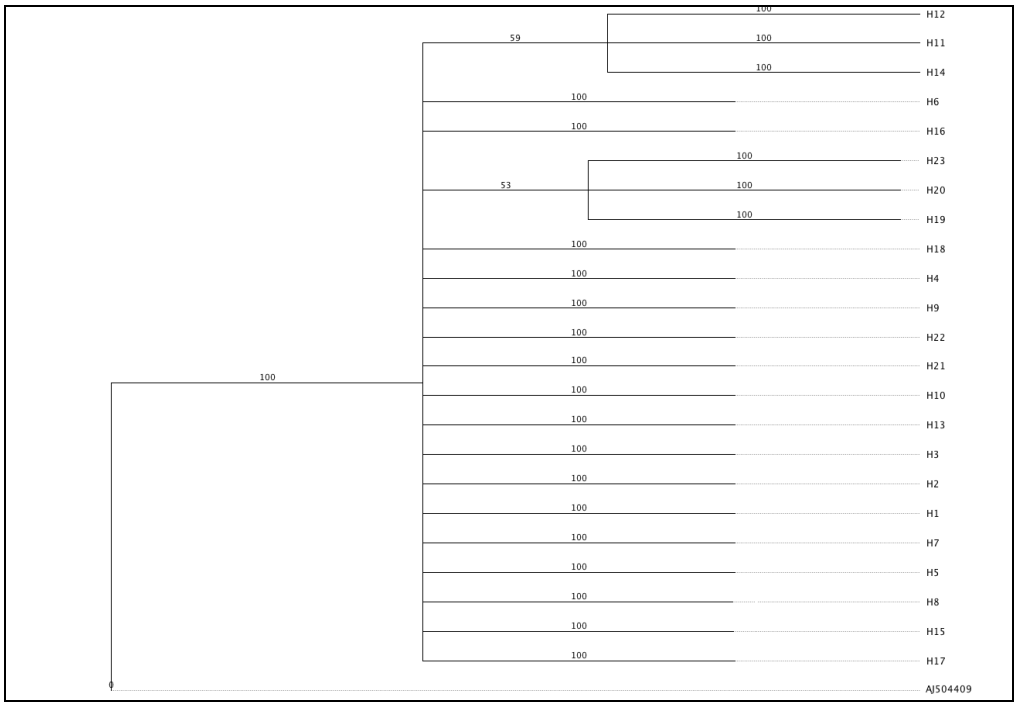
Myotis emargiantus, consensus NJ Tree



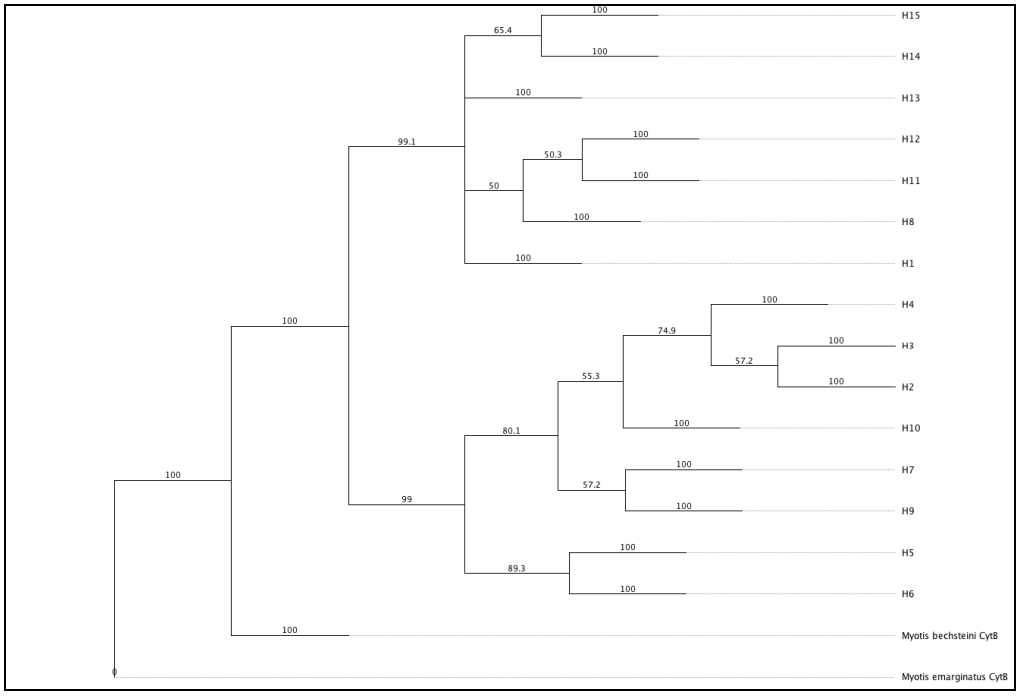
Myotis emarginatus, MrBayes Tree



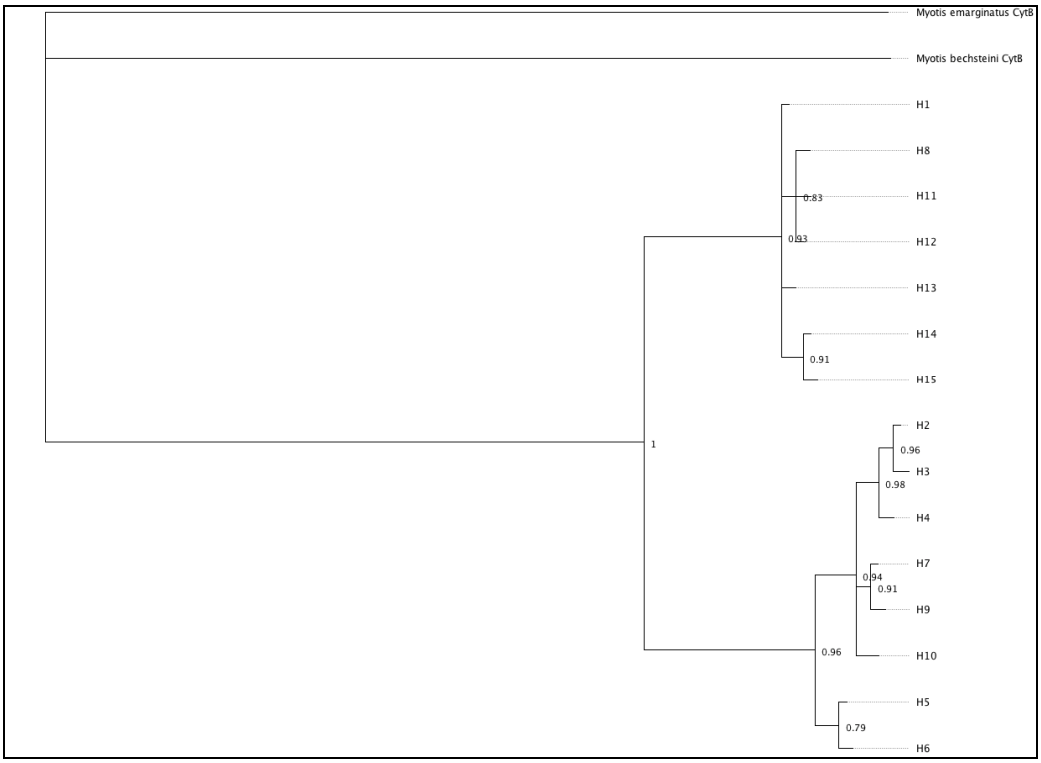
Myotis emarginatus, MP Tree



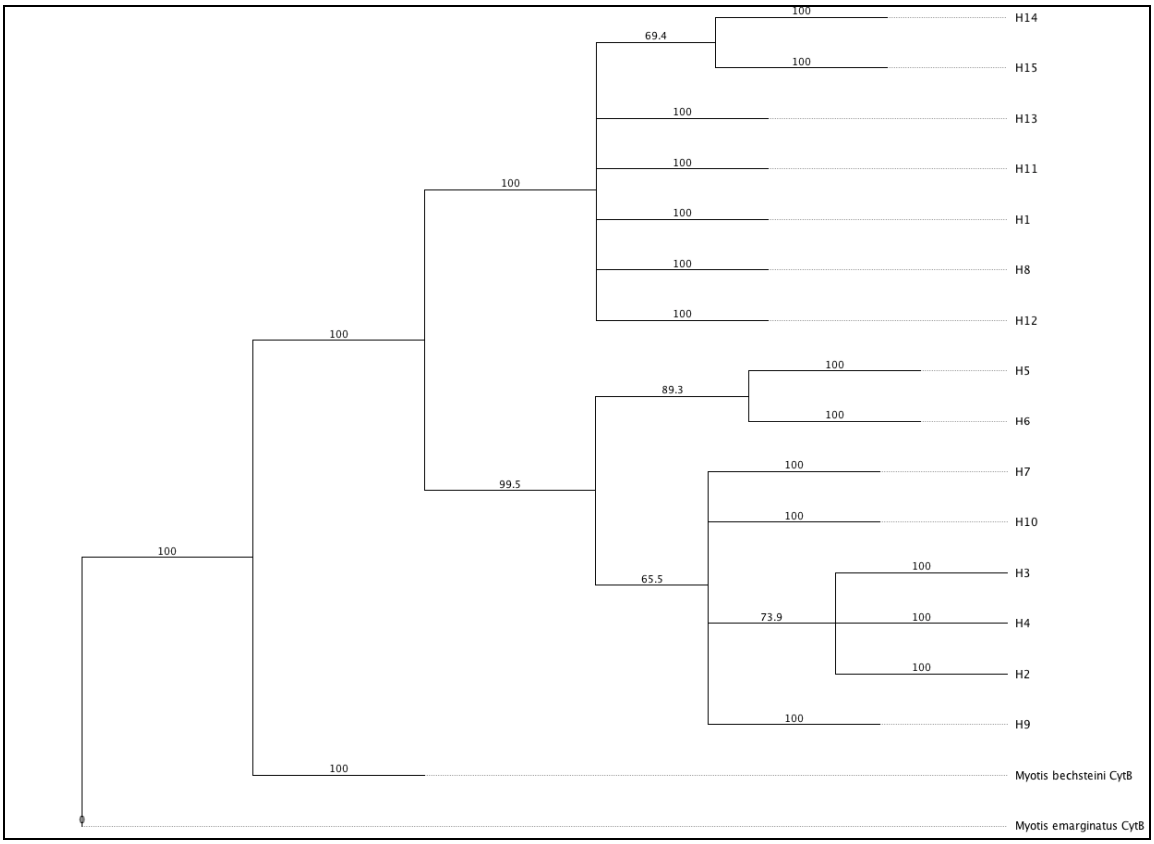
Myoti capaccinii, consensus NJ tree



Myotis capaccinii, Mrbayes tree



Myotis capaccinii, MP Tree



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ACKNOWLEDGEMENTS

I greatly thank everybody who allowed me the achievement of this PhD Thesis in a pleasant way: my Professor and Supervisor Ettore Randi who made possible the achievement of one of my career aims: the PhD; Romolo Caniglia who taught me most of my knowledge about Non-invasive Genetics and Microsatellites, during these three years he was not only laboratory supervisor but also a great friend, who considered me as a son during my whole PhD period; I want to thank Romolo also for many helpful advices to resolve any kind of technical laboratory problems and to organize this thesis.

I greatly thank also:

- Giulia who had the patience to wait for other three years of studies; she has always encouraged me and told me that I could do it.
- My parents and my sister who have strongly supported me and always helped me to face all sort of difficulties, without them I would have never achieved this goal, for the tacit or explicit support that gave me so many times, never hindering my choices, encouraging me always to go on my way.
- All my "old" friends in Alba; who, despite the absence from my hometown, have always been closed to me, being people to whom I could, I can and I can always count on.
- All my friends in Bologna at ISPRA Chiara, Federica, Elena, Francesca, Alessandra, Nadia, Silvana, Patrizia, Adriano, Andrea and Rita who contributed to make pleasant the laboratory experience and also the free time;
- Marco who divided with me the "hard" but very nice experience of PhD.
- Aritz "the basque" , who is "*efetivamente*" not only a good PhD researcher but also a great friend.
- Emma Teeling who gave me the chance to spend a period at UCD (University College Dublin) allowing me to analyze my data with the hope to produce a great PhD thesis: "*Go raibh míle maith agat*"; many thanks also to Sebastien Puechmaille and Emma Boston for their strong support and help in the six months at UCD, they taught me everything I know about the population size estimation of bats, revealing to be excellent and very patient teachers and good friends. Thanks to all the people of the "Batlab": Bruno, Stephen, Graham, Keith, Una and Nicole, it was a pleasure working with them.
- All the people of the "Skylab" at UCD, Bruno, Tilen, George, Jim, Elanie, Karl, Conor and of course all the people of the "Italian Group" Sergio, Silvia, Chiara, Angela, Mauro, Alexia e Carlotta. They made my period in Ireland amazing, sharing it between laboratory and pub.
- At my aunt Tiziana, a support for all the technical and organizing point of view, essential for the preparation of my thesis.
- Silvana Allario for the supervision of the English style of my thesis.
- Many thanks also to everybody who took care of the sample and field information collection:
  - ✓ *Paolo Agnelli (Museo Specola, Firenze)*
  - ✓ *Roberto Toffoli and Paola Culasso*
  - ✓ *Felice Farina and Roberto Facoetti (Provincia di Como)*
  - ✓ *Fabio Maltoni*
  - ✓ *Giovanni Mastrobuoni (Parco Nazionale del Circeo)*
  - ✓ *Simone Vergari (Centro Naturalistico e Archeologico dell'Appennino Pistoiese)*
  - ✓ *Adriano Martinoli (Università dell'Insubria)*
  - ✓ *Danilo Russo (Università Federico II)*
  - ✓ *Cristiano Spilinga (Studio Naturalistico Associato Hyla)*
  - ✓ *Mauro Mucedda (Centro Pipistrelli Sardegna)*
  - ✓ *Maurizio Gioiosa (Parco Nazionale del Gargano)*
  - ✓ *Martina Rossi (Università dell'Insubria)*
  - ✓ *Christian Drescher (Museo Scienze Naturali di Bolzano)*
  - ✓ *Elena Patriarca and Paolo DeBernardi (CRC, Centro Regionale Chirotteri)*
  - ✓ *Alessandra Palladini*

Thanks also to the people of other groups all over Europe who gave their samples to make this research project:

- *Javier Juste (Estación Biológica de Doñana; Seville, Spain)*
- *Rasit Bilgin (Boğaziçi University, Bosphorus University; Istanbul, Turkey)*
- *John Altringham (Univeristy of Leeds; Leeds, United Kingdom)*
- *Manuel Ruedi (Natural History Museum of Genève, Switzerland)*

Finally I want to remember my grandfather Ivio and my cousin Stefano, because they would have rejoiced at me and with me for this important moment of my life.