UNIVERSITY OF NAPLES FEDERICO II

DOCTORAL SCHOOL OF ENHANCEMENT AND MANAGEMENT
OF AGRO-FORESTRY RESOURCES (XXVII CYCLE)

Ecology and Phylogeography of the Riparian Habitat Specialist *Myotis daubentonii*

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Tutor: Dr. Danilo Russo
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“The end of the human race will be that
it will eventually die of civilization”

R.W. Emerson

ABSTRACT

Riparian ecosystems are known as habitats with high biodiversity levels which perform several ecological functions. The importance of riparian habitats for foraging and commuting of many bats species was observed in different geographic areas. Bats are highly vulnerable to habitat changes, in addition specialist taxa are more sensitive to small-scale environmental alterations. *Myotis daubentonii* constitutes an interesting model species widespread in Europe as riparian specialist. The objectives of this thesis were to study the effects of an altitudinal gradient on ecology and thermal physiology of *M. daubentonii* males of a population in Central Italy, for which intra-male spatial segregation was known, and investigate the genetic structure and phylogeography of this species in different river basins of Europe. The first study was carried out comparing habitat productivity, body condition, thermoregulation strategies, use of space and habitat selection between males from two altitude zones. Results show that the more selective and diurnally homoeothermic downstream males, show a better body condition in late spring (possibly as a consequence of higher food availability in that period and/or hibernating in milder climate) but then they lose weight. Upstream males prove more flexible as they forage in a broader range of habitats and make a larger use of daily torpor, strategies that in the long run appear rewarding as unlike downstream males at least they showed no body condition drop over summer. I conclude that the main benefit for downstream males is not energetic but probably reproductive as they may increase fitness by extra-mating. However, a better body condition following hibernation might also imply a higher survival likelihood of downstream males at that time of year. The second study was carried out analyzing the cytocrome *b* gene (Cyt *b*) and hypervariable non-coding domains *I* and *II* (*HVI* and *HVII*), from the control region (D-loop) of the mtDNA of respectively 157 and 123 samples of *M. daubentonii* from sixty three localities of Europe. The data show quite a remarkable differentiation with more than fifty different haplotypes. All phylogenetic reconstructions point to the distinction of three main lineages very differentiated and highly structured: a lineage spread throughout the Iberian Peninsula, in previous research identified as *M. d. nathalinae*; a lineage found in Italy, France, Switzerland, Germany, Sweden and in the Central and Northern Iberia; another lineage consisted of samples from Serbia, Montenegro, Greece, Netherlands and from the North of Spain and Portugal. My analysis demonstrated that the Mediterranean Peninsulas (Italy, Iberia, Balkan) acted as glacial refugia for *M. daubentonii* and its European populations have originated from the postglacial Palaearctic expansions of the Italian and Balkan lineages, while the Iberian lineage did not cross the Pyrenees with a possible pattern of *refugia-within-refugia* as a consequence of the climatic cycles from the Pleistocene.

ORGANIZATION OF THESIS

This thesis consists of an introductive chapter devoted to riparian habitats and a model specialist bat typical of these ecosystems in temperate regions, *Myotis daubentonii*. The next two chapters are two papers that have been or will be submitted to international scientific journals. The first is a study of the ecological and physiological intra-sexual differences of *M. daubentonii* along an altitudinal gradient. The second study investigates the genetic structure and phylogeography of Daubenton's bat populations of Europe. The last chapter contains the conclusions of the thesis.

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1. INTRODUCTION

1.1 Riparian habitat

Riparian habitats are interfaces (ecotones) between different environmental ecosystems, respectively aquatic and terrestrial. Because of their intrinsic dynamism, due to the continuous interactions between the neighbouring ecological systems, they are difficult to define in space and time (Naiman and Décamps, 1997). Riparian habitats are non-equilibrium systems in which there is a continuous flow of matter and energy between biotic and abiotic components of aquatic and terrestrial systems, making them among the most diverse, complex and dynamic habitats of the terrestrial portion of our planet (Nilsson, 1992; Naiman et al., 1993; Naiman and Décamps, 1997). Therefore, such ecosystems are different mosaics of landforms, environments and communities in a larger landscape (Gregory et al., 1991). The hydrology and its reciprocal action with local geomorphology is the main factor determining the composition, structure and spatial development of these naturally disturbed ecosystems (Mertes et al., 1985; Salo et al., 1986).

Currently, riparian habitats are often surrounded by human-altered areas, such as farmland, plantations of exotic tree species and urban areas; moreover since the nineteenth century, their natural disturbance regime has been modified due to both the engineering and hydroelectric progress, in addition to deforestation (Petts et al., 1989). Since hydrology is the main factor affecting the structure and development of freshwater habitats, its anthropogenic alteration involves changes in biotic communities, damaging them (Williams and Winget, 1979; Chatterbus et al., 1986; Décamps et al., 1988; Copp, 1990; Howe and Knopf, 1991; Nilsson et al., 1991; Nilsson and Berggren, 2000). It is well known that active natural disturbance is essential to maintain the high biological diversity of riparian ecosystems (Naiman et al., 1993; Ward, 1998; Ward et al., 1999).

Riparian habitats perform several ecological functions, which make them disproportionately more important than other habitats of the same size (Gregory et al., 1991): i) they provide shelter and fodd for wildlife, timber and water; ii) they represent natural ecological corridors for plants, humans and wildlife (Naiman et al., 1993; Naiman and Décamps, 1997; Bennett, 2003); iii) they act as natural filters of nutrients and debris, and stabilize the channel and the banks; iv) they are biological and physical buffers (Gregory et al., 1991). Besides the role of mitigating flood flows by intercepting rainfall and interacting with solar radiation, riparian vegetation affects the microclimate (e.g. light, temperature and humidity). In this way, it creates suitable conditions both for the growth of insects, other invertebrates and the thermal protection for vertebrates, thus performing an important role for wildlife (Gregory et al., 1991).

Riparian ecosystems are known as habitats with high biodiversity level (Brown et al., 1978; Harris, 1988; Doyle, 1990; Strong and Bock, 1990; Nilsson, 1992; Naiman et al., 1993; Dunham, 1994; Murray and Stauffer, 1995). The action of flooding and channel migration, operating as a continuous disturbance, allows a high degree of compositional and structural diversity in river plant communities (Gregory et al., 1991; Ward et al., 1999). Spatial-temporal structural heterogeneity of riparian vegetation provides a large spectrum of microhabitats and trophic niches. Not only do riparian habitats host species linked to water throughout the life-cycle, but are also home to those species that require both aquatic and terrestrial environments during their life cycle, such as amphibians, reptiles and aquatic insects. Baxter et al. (2005) estimated that 25-100% of the energy for terrestrial consumers, such as birds, bats, lizards and spiders is provided by the emergent adult insects from benthic communities. Furthermore, some species are closely related to riparian environments for foraging, such as some bats that hunt prey over the water surface (Vaughan et al., 1997) and other species that forage over water bodies and shelter in riparian vegetation, such as wetland birds.

1.2 *Myotis daubentonii* (Kuhl, 1817)

Figure 1. *Myotis daubentonii* (Photo by L. Cistrone).

Currently, more than 1200 species belonging to the order Chiroptera have been identified, accounting for about a quarter of all known mammals. Vespertilionid bats constitute the largest chiropteran family and the second largest mammal family, with about 44 genera (Corbert and Hill, 1991). *Myotis daubentonii* (Chiroptera, Vespertilionidae; Fig. 1) is a medium-size myotid bat specialised into riparian habitat. This species has a Eurasian geographical distribution including most Palaearctic regions. It occurs from Portugal to Ireland, Norway and Greece, also in Central and South-Western China, in Japan (Hokkaido) and Korea. *M. daubentonii* is one of the most widespread in Europe (Corbert, 1978; Bogdanowicz, 1994), it shows significant intraspecific morphological variability, so that several subspecies have been described (*M. d. daubentonii*, Kuhl, 1817; *M. d. nathalinae*, Tupinier, 1977; *M. d. volgensis*, Eversmann, 1840). Discriminant analysis on morphological data (Bogdanowicz, 1990) demonstrated that the variability is not sufficient to validate the three European subspecies, and that all morphotypes can be considered as *M. d. daubentonii,* although some individuals from marginal populations, such as those in the Iberian Peninsula, showed morphological characters larger than expected, thus indicating that further investigations are needed. Preliminary molecular studies revealed that the two morphotypes "*nathalinae*" and "*daubentonii*" correspond to distinct genetic lineages (Simões et al., 2007). This study suggests that during the Last Glacial Maximum *M. daubentonii* populations were confined to the peninsulas of Southern Europe, an event which may have had large influence on their genetic structure.

M. daubentonii bats have short, rounded and clearly separated ears; the wings are reddish or dark brown but never black and the short, dense fur is brown-gray to a slightly red dark bronze on the dorsum and silver-gray to white on the belly. Some diagnostic characteristics of *M. daubentonii* are its particularly large feet that are more than half the length of the tibia and its long calcar; the platopatagium fits the middle of the metatarsal. The biometric ranges for this species are: forearm length 33-42 mm, wingspan 240-275 mm, body mass 5-15 g. Usually, females are slightly larger than males (Bogdanowicz, 1994).

Bats have a high adaptability to a wide variety of habitats showing different morphologies also in relation to the structure of the habitat most frequented. The wing morphology of *M. daubentonii*, characterized by low wing loading along with rather short and rounded wingtips, indicates that this species has a slow flight, with efficient manoeuvrability (Jones and Rayner, 1988).

M. daubentonii is typically promiscuous, the mating period begins in August in the nursery roosts, when the young are weaned, and continues over winter during the hibernation period until April (Abelencev et al., 1956; Roer and Egsbaek, 1969; Bogdanowicz, 1994; Encarnação et al., 2004). Most mating occurs in October-November at swarming sites (Parsons and Jones, 2003). As many other bats, *M. daubentonii*, is characterized by delayed fertilization: in autumn after mating, females can store sperm cells without ovulating (so that fertilization will not take place), spermatozoa are preserved across the winter, and will fertilize the egg cells only once winter is over and females ovulate – a mechanism aimed to maximise the chance that births will take place in the months when food is most abundant. The gestation period lasts about 53-55 days (Abelencev et al., 1956), usually one female gives birth to one bat in a year (Kurskov, 1981). Lactation lasts about 35-45 days (Abelencev et al., 1956), the young generally begin to fly by the third week and reach adult size in 9-10 weeks (Bogdanowicz, 1994). Sexual maturity is reached in the second year (Bogdanowicz, 1994; Encarnação et al., 2004).

M. daubentonii is strictly associated to natural and artificial freshwater habitats, such as streams, rivers, ponds and canals for foraging. It was also detected hunting in deciduous and mixed forests (Ahlén and Gerell, 1990). Typically, summer roosts are given by tree holes, bridges, buildings, rock crevices and bat boxes, while in winter this species hibernates in underground sites. Nursery roosts include mainly adult females, and may consist of more than 100 individuals (Speakman et al., 1991).

In summer, adult males either form independent groups or share roosts with females (Speakman et al., 1991). For some geographical areas, *M. daubentonii* shows sexual segregation; it is reported that adult males are disproportionately abundant at higher elevations, while females are restricted to lower altitudes (Leuzinger and Brossard, 1994; Altringham et al., 1997; Russo, 2002). The actual value of the elevational threshold above which only males are found depend on the latitude of the area considered (Altringham and Senior, 2005; Russo, 2002). Downstream males share summer roosts with females and have been found to take advantage of this proximity by mating in summer besides autumn (Encarnação, 2012; Senior et al., 2005) with a higher reproductive success than upstreams males (Angell et al., 2013). To save energy, *M. daubentonii* uses torpor, a thermoregulation strategy, consisting of a reduction in body temperature and metabolic rate. During the activity season this species can thus adopt heterothermy in daytime (Geiser, 2004; Dietz and Hörig, 2011), while during its hibernation period it can remain uninterruptedly in torpor for many days; the maximum recorded period was 79 days (Průcha and Hanzal, 1989). In spring, when ambient temperatures and insects abundance increase, the bats awaken to forage and move to summer quarters. The dispersal season occurs in spring and autumn; the maximum distance travelled by a *M. daubentonii* in Europe is260 km (Urbańczyk, 1989).

This insectivorous bat, hunts mainly on small Diptera in the family Chironomidae (Racey and Swift 1983; Beck 1991; Sullivan et al. 1993; Vaughan 1997) that are caught flying low over the water surface (Jones and Rayner, 1988; Kalko and Schnitzler, 1989); further prey comprises other Diptera, Trichoptera, Ephemeroptera, Lepidoptera, Coleoptera and perhaps small fish (Brosset and Delmare, 1966; Kurskov, 1981; Swift and Racey, 1983). *M. daubentonii* can capture prey on the wing, scooping it with the tail membrane or trawling on the water surface by using feet and or the wing membrane (Jones and Rayner, 1988).

This species uses echolocation to forage and orientate. It emits short frequency-modulated (FM) calls, characterized by start frequency between 70 and 95 KHz and final frequency between 25 and 30 KHz lasting 3-4 milliseconds, with a mean detection distance of 128 cm and a mean reaction distance of 112 cm (Kalko and Schnitzler, 1989; Bogdanowicz, 1994; Russo and Jones, 2002). *M. daubentonii* prefers foraging over smooth water surfaces, avoiding cluttered spots, because ripples interfere with echolocation (Rydell et al., 1999; Warren et al., 2000; Siemers et al., 2001). According to Limpens and Kapteyn (1991) in the Netherlands, *M. daubentonii* avoids artificial light during foraging and strictly follows linear landscape elements, hesitating to cross open spaces. Although several authors have observed foraging by more bats in the same patch (Wallin, 1961; Bogdanowicz, 1994; Ciechanowski et al., 2007; Dietz and Kalko, 2007), Encarnação et al. (2010) reported on aggressive and territorial behaviours (e.g. chases) that lead to the exclusion of one of the two competitors from a given foraging area. *M. daubentonii* is an opportunistic predator, with a high rate of attacks and approximately 50% of success (Kalko and Braun, 1991). One individual can capture up to 4.9 g of insects on a single night; pregnant females and males undergoing spermatogenesis consume approximately 8.0 g of insect for night (Holweg and Wolters, 2005). Therefore this species plays an important role in riparian ecosystems, controlling aquatic insects populations, also comprising many pest species (Holweg and Wolters, 2005).

1.3 Aims

The management and conservation of ecosystems is a priority of the new millennium in landscapes which are being increasingly altered by human action (Hobbs and Harris, 2001). The importance of riparian habitats for foraging and commuting of many bats species was observed in different geographic areas (LaVal et al., 1977, Vaughan et al., 1997; Racey 1998; Grindall et al. 1999; Russo and Jones, 2003; Menzel et al., 2005; Biscardi et al., 2007; Akasaka et al., 2012). Chiroptera is among the most threatened mammal taxa in the world; for instance all Italian bat species feature in the I, II annexes of 92/43/EEC “Habitat Directive” and at least part of this endangerment is linked with the alteration of watersites. Bats are in fact highly vulnerable to habitat changes, since they are especially sensitive to even small-scale environmental alterations (Brouat et al., 2004; Ober and Hayes, 2008; Campbell et al., 2009).

Several studies showed that bats use linear landscape elements, such as rivers and riparian vegetation, as a landmark for migration and dispersion (Fenton and Thomas, 1985; Fleming et al., 2003; Furmankiewicz and Kucharska, 2009). Over long time spans, hydrography and basin morphology may therefore influence genetic population structure by facilitating or countering gene flow. Therefore, the genetic patterns of each population or species could be influenced by geographic elements (Castella et al., 2000; Ruedi et al., 2008; Campbell et al., 2009).

Phylogeography is a multidisciplinary science that investigates the relationships between the history of the Earth, ecology and biodiversity. It is identifies and analyzes the geographic distributions of gene lineages within or between species, processing the historical mechanisms and factors that led to the observed distributions (Avise, 2000). Understanding phylogeographic patterns, as well as providing information on the evolutionary history and population dynamics, can lead to the recognition of cryptic species and subspecies. Therefore phylogeographic studies can be essential tools for the management and conservation of threatened species (e.g., Avise et al., 1987; Avise, 2000; Frankham et al., 2002).

The main objective of this thesis was to investigate the degree of habitat influence on ecological and genetic variability of the riparian habitat specialist *M. daubentonii* at different spatial and temporal scales. First of all, I examined the effects of an altitudinal gradient on ecology and thermal physiology of *M. daubentonii* males in a population in Central Italy, for which spatial segregation has been observed (Russo, 2002). On a larger scale, I also explored the genetic structure and phylogeography of *M. daubentonii* from different river basins of Europe. The latter was carried out in collaboration with the Murciélagos Research Group of the Estación Biólogica de Doñana (Seville, Spain).

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2. HOW TO BE A MALE AT DIFFERENT ELEVATIONS: ECOLOGY OF INTRA-SEXUAL SEGREGATION IN THE TRAWLING BAT *MYOTIS DAUBENTONII*

2.1 Introduction

Several forces drive social segregation (i.e. the tendency to form separate social groups; Bon and Campan, 1996) in vertebrates. Sexual segregation is a common form of social segregation and in vertebrates may be often explained in terms of sex-specific habitat requirements and/or sociality. Differences in energy demands, body size, social behaviour, antipredatory needs and breeding phenology are all closely associated with spatial, social and habitat selection differences between sexes (Bonenfant et al., 2004; Dietz et al., 2006; Ruckstuhl, 2007). The two sexes may either segregate spatially or temporally (Russo et al., 2011); segregation has been advocated at least in certain cases as a mechanism to mitigate intersexual competition, yet this might only be an effect, rather than the driver, of the phenomenon.

Besides sexual segregation, another (subtler) form of social segregation recorded in vertebrates is intrasexual segregation (Archer, 1988). The two social patterns may be related for several reasons. For instance, the presence or absence of one sex in social groups may influence within-sex behavioural rates of aggression in the other, leading to different degrees of intrasexual segregation (Weckerly et al., 2001), or some males may associate with females as a form of antipredatory mimicry (Geist and Bromley, 1978) or to increase reproductive success (Senior et al., 2005). A common explanation for intrasexual segregation is sex-specific aggressiveness, so that males will only displace males from their vital space, females only females (Zabala et al., 2007; Ancillotto and Russo, 2014), but this is unlikely to apply to all known cases.

Sexual segregation in bats is often attributed to different microclimate requirements (Ruckstuhl, 2005) or prey availability (Angell et al., 2013). Many temperate bats exhibit sex-biased segregation with females occurring at lower altitudes than males during the activity season

(McGuire and Boyle, 2013). This spatial segregation is explained in terms of different energetic requirements of the two sexes and often expressed by a biased sex ratio over elevational gradients, with males being more frequent higher up (Grindal et al., 1999; Russo, 2002). In the breeding season, females need to preserve homeothermy for foetal development and the increased energy demands posed by this condition or by subsequent lactation lead them to congregate at lower altitude, where warmer roosts and more productive foraging habitats are found (Erickson and Adams, 2003; Senior et al., 2005; Lintott et al., 2014).

The Daubenton's bat *Myotis daubentonii* (Vespertilionidae. Kuhl, 1817) is medium-sized vespertilionid strictly associated to aquatic habitat, where prey is either caught on the wing or "trawled" from the water surface by using feet and/or the wing membrane (Kalko and Schnitzler, 1989). This species mainly preys upon small dipterans in the Chironomidae family, which constitute the bulk of its diet (Vaughan, 1997; Vesterinen et al., 2013). *M. daubentonii* represents a very interesting model species to investigate social segregation both between and within sexes: in several regions of Europe adult males are disproportionately abundant at higher elevations, while females are restricted to lower altitudes (Leuzinger and Brossard, 1994; Altringham et al., 1997; Russo, 2002). The actual elevational threshold above which only males are found depends on latitude (Altringham and Senior, 2005; Russo, 2002). Downstream males share summer roosts with females and have been found to take advantage of this proximity by mating in summer besides autumn (Encarnação, 2012; Senior et al., 2005) thus achieving a higher reproductive success than upstream males (Angell et al., 2013). From an energetic point of view, such males should be able to exploit more productive foraging habitats as insects are known to be more abundant in warmer sites (Taylor, 1963) and also get access to warmer roosting sites. It is unclear why only some males are allowed to share habitats with females, yet a plausible hypothesis is territoriality, i.e. downstream bats (males and/or females) would actively exclude some males restricting them to higher altitudes in less productive environments (Russo, 2002; Encarnação et al., 2005; Senior et al., 2005).

The energetic costs of living at different elevations may be largely influenced by the frequency and depth of daytime torpor (Wang, 1989; Turbill et al., 2003; Willis, 2005). In summer, when prey is scarce and/or temperatures are low, unlike females, *M. daubentonii* males may use daily torpor (Hamilton and Barclay, 1994; Turbill et al., 2003; Dietz and Kalko, 2006; Becker et al., 2013) but clearly the energetic significance of torpor will depend on roost temperature (in turn, an effect of altitude-dependent temperature) as well as on how much energy is gained by foraging (Dietz and Hörig, 2011; Becker et al., 2013).

Although much work has been done on inter- and intrasexual segregation in *M. daubentonii* (Russo, 2002; Senior et al., 2005; Encarnação, 2012; Angell et al., 2013), the ecology of male segregation along an altitudinal gradient has yet to be fully unveiled. I tackled this goal by looking at the effects of altitude on thermal and foraging ecology of male *M. daubentonii* and compared habitat productivity, body condition, thermoregulation strategies, use of space and habitat selection between two altitude zones. Specifically, I tested the following predictions:

1. Foraging areas located downstream will have higher prey availability so foraging there will be more profitable;
2. To minimize energy loss in less productive, colder environments, daily torpor in upstream males will be deeper than in downstream males;
3. If prediction 1, is true, downstream males will attain a better body condition because they feed in more productive areas;
4. To cope with a less productive and predictable environment and track potentially ephemeral food concentrations, upstream males will be more flexible in habitat selection;
5. One controversial issue is whether upstream males are excluded from lower elevations by intraspecific competition with resident bats (Russo, 2002; Senior et al., 2005). To test this, I translocated upstream males to low altitude. I predict that if the competition hypothesis is

false, then translocated upstream males will remain in the new area exhibiting no substantial behavioural difference from typical downstream males.

2.2 Materials and methods

Study area

The *M. daubentonii* population I investigated was found along a 28-km stretch of the Sangro River (Fig. 2), in the Abruzzo, Lazio and Molise National Park and its buffer zone, in Central Italy (41° 45′ 46.8″ N, 13° 58′ 8.4″ E). In the study area the river stretches along an altitudinal gradient of 300 m comprised between 1100 m and 800 m a.s.l. and is mostly characterized by laminar flow with limited turbulence spots. At ca. 950 m a.s.l the river is blocked by a dam used to produce hydroelectric power, forming an artificial lake (Barrea Lake). The riparian vegetation is well developed along much of the course and is dominated by *Salix* spp.

Female *M. daubentonii* do not occur > 900 m a.s.l. and males at lower altitudes are far less numerous than uphill (Russo, 2002). Hereafter I refer to "high" and "low" elevation (or "upstream" and "downstream") as to the altitudinal zones respectively above 1000 m a.s.l. and below 900 m a.s.l.; the Barrea Lake is located at ca. 950 m a.s.l. (Fig. 2).

Prey availability

During summer 2013 I sampled nocturnal aerial insects using sticky traps (Kunz, 1988; Becker et al., 2013). Based on radiotracking data (see below) I selected three main foraging areas for each of the high and low altitudinal zones as well as at the lake. For each of them, on the river, I chose three trapping sites, at least 50 m apart. At each site I installed two sticky traps, one for each bank, while for each of the three areas on the lake I placed six traps along the bank at least 50 m from each other. The traps were installed 10 cm to the water surface. They consisted of a circular panel (22 cm of diameter) sprayed with glue (Vebi Colla Spray, Vebi Istituto Biochimico s.r.l., Italy). To sample nocturnal insects only, sticky traps were set up at sunset and removed at sunrise over three days. I identified insect families with a stereo-microscope using an identification key (Venturi, 1982) and reference material.

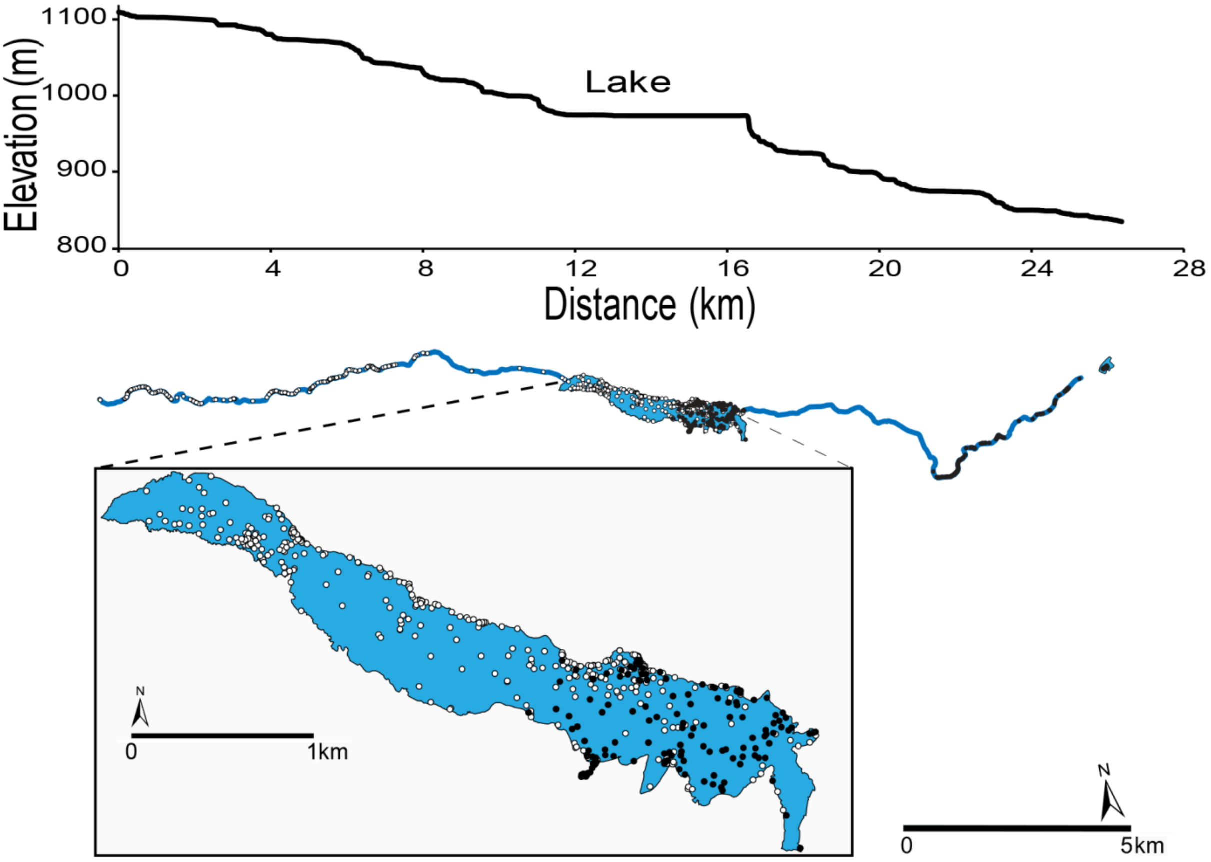

Figure 2. Elevation gradient (above) and map (below) of study area (Sangro river and Barrea Lake, Abruzzo Lazio and Molise National Park and its buffer zone, Central Italy) with locations (fixes) of 23 *Myotis daubentonii* detected by radio tracking. Note the overlap of foraging areas used by males from the two altitudinal zones in the lake area.

Daytime thermoregulation

I used temperature telemetry (Becker et al., 2013; Willis et al., 2005; Willis et al., 2006) to investigate the thermoregulation strategies of *M. daubentonii* adult males in the two altitude zones. During the summers of 2012 and 2013 I mistnetted bats over the river in the surroundings of known roosts. For each individual I assessed age and sex (Anthony, 1988; Racey, 1988). Adult males were fitted with temperature-sensitive radio-transmitters (LB-2NT, Holohil Systems, Ontario, Canada) using Skinbond$^{(R)}$ surgical cement. The combined mass of the transmitter (0.36 g) and glue did not exceed 5% of the bat's body mass. In the temperature sensitive tags I used, pulse emission rate changes according to skin temperature (T_s) and the latter can be inferred by recording pulse rate and consulting unit-specific calibration curves provided by the manufacturer (Becker et al., 2013; Willis et al., 2005; Willis et al., 2006). Tag signal was detected by a R-1000 telemetry receiver (Communications Specialists, Inc., CA) connected with a Yagi antenna. In daytime (meant as the time between a bat's return to roost and its subsequent emergence) I clocked 21 pulses for 3 times every 15 minutes for all bats (Becker et al., 2013). I also measured ambient temperature (T_a) with a digital thermometer (±0.1°C) placed in the shade near the roost at a height of 1.5 m. In no case did roost structure allow us to measure roost's internal temperature.

A previous study (Willis and Brigham, 2003) showed that T_s and body core temperature are strongly correlated and do not differ > 6°C. I categorized as torpor the thermal state of a bat whose T_s was < 6 °C relative to the temperature measured 15 minutes before emergence from roost (Dietz and Kalko, 2006); the latter was assumed to be typical of an active, fully homeothermic bat. I calculated the heterothermy index (HI, Boyles et al., 2011) over the time interval 6:00 AM – 8:00 PM.

$$HI = \sqrt{\frac{\Sigma\left(T_{b-opt} - T_{b-i}\right)^2}{n-1}}$$

This index expresses the temporal variation of the detected skin temperatures in a certain sampling period in relation to the active skin temperature detected in active bats 15 minutes before roost emergence (T_{b-opt}). Higher HI values express a grater magnitude of heterothermy.

Sunrise and sunset times changed > one hour during the sampling period. To make sure our HI referred to bats in the roost I restricted its calculation to the time comprised between 6.00 AM and 8:00 PM when all bats were day-roosting.

Body condition

I assessed body condition using forearm length (FAL, mm) and body mass (g) of 198 *M. daubentonii* adult males mistnetted in 2000-2013 within the boundaries of the study area along the Sangro river's altitudinal gradient. FAL and body mass were measured respectively with a calliper to the nearest 0.1 mm and a digital scale to the nearest 0.1 mm. For each bat I calculated the Scaled Mass Index (SMI, Peig and Green, 2009). I chose to use SMI because for both small terrestrial mammals (Peig and Green, 2009) and bats (Puechmaille et al., 2014) it is regarded as a robust indicator of the body condition which best accounts for variation linked with size, age and sex (Peig and Green, 2010).

$$SMI = M_i \left[\frac{FAL_0}{FAL_i}\right]^{b_{SMA}}$$

Where, M_i and FAL_i are the body mass and the forearm length of individual *i* respectively; FAL_0 is the arithmetic mean value for the study population; b_{SMA} is the scaling exponent estimated by the standardized major axis regression of M on FAL.

Use of space and night activity

I radiotracked male bats mistnetted in the summers 2012-2013 and fitted them with temperature-sensitive radio-transmitters (model LB-2NT, Holohil Systems, Ontario, Canada) from dusk emergence to their return to the roost (Table 1). Bat locations (hereafter termed fixes) were obtained by cross-bearing and, where applicable, “homing-in” (White and Garrott, 1990). I assessed the degree of accuracy by locating stationary active tags in several sites across the study area and comparing their actual position with that estimated by radiotracking. In all cases the error was negligible (< 5 m). Spatial analysis was carried out with ArcView 3.1 (ESRI). All fixes were mapped using the ArcView Extention “Radiating Line” (Jenness Enterprises, http://www.jennessent.com/arcview/radiating_lines.htm).

A 2380 km^2 small-scale vegetation map was generated *a posteriori* by carrying out photo-interpretation of the riparian vegetation within 10 m from the banks of all water bodies. Photo interpretation relied on digital ortophotos (0.2 m/px) at a nominal scale of 1: 5.000. I classified the habitats potentially relevant for *M. daubentonii* as follows: river with riparian vegetation on both banks (17%), river with riparian vegetation on one bank (2%), river with no riparian vegetation (2%), lake shore with riparian vegetation (7%), lake shore with no riparian vegetation (2%), lake-interiors (64%) and flooded *Salix* spp. woodland (6%).

To assess habitat selection I considered only foraging fixes and included bats for which ≥ 50 fixes had been obtained. The number of fixes falling in each habitat category was defined by carrying out a GIS spatial join operation between the shape file containing bat fixes and that of habitat types. Our radiotracking data clearly showed that when foraging or commuting bats never left the main river or other minor water bodies in its immediate surroundings: I thus refrained from using Minimum Convex Polygons for habitat selection analysis as these would have included large proportions of unused habitat. Instead, I concentrated on the habitat actually suitable to bats comprised within the spatial buffer used for photointerpretation.

To calculate the maximum distance travelled on a night from the roost, I considered: 1) the straight distance between the latter and the farthest location reached on a given night; and 2) the length of the actual route covered by bats along the waterways they followed.

Finally, I compared the time spent night-roosting between upstream and downstream males, meant as the total amount of time a bat spent inactive from emergence to sunrise.

Testing the competition hypothesis

In August 2013, I mistnetted 10 *M. daubentonii* adult males at a high altitude roost, fitted them with temperature-sensitive radio-transmitters (model LB-2NT, Holohil Systems, Ontario, Canada) and promptly transferred them to a downstream roost hosting over 100 adult bats of both sexes. Bats were manually introduced in the downstream roost within 3 hrs to capture. Over the subsequent 12 days I radiotracked them to follow their nightly movements and record daytime skin temperature.

Table 1. Date of capture, altitude of capture and roosting, biometry (FAL=forearm length, BM=body mass) and tracking details of 23 male *Myotis daubentonii* radiotracked at the Abruzzo, Lazio and Molise National Park and its buffer zone, in Central Italy. *N* fixes = number of foraging locations recorded for each bat.

Bat code	Date of capture	Altitude	FAL (mm)	M (g)	Days tracked	*N* fixes
170433	04/07/2012	high	37.3	7.1	7	66
170438	04/07/2012	high	35.4	7.1	3	66
170443	04/07/2012	high	36.4	6.9	9	56
170448	15/07/2012	high	36.2	7.1	3	89
174706	25/08/2012	high	35.9	6.0	6	73
174703	25/08/2012	high	37.3	6.8	4	79
174710	25/08/2012	high	36.8	6.5	5	64
180032	09/07/2013	high	37.1	5.3	6	69
180027	10/07/2013	high	37.8	7.1	4	66
180038	10/07/2013	high	37.2	7.7	4	60
180037	22/08/2013	high	37.0	6.5	6	57
180029	22/08/2013	high	38.0	7.2	2	80
180028	22/08/2013	high	38.6	7.0	3	69
180022	22/08/2013	high	38.4	6.1	5	62
170439	07/07/2012	low	35.9	7.1	7	53
170449	07/07/2012	low	38.4	7.1	7	60
174701	20/08/2012	low	37.0	6.9	7	91
170446	20/08/2012	low	36.7	6.5	7	69
170440	20/08/2012	low	37.5	6.1	7	59
180023	01/07/2013	low	36.5	6.5	3	64
180040	01/07/2013	low	37.3	6.9	3	77
180021	01/07/2013	low	36.9	7.8	3	81
180030	01/07/2013	low	34.2	6.3	4	80
180031	22/08/2013	transferred	36.7	7.7	8	56
180035	22/08/2013	transferred	36.5	6.5	5	85
Mean ± SD			36.9 ± 1.0	6.8 ± 0.6	5.1 ± 1.9	69.2 ± 10.9

Statistical analysis

I applied General Linear Models (GLM ANOVA) followed by Tukey's post-hoc tests to compare the number of chironomids (Diptera Chironomidae) caught along the altitudinal gradient. I focused on such insects because they represent *M. daubentonii*'s staple food (e.g. Vaughan, 1997; Vesterinen et al., 2013) and can thus be used to assess the productivity of foraging areas.

The relationship between HI and T_a was explored with Pearson correlation tests; HI was also compared between upstream and downstream males by a GLM ANOVA. The same test followed by Tukey's post-hoc comparisons was also used to explore the effects of altitude and reproductive season on SMI. Reproductive season was categorized respectively as either pre-birth (from April to June) or post-birth (from July to September) time.

To assess habitat selection I performed a compositional analysis (Aebischer et al., 1993) in which the percent foraging fixes recorded for each habitat represented the "used" portion whereas the percent habitat occurrence expressed habitat availability.

I compared the mean and maximum distance travelled on a night and night-roosting time between upstream and downstream males by GLM ANOVA. All analyses except compositional analysis were performed with Minitab 13.1 (State College, PA: Minitab, Inc.). Compositional analysis was performed with R's "*adehabitat*" package (R Core Development Team, Calenge et al., 2005). Statistical significance was set at $p = 0.05$.

2.3 Results

Prey availability

I collected a total of 3111 chironomids. The mean number of chironomids caught differed significantly among the three study area sectors, and was highest at the lake, intermediate downstream and lowest upstream (lake 222 ± 137, downstream 80 ± 33, upstream 44 ± 23; GLM ANOVA, $F_{2,24} = 22.95$, $P < 0.005$ and Tukey's post-hoc tests).

Daytime thermoregulation

I recorded daytime skin temperatures of 22 adult males roosting at different altitudes (n upstream = 12; n downstream = 10). Typically, when upstream males returned to the roost**,** I recorded a body temperature drop leading to heterothermy, whereas downstream males remained mostly homeothermic in daytime (Fig. 3). The Heterothermy Index (HI) was significantly higher (7.61 ± 3.00) for upstream males than for those downstream (4.39 ± 2.92) (GLM ANOVA, $F_{1,20} = 6.39$, $P < 0.05$). HI also showed a negative correlation ($r = -0.57$) with ambient temperature ($P < 0.005$).

Body condition

Scaled Mass Index measured for 198 males captured in 2000-2013 was significantly higher for downstream males than for those upstream (GLM ANOVA, $F_{1,194} = 12.52$, $P < 0.005$); season had no effect on it but showed a significant interaction with altitude ($F_{1,194} = 7.64$, $P < 0.01$), reflecting the fact that body condition dropped in downstream males so that by July its value approached that recorded for upstream males.

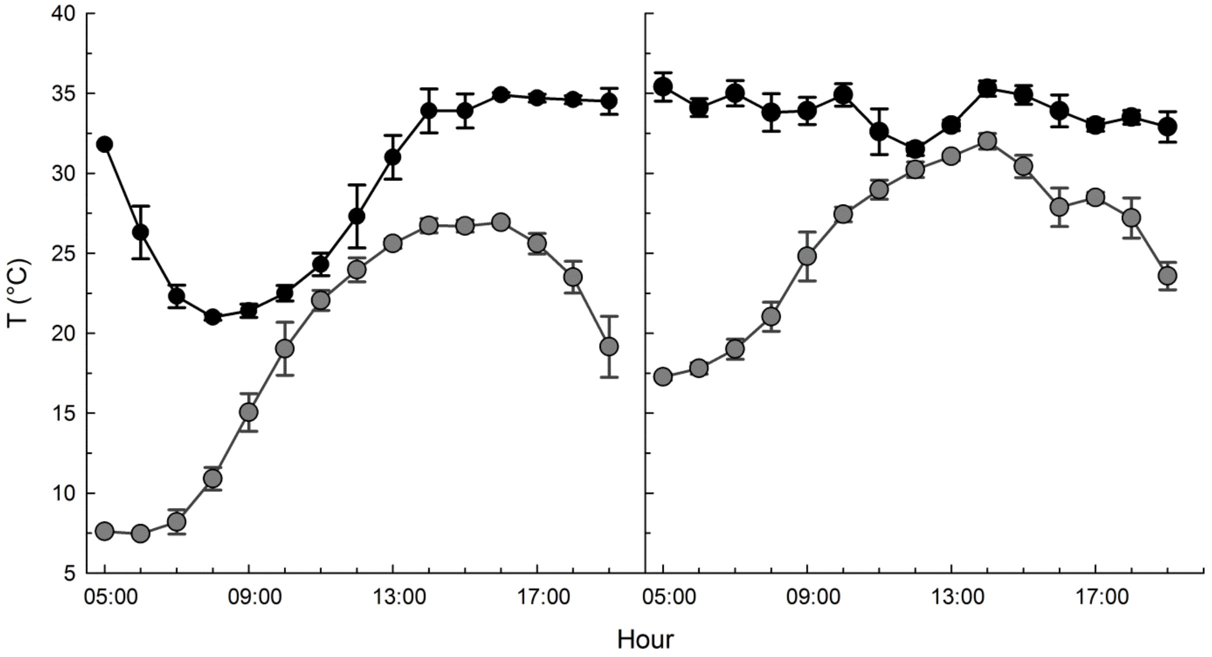

Figure 3. Two daytime skin temperature patterns respectively representative of an upstream (left) and a downstream male (right) *Myotis daubentonii* recorded at the Abruzzo Lazio and Molise National Park and its buffer zone (Central Italy). Skin temperature is given by the black line and ambient temperature by the grey line. When upstream males entered the roost, a body temperature drop was typically observed, leading to heterothermy, while downstream males remained mostly homeothermic in daytime.

Use of space and night activity

I assessed habitat selection by radiotracking for 23 bats. Bats were only recorded over water or near riparian vegetation (Fig. 2). Upstream males never switched to downstream roosts and vice versa. For upstream males, compositional analysis led to the following ranking (where significant differences occur, habitats are separated with >>>): river with riparian vegetation on both banks > lake shore without riparian vegetation > lake shore with riparian vegetation > lake interiors > river with riparian vegetation on one bank > flooded *Salix* spp. woodland >>> river without riparian vegetation on banks. For downstream males I obtained what follows: river with riparian vegetation on both banks >>> lake shore with riparian vegetation > lake shore without riparian vegetation > river without riparian vegetation on banks > river with riparian vegetation on one bank > lake-interiors > flooded *Salix* spp. woodland. Noticeably, foraging areas of upstream and downstream males on the lake overlapped (Fig. 2). Upstream males covered longer nightly distances than downstream males (GLM ANOVA, Table 2) and night-roosted for a longer time than the latter (GLM ANOVA, $F_{1,11} = 7.42$, $P < 0.05$; Table 2). Three of the upstream males switched roosts every day while the remaining were loyal to the same site; downstream males kept sharing the same roost with females and never switched elsewhere.

Table 2. Distances travelled and night-roosting time of 14 upstream and 9 downstream *Myotis daubentonii* males radiotracked at the Abruzzo, Lazio and Molise National Park and its buffer zone, in Central Italy. I considered both the straight distance from the root to the farthest location reached on a given night and the length of the actual route covered by bats following the waterways; inactivity time is the total amount of time a bat spent inactive from emergence to sunrise.

	Actual route length covered (km)	Straight distance (km)	Max actual route length covered (km)	Max straight distance (km)	Inactivity time (% night)
Upstream	7.3 ± 2.9	6.5 ± 2.8	10.3 ± 4.1	9.2 ± 3.8	33 ± 12
Downstream	2.4 ± 2.1	2.1 ± 1.8	3.6 ± 2.9	3.1 ± 2.5	18 ± 7
GLM, ANOVA	$F_{1,21} = 19.12$	$F_{1,21} = 17.84$	$F_{1,21} = 17.86$	$F_{1,21} = 18.04$	$F_{1,11} = 7.42$
	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.05$

Testing the competition hypothesis

Eight of ten upstream males I translocated downstream returned to high altitude areas in one or two nights. One bat roosted with downstream males and females only on the day following translocation, then moved to a nearby bridge where it apparently roosted alone. The other male stayed two days at the roost to where it had been translocated, then moved ca. 5.5 km farther downstream where it spent at least another 8 days (data collection ended due to battery failure). Both subjects night-roosted longer (60 ± 23) than upstream (33 ± 12) and downstream (18 ± 7) males (GLM ANOVA, $F_{2,12} = 11.94$, $P < 0.005$). Of the remaining translocated bats, one stayed downstream for 5 days before returning upstream.

2.4 Discussion

Our study offers a comprehensive picture of thermal and foraging ecology of male *M. daubentonii* living upstream and downstream with respect to a lake, along an elevational gradient that despite its limited slope (ca. 300 m) leads bats to adopt completely different strategies. Our altitude gradient is greater that those considered in UK studies, where intrasexual segregation of males in *M. daubentonii* was also analysed (Senior et al., 2005; Angell et al., 2013). One study (Senior et al. 2005) recorded spatial use differences between upstream and downstream males along a ca. 100 m gradient, whereas a more recent analysis (Angell et al., 2013) was extended farther downstream, categorizing bats according to three elevational zones (upper-elevation > 200 m a.s.l.; mid-elevation 100-200 m a.s.l.; low-elevation < 100 m a.s.l.). I assume that our "upstream" and "downstream" males correspond to the extremes of the altitude gradient considered by the latter study (Angell et al., 2013). The situation I studied also differed from the British ones because in our study area the dam and the artificial lake physically separated the upstream and downstream river stretches in our study area, creating a further habitat type available to bats.

According to our first prediction, I found that downstream areas are more productive, so bats roosting there potentially have an energetic advantage as they move less to reach profitable feeding sites and cover shorter distances between foraging sites (Senior et al., 2005). This sets the scene for the hypothesis that females and/or dominant males would select more productive foraging areas at low altitudes excluding subordinate males at higher elevations (Grindal et al., 1999; Russo, 2002; Encarnacão et al., 2005; Senior et al., 2005).

By sampling chironomids, I provided direct confirmation of what indirectly assessed in the UK (Angell et al., 2013) by counting feeding buzzes – sequences of echolocation pulses broadcast when attempting to catch prey (Griffin, 1958).

Chironomids are found in a range of habitats and water conditions; however, many species in this genus are tolerant to organic pollution and may be favoured by eutrophication (Saether, 1979; Rae, 1989). In addition, in their larval stages they are major components of benthos in backwater, therefore abound in lakes where they may colonize both microphytes and soft sediment (Pinder, 1986), which explains why in our study area they peaked at the lake as well as downstream, where human settlements are more widespread and river water more subjected to organic input. Chironomids also concentrate where air and water temperatures are higher (Walker and Cwynar, 2006) so they are more likely to occur downstream, favouring foraging activity there. Only a previous study (Senior et al., 2005) besides ours radiotracked upstream males and downstream males and unlike in our case found no foraging area overlap. Our situation may differ because our sample size was larger than in that case, or perhaps because of the presence of the lake connecting the areas upstream and downstream – an especially profitable foraging habitat for bats from both elevational zones. It is also important to notice that although downstream males in several cases moved upstream (to the lake) to forage, upstream males never moved downstream of the lake. Whether foraging sites downstream of the lake (however productive) were too distant to represent convenient destinations for upstream males, or the latter's access to those areas was restrained by competition with resident bats has yet to be ascertained. Noticeably, downstream males trespassed the dam to reach lake, so the dam was not an impermeable barrier (Rebelo and Rainho, 2008).

Upstream males exhibited more prolonged and deeper torpor than did those at low altitudes, in agreement with our second prediction. Besides hibernating in winter, bats from temperate areas use torpor to minimize energy loss year round (Altringham, 2011), yet torpor's depth and duration depend on environmental conditions (Willis et al., 2006). In this study it was technically impossible to measure roost temperature because the actual roosting spaces were not accessible to place dataloggers. However it is sound to assume that roost's internal temperatures reflected those I measured outside. The colder ambient temperatures found upstream allow upstream males to make an effective use of heterothermy and thus save significant energy. Downstream males were probably unable to do so because of the warmer roost temperatures, which would partly explained why they remained homeothermic in daytime. Our results agree with those of another study (Encarnação et al., 2012) which related depth and duration of torpor in male *M. daubentonii* with altitude.

The negative relationship I found between the heterothermy index and ambient temperature also confirms that thermoregulation strategies in male *M. daubentonii* are mainly influenced by the latter variable (Turbill and Geiser, 2006; Willis et al., 2006). As in previous studies (Russo, 2002; Senior et al., 2005) males caught downstream had a better body condition yet I recorded a drop of the latter which by the end of summer tended to equal that of upstream males. Assuming downstream and upstream males are loyal to the same areas year round, I suggest that the better body condition of the former early in the season is due to the higher productivity of the feeding sites they may exploit soon after hibernation, or to the milder winter temperatures they face, allowing more frequent arousals from hibernation to forage (Park et al., 1999, 2000; Zahn and Kriner, 2014). Reduced opportunities to adopt torpor in summer, the onset of spermatogenesis and mating activity at that time (Encarnação, 2012; Senior et al., 2005) or perhaps stronger competition at feeding sites with females and volant juveniles may all potentially explain why body condition of downstream males dropped during the season. In male colonies of *M. daubentonii* in Germany a further pattern was observed, with body mass increasing over the year (Encarnação et al., 2004).

The maximum distance travelled during the night by radiotracked bats are longer than those observed in the other telemetry study that looked at intra-male segregation of *M. daubentonii* (Senior et al., 2005), although the greatest distances travelled overnight from upstream males are similar to those I recorded. Besides, unlike in that case (Senior et al., 2005), in ours upstream males were less active during the night than downstream ones.

Upstream males returned to roosts earlier than downstream ones and quickly got torpid. This is likely to be a sound strategy to preserve energy in a cool roosting environment when prey availability is too scarce to make active foraging convenient.

As found in other studies (Rydell et al., 1999; Russo & Jones, 2003), downstream *M. daubentonii* preferred to forage where riparian vegetation is available. The latter shelters foraging spots from wind, keeping water surface smoother (which helps echolocation in trawling bats: Rydell et al., 1999; Holland and Waters, 2007; Greif and Siemers, 2010) and allowing insects to congregate in swarms (Delettre and Morvan, 2000). Besides, riparian vegetation is also an important habitat for insect reproduction (Delettre and Morvan, 2000). This behaviour is also known for another trawling species, *Myotis capaccinii* (Biscardi et al., 2007 – but see Almenar et al., 2006, 2009).

Downstream males showed a strict selection pattern as they mainly hunted at river spots with abundant riparian vegetation whereas upstream ones were more generalist simply preferring least river areas without vegetation on banks and making a large use of all other habitats. By selecting a broader range of habitats and covering longer distances to find suitable foraging areas (Senior et al., 2005), upstream males may get access to temporarily available food concentration – an important strategy in a less productive and unpredictable foraging environment such as that found at higher altitudes.

Our translocation experiment could not disprove the competition hypothesis as upstream males mostly homed back to their upstream quarters after being translocated to low altitude except two, which stayed where they had been moved but got access to sub-optimal roosting and foraging resources.

M. daubentonii is, according to the available literature, territorial at least in foraging sites, where chases are commonly observed (Encarnação et al., 2010). Social groups of *M. daubentonii* are centred on females, which have been hypothesized to be dominant over males, excluding them from territories with higher quality foraging habitats (Grindal et al., 1999; Russo, 2002; Encarnacão et al., 2005; Senior et al., 2005). The inter-sex associations of *M. daubentonii* are less frequent than in other bat species and have been found to be restricted to one year, but males can be tolerated in the roosting areas of female social groups if they comprise high-quality foraging habitats (August et al., 2014).

In summary, I showed that downstream areas are more productive and that the best foraging sites are closer to downstream roosts, yet this only confers a moderate advantage to male *M. daubentonii* dwelling those areas in terms of body condition, which tends to decline over the active season and reaches the values recorded in upstream males. The latter's strategies, including a larger use of daytime torpor, frequent night-roosting and less selective habitat selection, seem to mostly compensate for the disadvantages linked with foraging in less productive areas. Overall this picture is in agreement with the fact that the main advantage for downstream males is reproductive, since those bats are offered an extra-chance for mating (Senior et al., 2005; Angell et al., 2013) besides autumn swarming (Parsons and Jones, 2003; Senior et al., 2005). However, it cannot be ruled out that living downstream also confers a survival benefit as by getting access to more productive areas early in the active season bats may quickly replenish their fat stores and thus mitigate mortality following hibernation.

When translocated to low altitude, upstream males either returned promptly to their original areas or used low-quality roosting or foraging sites. Whether this is the effect of those males being displaced by resident individuals has yet to be ascertained, but our experiment cannot confute this scenario. If resident males displace those residing upstream, competition for females could be the driving factor behind male-male aggressiveness (Ancillotto and Russo, 2014); alternatively, males found upstream might be confined there by females, perhaps based on fitness clues that are, to date, unknown. Should this be the case, body condition would not be a suitable proxy for individual fitness as its difference between the two elevational zones is only transient and disappears ahead in summer.

2.5 References

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3. A FIRST APPROACH TO THE PHYLOGEOGRAPHY OF DAUBENTON'S BAT *MYOTIS DAUBENTONII*

3.1 Introduction

The current geographical distributions, demographics and genetic differentiation of plant and animal species have been strongly influenced by climatic oscillations occurred - at least - during the Quaternary era (Avise, 2000; Hewitt, 2004). In cold periods (glacial), the loss of habitat caused the extinction of local populations and/or their confinement in favourable areas (glacial refugia). During warmer periods (inter-glacial), with the availability of new territories and habitats, populations expanded again (Avise, 2000; Schmitt, 2007). The most recent ice ages in Europe date back to the late Pleistocene, during which the major glacial refugia in the Western Palaearctic were located in the Italian, Iberian and Balkan peninsulas (Taberlet et al, 1998; Hewitt, 1996, 1999, 2004; Randi, 2007). Cyclic alternating of contraction to the lower latitudes and rapid northwards re-expansion of the species ranges is probably at the basis of genetic paucity in northern populations (founder effect) compared to the southern ones, which due to their relatively large stability show more polymorphisms and genetic variability (Hewitt, 2000, 2004). Furthermore, recent studies have identified small geographically isolated areas that acted as refugia within the southern areas traditionally identified as glacial refugia (Gómez and Lunt, 2007; Canestrelli and Nascetti, 2008; Grill et al., 2009). This pattern of "refugia-within-refugia", characterized by isolated populations in allopatric shelter areas, is thought to have prompted the high number of endemisms and large genetic differentiation we now find in the southern areas. Therefore, the current geographic distribution of genetic variability of a species and the genetic structure of its populations reflect how the species responded to the contraction and expansion cycles during the Ice Ages. This genetic signature will depend on several factors, such as the species' dispersal ability, the characteristics of the refuge, the re-colonization rate from other refugium, gene flow levels and demographic aspects related to the particular life history of the species, etc., as shown by the large amount of phylogeographic studies published world-wide and particularly for European specie in recent years (Hewitt, 1999; Avise, 2000; Schmitt, 2007). Nevertheless, and despite this recent advance, the phylogeography of many mammals widespread in the European Palaearctic region still remains little explored in this context (e.g. Hewitt, 2011).

The Order Chiroptera is a very diversified group, being the second largest among mammals. Bats are present all over the World except for a few distant oceanic islands and the polar regions (Kunz and Pierson, 1994). Bats are adapted to a large variety of habitats (Kunz and Lumsden, 2003; Patterson et al., 2003), showing different morphology according (among other things) to the structure of the most frequented habitat. Particularly wing morphology, in addition to reflect preferred habitat characteristics, is also indicative of dispersal ability, which varies among species (Norberg and Rayner, 1987).

The Daubenton's bat, *Myotis daubentonii* (Chiroptera, Vespertilionidae; Kuhl 1817) is a medium-size *Myotis* among the most widespread bats in Europe (Corbert, 1978; Bogdanowicz, 1994), and is known for being strictly linked with aquatic habitat for foraging (Jones and Rayner, 1988; Kalko and Schnitzler, 1989; Siemers et al., 2001). It catches insect flying low on water surface and its wing morphology is characterized by low wing-loading along with rather short and rounded wingtips, characteristics linked with slow flight and efficient manoeuvrability (Jones and Rayner, 1988). This species has Eurasian distribution comprising most of the Palaearctic regions (Bogdanowicz, 1994). *M. daubentonii* occurs from Portugal to Ireland, from Norway to Greece, all the way to Central and South-Western China, Japan (Hokkaido) and Korea (Bogdanowicz, 1994). *M. daubentonii* shows a great morphological variability, so that several subspecies have been recognized such as *M. d. nathalinae*, (Tupinier, 1977) and *M. d. volgensis*, (Eversmann, 1840). Recently, morphological analyses have shown that genetic variability is not sufficient to support the three European subspecies, and that on the contrary, all should be considered as belonging to the nominal form *M. daubentonii,* except for maybe marginal populations, as from the Iberian Peninsula, which are larger in some characters and need further investigations (Bogdanowicz, 1990). Mayer and von Helversen (2001) showed the existence of genetic differentiation across European *M. daubentonii* populations. Simões et al., (2007) have shown that the two morphotypes "*nathalinae*" and "*daubentonii*" corresponded to distinct genetic lineages but not quite and suggest that, during the Last Glacial Maximum (LGM, 23˙000 – 18˙000 years ago), *M. daubentonii* populations were confined to the peninsulas of Southern Europe, an event which may have had large influence on their genetic structure. Still, the available information is local and restricted to few samples (Mayer and Von Helversen, 2001; Simões et al., 2007; Ngamprasertwong et al, 2008). My work aimed to understand the phylogeography and historical processes beyond *M. daubentonii* populations genetic variability from different regions of Europe. I have analyzed genetic diversity of the most informative markers at this evolutionary level such as the cytocrome *b* gene and the hypervariable domains *I* and *II* of the mitochondrial DNA (mtDNA).

The specific objectives were: a) to obtain the first accurate description of the hypervariable domains *I* and *II* for this bat species; b) to identify the main lineages within the European *M. daubentonii* populations; c) to analyze the variability within and between the different lineages; d) to identify *M. daubentonii* European glacial refugia.

3.2 Materials and methods

I analyzed a total of 157 samples of *M. daubentonii* from 63 localities of France, Germany, Greece, Italy, Montenegro, Netherlands, Portugal, Serbia, Spain, Sweden and Switzerland (Fig. 4). Samples were non-invasively collected with wing punches and stored in 96% ethanol (Worthington Wilmer and Barratt, 1996). In the laboratory, tissues were digested with proteinase K and DNA was extracted using phenol/chloroform and ethanol precipitation (Sambrook et al., 1989). A cytocrome *b* (Cyt *b*) gene fragment was amplified with the primers Molcit-F (5’-AATGACAT-GAAAAATCACCGTTGT-3’) and MVZ-16 (5´-AAATAGGAARTATCAYTCTGGTTTRAT-3´) to obtained. The PCR cocktail (20 µl final reaction volume) included 2 µl of DNA extract, 1 µl of each primer (10 µM), 0.8 µl of $MgCl_2$ (50 mM), 0.16 µl of dNTP (25 mM), 0.5 units of taq-polymerase. Thermocycling consisted in a 4' initial denaturation at 94°C followed by 35 cycles of 30" at 94°C, 30" at 52ºC and 60" at 72°C, and a final extension of 10' at 72°C. For each samples, the hypervariable domain *I* (*HVI*) and *II* (*HVII*) from control region (D-loop) of mtDNA were amplified by a polymerase chain reaction (PCR) with primer pairs L15926 (5′-TCAAAGCTTACACCAGTC1TGTAAACC-3′; Kocher et al., 1989) and CSBF (5′-GTTGCTGGTTTCACGGA GGTAG-3′; Wilkinson and Chapman, 1991), L16517 (CGACATCTGGTTCCTACTTCAGG; Fumagalli et al., 1996) and H607 (5′-AGGACCCATCTAAGCATTTTCAGTG-3′; Worthington Wilmer et al., 1994) respectively. The reaction was performed in a total volume of 20 µl containing 2 µl DNA extract, 0.75 µl primers F/R (10 mM), 0.8 µl $MgCl_2$ (50 mM), 0.3µl BSA (10 mg/ml) 0.16 µl dNTPs (25 mM), 0.12 µl *Taq* DNA polymerase (5 Ʊ) with 2 µl of appropriate buffer (10X; QIAgen, Inc.) and distilled H_2O. Amplification of HVI included 4' initial denaturation at 94°C, followed by 30 cycles at 94°C (45"), 60°C (45") and 72°C (1'), with a final extension at 72°C (7'). The thermal profile of *HVII* amplification was: 4' initial denaturation at 94°C, followed by 30 cycles at 94°C (45"), 52°C (45") and 72°C (1'), with final extension at 72°C (7'). PCR products were purified and sequenced using an ABI 3100 automated sequencer (PE Biosystems, Warrington, UK). All sequences were aligned and edited visually using Sequencher 4.5 (Gene Codes Corp., Ann Arbor, MI, USA).

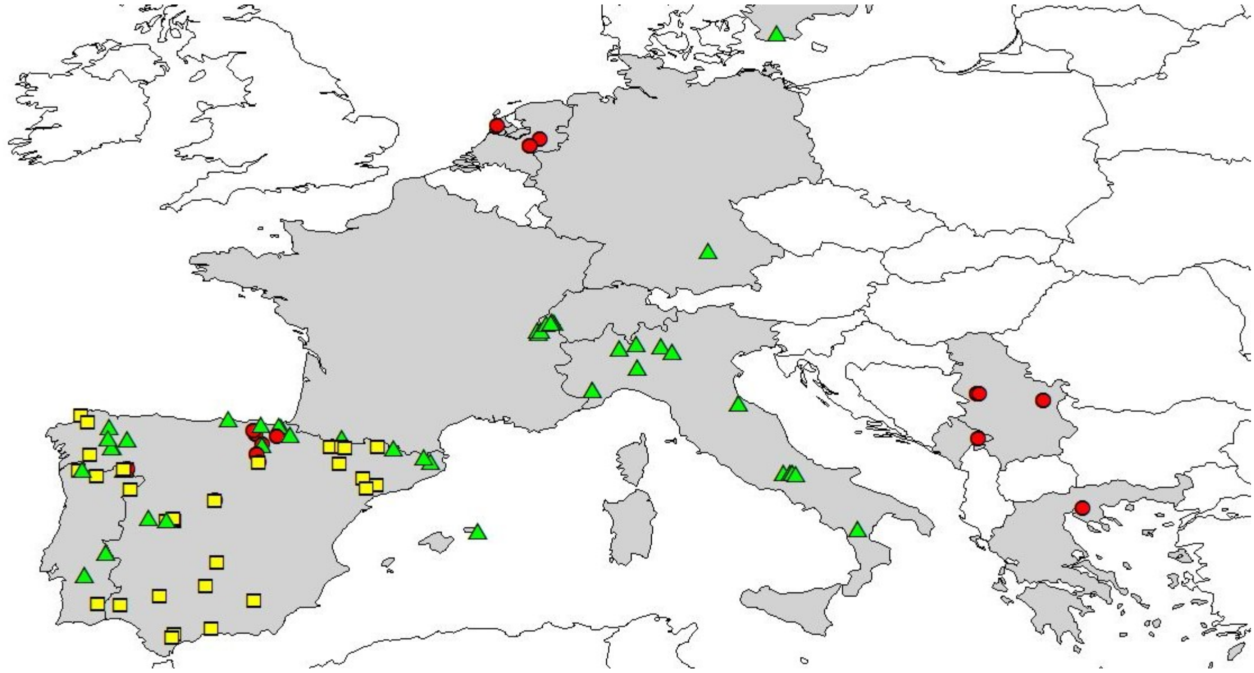

Figure 4. Map showing the sampling locations of *Myotis daubentonii*. Symbols represent the three main lineages: 🔴 Balkan, 🟨 Iberian and ▲ Italian.

Phylogenetic hypotheses were elaborated from the Cyt *b* sequences under different optimality models: Maximum Parsimony (MP) was implemented using PAUP* 4.0b10 (Swofford, 2003), Maximum Likelihood (ML) was performed with the software PHYML (Guindon and Gascuel, 2003) and Bayesian inference (BI) was implemented using MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fitting models of sequence evolution used in ML and BI analysis were identified according to the Bayesian information criterion (BIC, Schwarz, 1978) using the software JMODELTEST (Posada, 2008). The Bayesian topologies were obtained after five simultaneous Markov chains which were run for 2 million generations; trees were sampled every 300 generations. The resulting burn-in values were determined empirically after tree likelihood scores reached stationary values. Analyses were repeated in two separate runs to ensure that trees converged on the same topology and similar parameters. Under MP, trees were obtained after heuristic search with an initial tree obtained by stepwise addition (random input order) of the taxa, followed by a complete tree-bisection–reconnection (TBR) branch swapping. This process was repeated 25 times. Topologies were obtained differentially weighting transversions according to likelihood estimates of ts/tv ratios to take into account the heterogeneity of the sequences. The robustness of the topology was assessed through bootstrapping (Felsenstein 1985) after 2,000 replicates. Confidence in the topologies of the ML was assessed using nonparametric bootstrap analysis (Felsenstein, 1985) based on 1000 pseudoreplicates. In order to calculate net-pairwise distances within and between the major clades identified by the phylogenetic analyses, I used a Kimura's 2-parameter model (K2P) with MEGA4 (Tamura et al., 2007). For the main groups described in the phylogenetic reconstructions, I computed the number of haplotypes (*h*), number of mutations (*g*), segregating sites (*S*), haplotype diversity (*Hd*) and nucleotide diversity (π) of Cyt *b* using DNASP 4.5 (Rozas et al., 2003), to evaluate genetic variability and understand historical processes.

For *HVI* and *HVII*, I implemented in PAUP* 4.0b10 (Swofford, 2003) a partition-homogeneity test (incongruence length difference, ILD) to test the congruence of the phylogenetic information in the two fragments. A repeated motif of 81 bp (R1 repeat) within *HVI*, which varies in number of repeated copies among individual bats in Vespertilionidae family (Wilkinson et al., 1997), was excluded from the analysis due to its uncertain evolution. Sequences of *HVI* and *HVII* were then combined to perform the phylogenetic and demographic analyses. Bayesian inference (BI) for the main lineages resulted by phylogenetic reconstructions of Cyt *b* was performed using MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003). I also obtained haplotypes Parsimony networks using the combined *HVI* and *HVII* through the median-joining algorithm with the software NETWORK 4.5 (Bandelt et al., 1999) for the major clades observed by the phylogenetic analysis of Cyt *b*. I computed the number of haplotypes (*h*), number of mutations (*g*), segregating sites (S), haplotype diversity (Hd) and nucleotide diversity (π) using DNASP 4.5 (Rozas et al., 2003) to evaluate genetic variability of *HVI* and *HVII* for the principal lineages. In order to explore the demographic history of lineages, I also calculated Fu's F_S statistic (Fu, 1997) and R_2 index (Ramos-Onsins and Rozas, 2002), which have been advised to be the most powerful tests for detecting expansion events (Ramos-Onsins and Rozas, 2002), as well as the more conservative Tajima's D test (Tajima, 1989). These indices are widely used as 'indicators' of demographic change assuming that if a population is in mutational neutral equilibrium it maintains a constant size. Negative and statistically significant values of these indices reveal selection or demographic expansion. In addition, I implemented the mismatching distribution analysis, which evaluates pair-wise differences between sequences. This distribution is irregular and usually multimodal in populations that have experienced a prolonged demographic stasis, whereas it is regular and unimodal in populations that have been increased for a long time or have undergone a rapid population expansion. I considered the raggedness statistic (r; Harperding, 1994), which measures the irregularity of the mismatch distribution and tau parameter (τ) to estimates the time elapsed since the start of the event expansion.

3.3 Results

The alignment for the Cyt *b* fragment of European *M. daubentonii* consisted of 157 sequences (Appendix 1), each 708 bp long with 587 invariable, 116 variable, of which 64 were singletons and 52 parsimony-informative sites for a total of 51 different haplotypes. For the *HVI* and *HVII* fragments a total of 123 sequences were obtained (Appendix 1), which were trimmed at the beginning of the repeats for region of the Dloop, to 370 bp and 318 bp respectively (Fig. 5; Fig. 6). I found a great variability in repeated fragments and similarities corresponding to the geographical origin. For the *HVI*, the repeats are preceded by a fragment of 18 bp with a few differences, except for the 3 samples from Huelva and one from Sevilla, in which it is of 21 bp. Subsequently, all the sequences have a fragment of 7 bp (ACACAAT), which is repeated 10 times only in an Italian sample (ITAQ04) and 2 times in 2 samples from Ávila and one from Burgos. After the latter fragment, there is a fragment of 7 bp (ACATAAT) only in all Italian sequences that is repeated 5, 9 or 12 times, this being absent in all other samples. In all sequences the following fragments are one of of 7 bp highly variable and one of 81 bp in 36 sequences before the start of the 81 bp fragment (R1). For *HVII*, a fragment of 22 bp (TTAATGGTTACAGGACATAACA) precedes the repeats in all sequences except 3 samples from Málaga for which the fragment consists of 17 bp. Before repeated fragment common to all samples, two fragments of 6 bp follow: these are absent in 3 samples from Málaga. In addition, the final repeated fragment consists of a motif of 12 bp (TACGCATAACGC) only in the 3 individuals from Málaga and one individual from Léon, while for all others the repeats consisted in a typical motif of 6 bp (TACGCA).

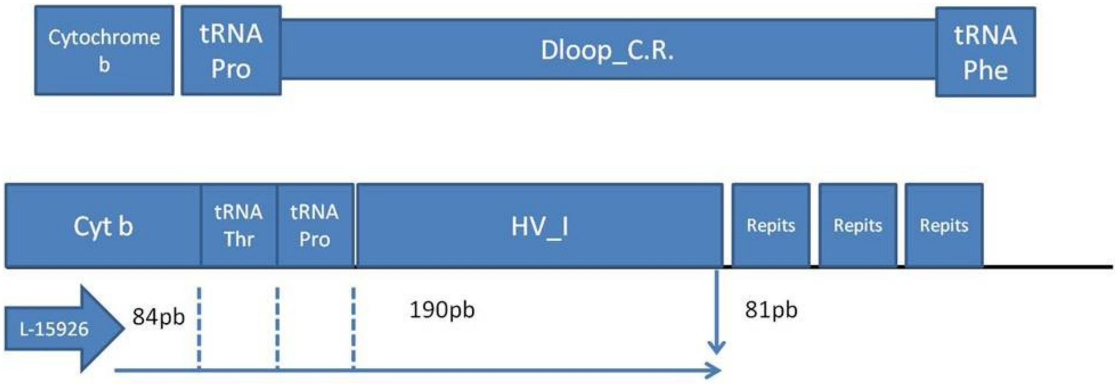

Figure 5. Descriptive diagram of hypervariable domain *I* (*HVI*) from the control region in mtDNA.

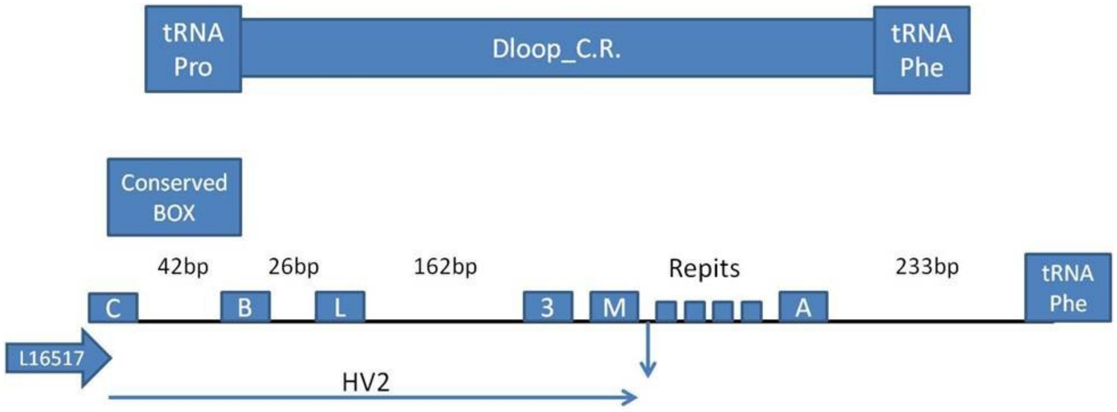

Figure 6. Descriptive diagram of hypervariable domain *II* (*HVII*) from the control region in mtDNA.

Overall Genetic Diversity and Phylogenetic reconstruction

The indices of genetic diversity and polymorphism showed high values of haplotype and nucleotide diversity for the total *M. daubentonii* data set (Cyt *b*: *Hd* = 0.931; $\pi = 0.014$; Table 3; *HVI-HVII*: *Hd* = 0.983; $\pi = 0.032$; Table 4).

For the Cyt *b* alignment, the best fitting substitution model was a HKY + G with gamma shape (G = 0.13). The different phylogenetic criteria (BI, ML and MP) produced similar and well-supported reconstructions for most of the nodes (Fig. 7). All phylogenetic reconstructions pointed to the distinction of three main lineages very differentiated and structured and highly supported. First, a small lineage made up by samples from Greece, Serbia, Montenegro, The Netherlands and North Iberia. Secondly, a lineage widely distributed in Italy but also in the Iberian Peninsula and in Central Europe, which also shows internal structure with subgroups in North and Central Italy, France, Switzerland and Iberia. In these two lineages, the Cyt *b* fragment showed respectively higher genetic variability within the Italian and Balkan peninsulas than within the rest of their geographic areas outside the peninsulas (Table 3), supporting the consideration of these lineages as 'Italian' and 'Balkan' hereafter (respectively green and red colours in Fig. 4). Finally, a third well supported lineage was restricted to the Iberian Peninsula and it is hereafter named as 'Iberian' (yellow colour in Fig. 7). This lineage is spread across Iberia and is highly structured, being subdivided in further lineages with relative geographic meaning. The longest K2P genetic distance (Fig. 7) was found between the Balkan lineage and Iberian lineage (2.38%), while the shortest distance was found between the Balkan and Italian lineages (1.83%). The highest value of K2P distance found within Iberian lineages (0.93%) reflects the deep structure detected by phylogenetic approaches.

For the DLoop markers, the partition-homogeneity test indicated congruence among the genetic information of the two *HVI* and *HVII* fragments (P=0.03) allowing the concatenation of these two fragments in the following analyses. Still, the Bayesian-based reconstructions of the concatenated *HVI* and *HVII* fragments were obtained by partitioning the data set by fragment allowing independent estimates of parameters. The Bayesian topologies were again obtained after five simultaneous Markov chains run for 2 million generations; trees were sampled every 300 generations. The resulting burn-in values were determined empirically after tree likelihood scores reached stationary values. The analyses were repeated in two separate runs to ensure that trees converged on the same topology and similar parameters. These phylogenetic reconstructions were carried out for each of the three main lineages depicted by the Cyt *b* reconstructions and showed high structure (Fig. 8; Fig. 9; Fig. 10).

In the Balkan lineages, a first split separated the Greek sample from those from Serbia and Montenegro whereas the samples from the Netherlands and Iberian Peninsula constituted separate subgroups (Fig. 8). The Iberian lineage was the best supported and most structured one (Fig. 9). This clade split in two principal groups: one from South Spain, made up of samples from Málaga, Huelva, Granada, Seville and Córdoba, and one widespread across the Peninsula with subgroups located in Portugal and in Central and Northern Spain. Finally, the Italian lineage showed supported subgroups from Central Italy, France and Spain; a unique haplotype from Navarra resulted detached from all others.

The main lineages and haplogroups resulting from the phylogenetic reconstructions were also clearly supported by the median-joining networks (Fig. 11; Fig. 12; Fig. 13). In the Balkan lineage, the Greek haplotype remained highly differentiated from the rest of haplotypes both in the network (Fig. 11) as well as in the phylogenetic reconstructions and connected in the network the Eastern haplotypes with the Iberian group. In the Iberian network, haplotypes from the same or nearby regions tended to be more similar than those from more geographically distant populations (Fig. 12). The network for the Italian lineage (Fig. 13) revealed a star-like topology with the haplotype in the centre of the star being the most abundant and found from Vizcaya, Lugo, Cantabria, León (Spain) to Cuneo (North Italy). The haplotype from Navarra stuck out again within the network of the Italian lineage and remained isolated but linked with the main groups (Fig. 13).

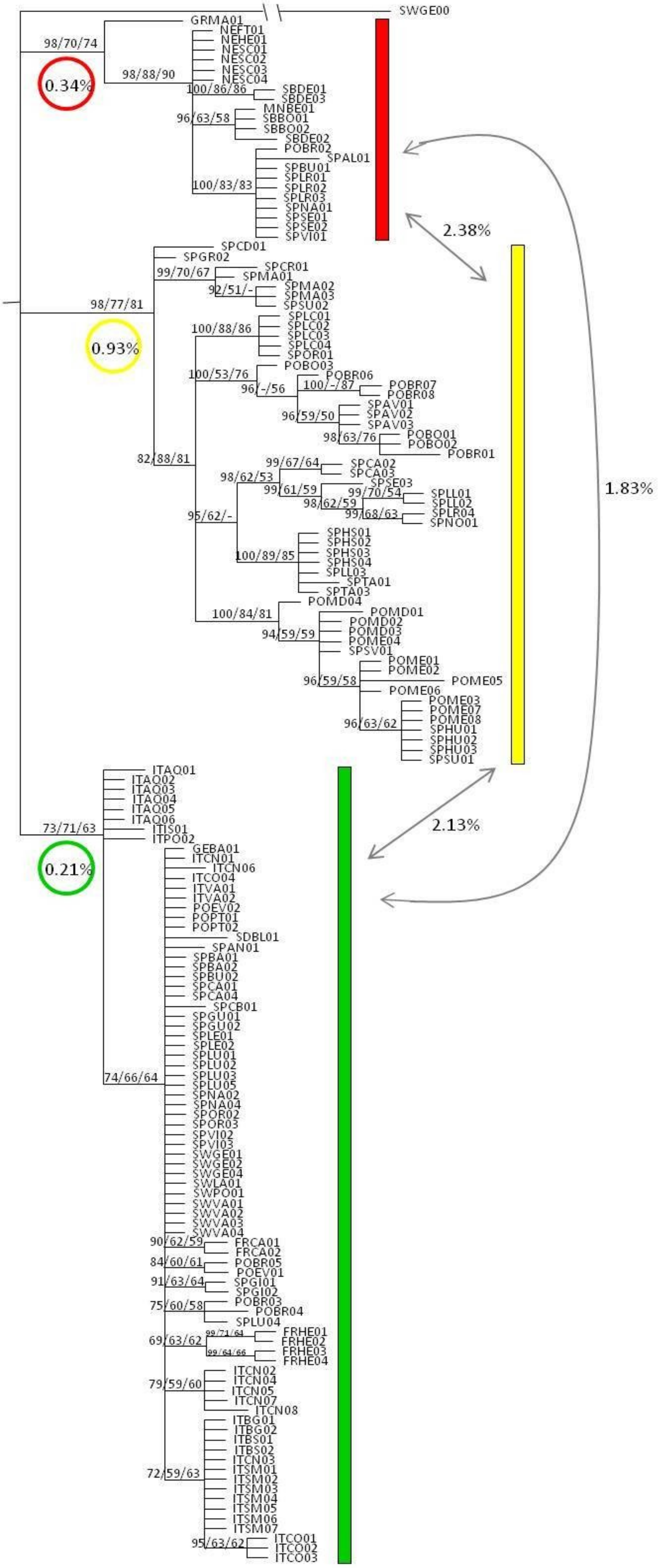

Figure 7. Phylogenetic reconstruction of the *Myotis daubentonii* samples from different regions of Europe and based on a Cyt *b* gene fragment and under the Bayesian criterion. Numbers on branches indicate, respectively, posterior probability and bootstrap supports for maximum likelihood, minimum evolution and maximum parsimony for that particular node. The name of each haplotype is the combination of the abbreviations of the countries and districts in which it is present: France (FR), Germany (GE), Greece (GR), Italy (IT), Montenegro (MN), Netherlands (NE), Portugal (PO), Serbia (SB), Spain (SP), Sweden (SD), Switzerland (SW). The K2P corrected genetic distances within (in circles) and between (with the arrows) lineages are also indicated. Colours correspond to those in Fig. 4.

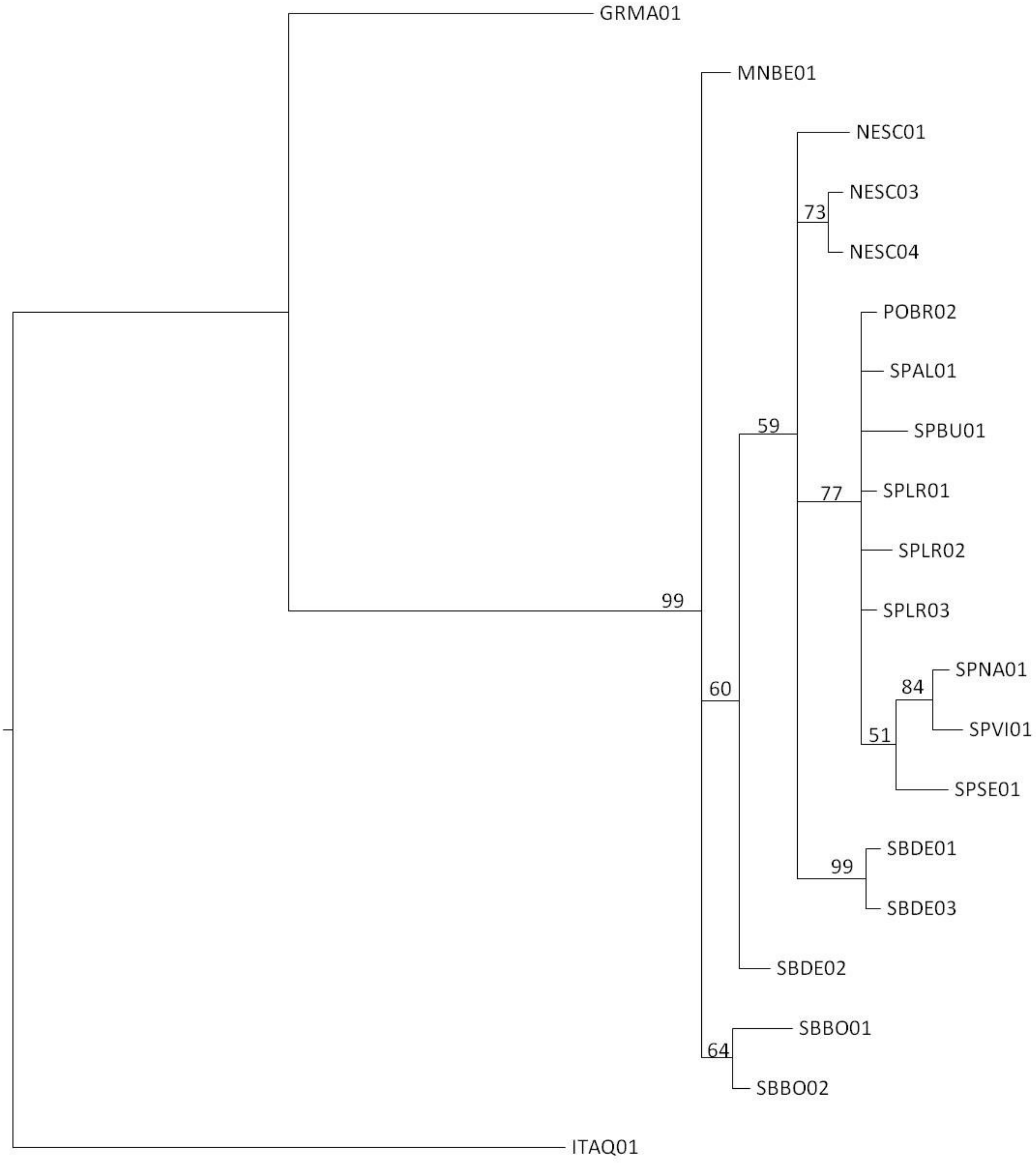

Figure 8. Phylogenetic reconstructions within the *Myotis daubentonii* Balkan lineage obtained by mtDNA fragments of the hypervariable domains *I* and *II* under a Bayesian criterion. Numbers on branches indicate posterior probabilities. The name of each haplotype is the combination of the abbreviations of the countries and districts in which it is present: Greece (GR), Italy (IT), Montenegro (MN), Netherlands (NE), Portugal (PO), Serbia (SB), Spain (SP).

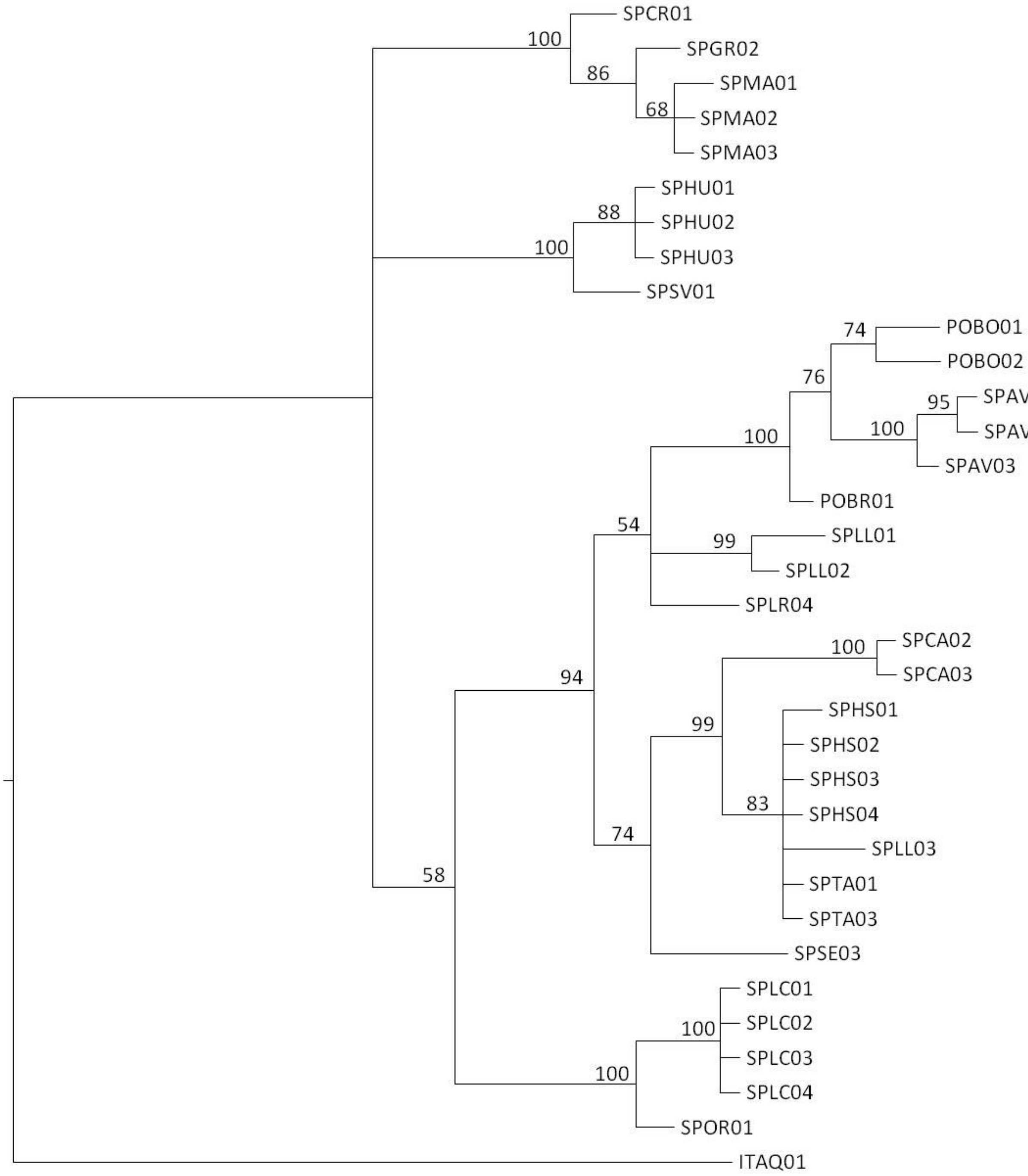

Figure 9. Phylogenetic reconstructions within the *Myotis daubentonii* Iberian lineage obtained by mtDNA fragments of the hypervariable domains *I* and *II* under a Bayesian criterion. Numbers on branches indicate posterior probabilities. The name of each haplotype is the combination of the abbreviations of the countries and districts in which it is present: Italy (IT), Portugal (PO), Spain (SP).

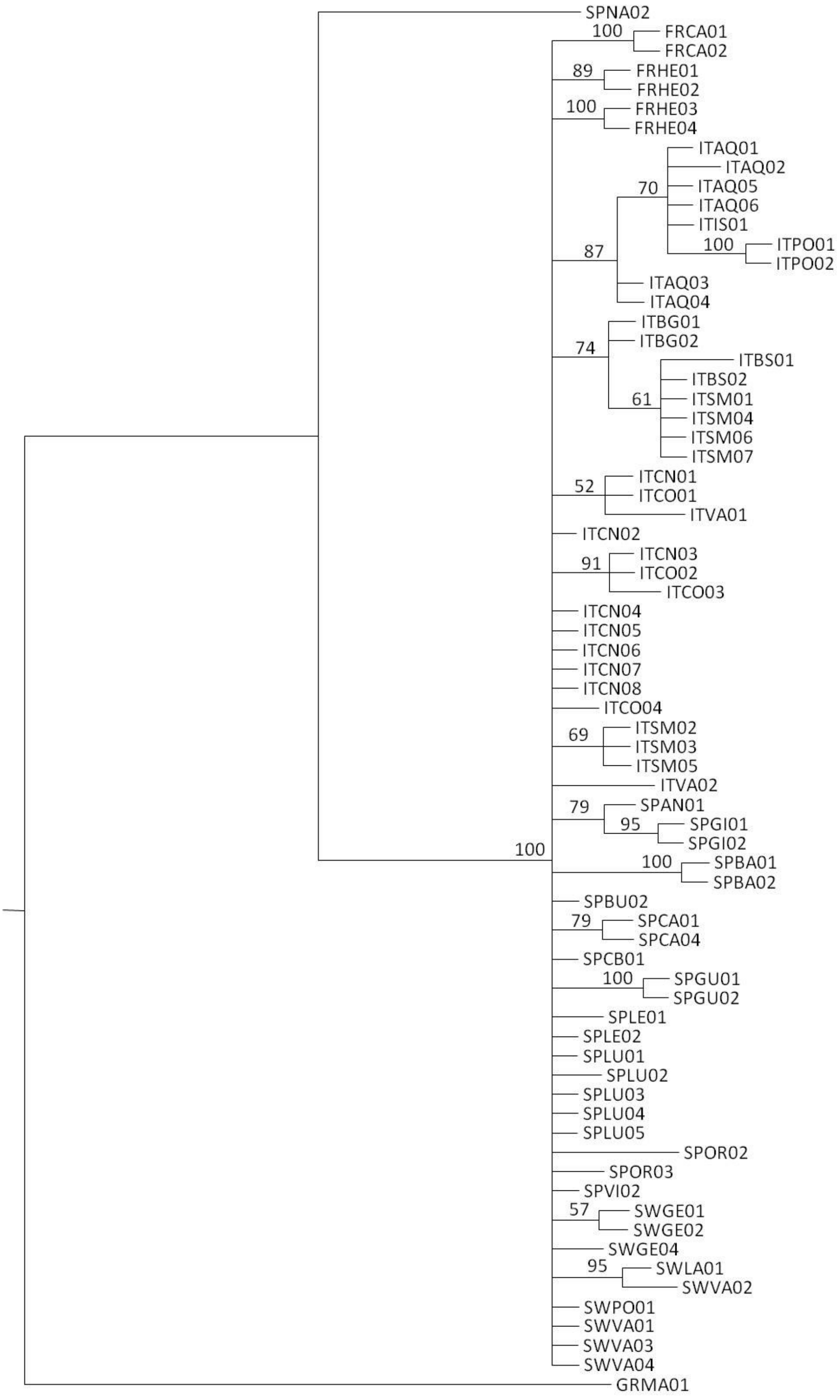

Figure 10. Phylogenetic reconstructions within the *Myotis daubentonii* Italian lineage obtained by mtDNA fragments of the hypervariable domains *I* and *II* under Bayesian criterion. Numbers on branches indicate posterior probabilities. The name of each haplotype is the combination of the abbreviations of the countries and districts in which it is present: France (FR), Germany (GE), Greece (GR), Italy (IT), Portugal (PO), Spain (SP), Sweden (SD), Switzerland (SW).

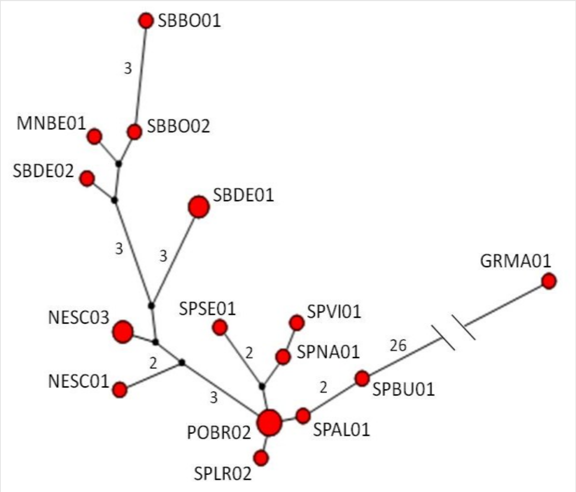

Figure 11. Parsimony haplotype network for the Balkan lineage of *Myotis daubentonii* in Europe. Circles represent haplotypes, with size proportional to its frequency. Solid lines represent inferred connections and black dots missing or un-sampled haplotypes. Numbers near a branch indicate the number of mutations (when more than one are present). The colours of the circles are the same as in Fig. 4.

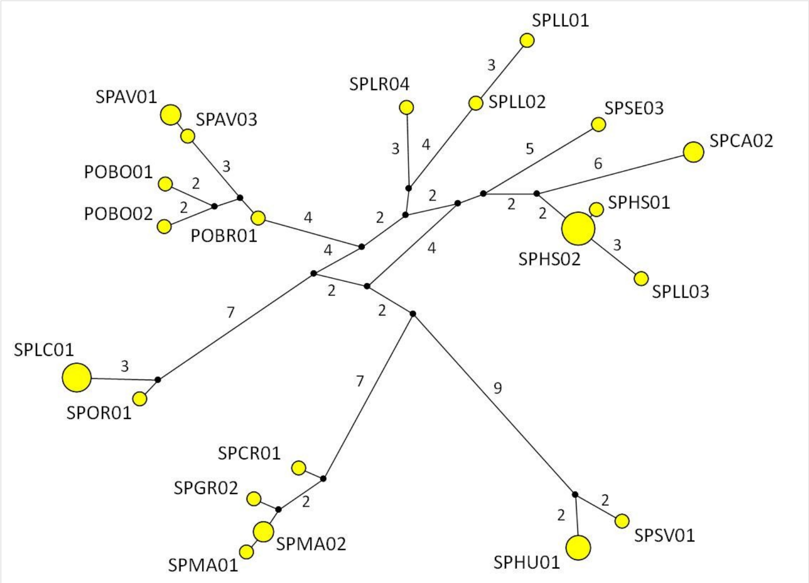

Figure 12. Parsimony haplotype network for Iberian lineage of *Myotis daubentonii* in Europe. Circles represent haplotypes, with size proportional to its frequency. Solid lines represent inferred connections and black dots missing or un-sampled haplotypes. Numbers near a branch indicate the number of mutations (when more than one are present). The colours of the circles are the same as in Fig. 4.

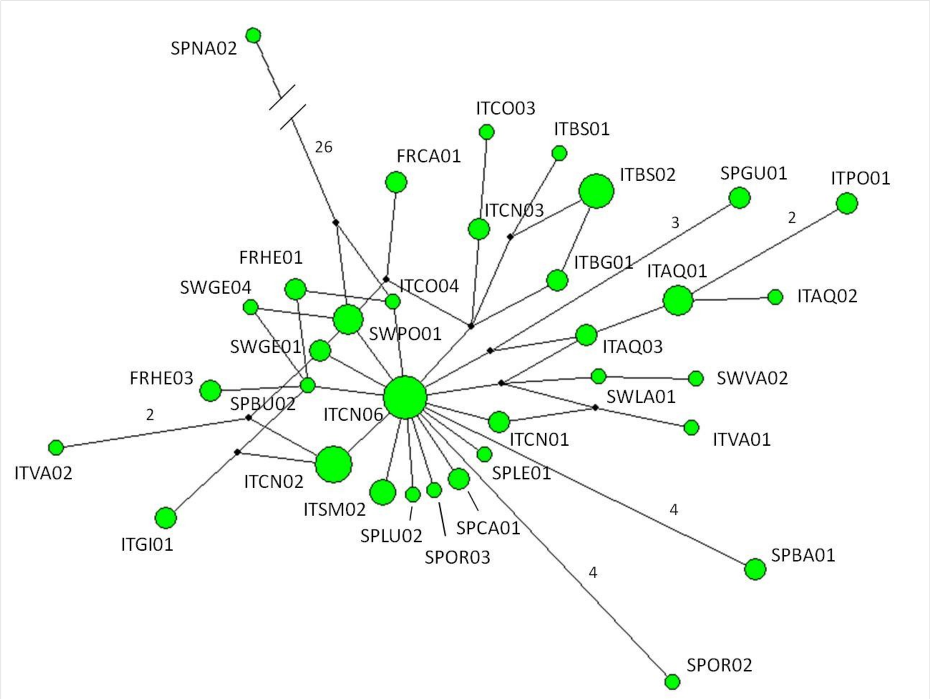

Figure 13. Parsimony haplotype network for Italian lineage of *Myotis daubentonii* in Europe. Circles represent haplotypes, with size proportional to its frequency. Solid lines represent inferred connections and black dots missing or un-sampled haplotypes. Numbers near a branch indicate the number of mutations (when more than one are present). The colours of the circles are the same as in Fig. 4

Genetic Variation and Demographic History within Lineages

The values of the indices of genetic diversity and polymorphism were high not only for the total *M. daubentonii* data set but also for the combined *HVI* and *HVII* fragments and for the three main lineages (Table 4). The estimated demographic indices (*Fs*, R_2, *D*) were all non-significant with values other than 0 and mismatching distribution multimodal for the Iberian lineage, indicating that there has been no expansion (Table 5) for this lineage. On the contrary, possible past events of demographic expansions were supported by the genetic variation pattern for the Balkan lineage, showing a negative and statistically significant *Fs* index ($Fs = -4.121$; $P < 0.05$) and a significant *r* value of mismatching distribution ($r = 0.013$; $P < 0.05$; Table 5; Fig. 14). This distribution appeared smooth and unimodal, as expected in cases of rapid population expansion, for the Italian lineage (Fig. 14), for which, although the *r* statistic was not statistically significant, neutrality tests supported demographic expansion events in the recent past (Table 5).

Table 3. Genetic variability for the cytocrome *b* for the main European groups of *Myotis daubentonii*: sample sizes (*n*), number of haplotypes (*h*), number of mutations (*η*), number of segregating sites (*S*), haplotype diversity (*Hd*), nucleotide diversity (*π*).

Lineages	*n*	*h*	*η*	*S*	*Hd*	*π*
Balkan	7	4	10	10	0.810 ± 0.130	0.004 ± 0.001
Balkan (European)	16	3	4	4	0.575 ± 0.080	0.001 ± 0.0003
Iberian	33	16	29	29	0.934 ± 0.023	0.008 ± 0.0005
Italian	33	10	10	10	0.828 ± 0.048	0.002 ± 0.0003
Italian (Iberian)	31	7	6	6	0.497 ± 0.108	0.0008 ± 0.0002
Italian (European)	17	5	6	6	0.647 ± 0.119	0.001 ± 0.0004
Total	157	51	63	63	0.931 ± 0.014	0.014 ± 0.0005

Table 4. Genetic variability for the hypervariable domains *I* and *II* for the three main European lineages of *Myotis daubentonii*: sample sizes (*n*), number of haplotypes (*h*), number of mutations (*η*), number of segregating sites (*S*), haplotype diversity (*Hd*), nucleotide diversity (*π*).

Lineages	*n*	*h*	*η*	*S*	*Hd*	*π*
Balkan	19	15	42	42	0.971 ± 0.027	0.012 ± 0.003
Iberian	33	21	63	62	0.958 ± 0.019	0.024 ± 0.001
Italian	71	33	41	39	0.963 ± 0.009	0.005 ± 0.0006
Total	123	67	105	101	0.983 ± 0.004	0.032 ± 0.001

Table 5. Demographic indices for the three main lineages of *Myotis daubentonii* in Europe: Fu's (F_S), R_2, Tajima's (*D*) neutrality indices with their respective *P* value, raggedness (*r*) and tau (*τ*). Asterisks (*) indicate significance at the 95% confidence interval.

Lineages	F_s (*P*)	R_2 (*P*)	*D* (*P*)	*r* (*P*)	*τ*
Balkan	-4.121 (0.037)*	0.121 (0.387)	-1.220 (0.098)	0.013 (0.025)*	1.504
Iberian	-1.308 (0.328)	0.128 (0.681)	0.253 (0.670)	0.028 (0.755)	11.122
Italian	-24.991 (0.000)*	0.051 (0.054)	-1.899 (0.011)*	0.029 (0.138)	1.430

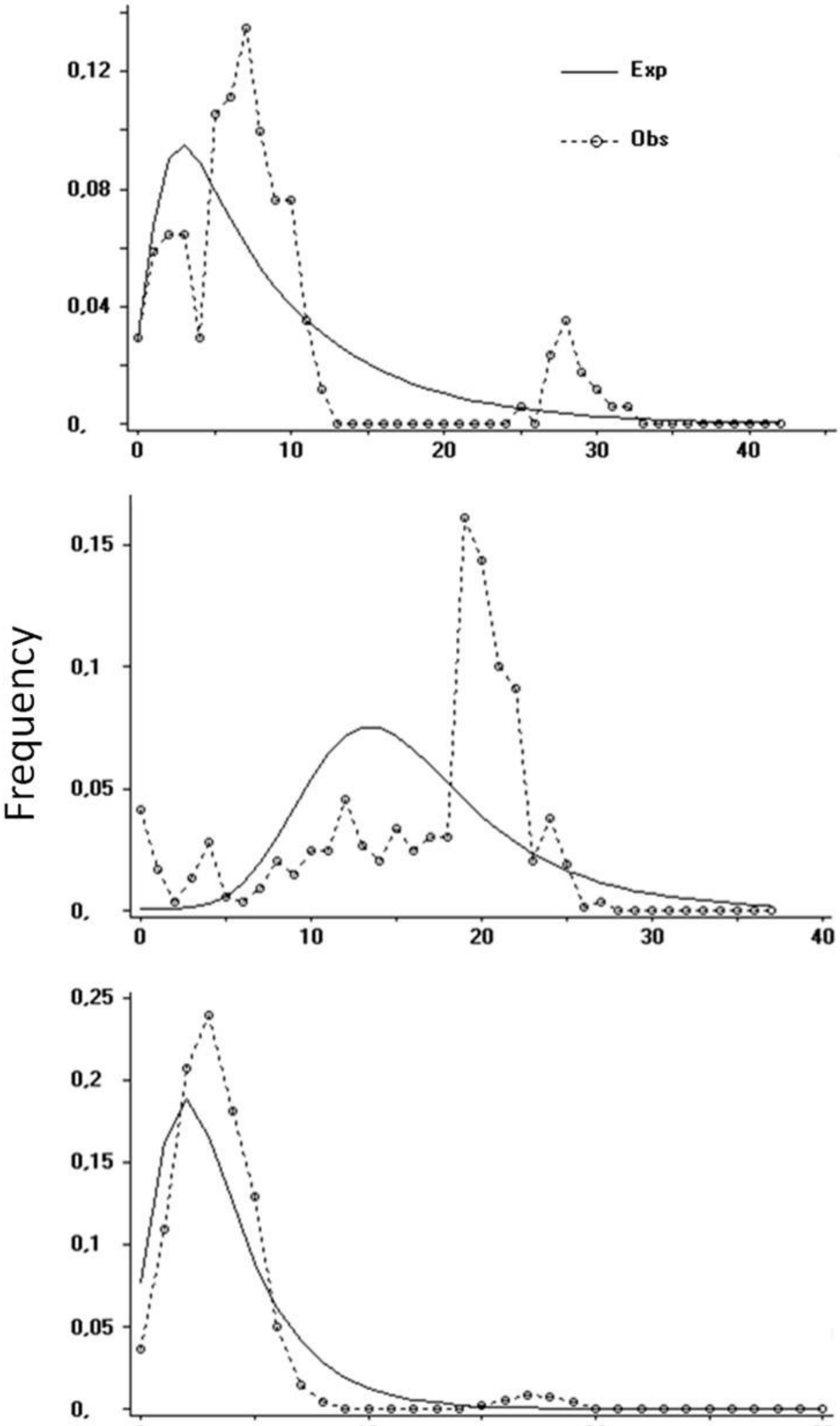

Figure 14. Mismatching Distributions of the three main lineages of *Myotis daubentonii* in Europe: Balkan (above), Iberian (centre) and Italian (below). The dashed line represents the observed distribution, while the solid line represents the expected distribution in the case of demographic expansion of the population.

3.4 Discussion

This study shows a broad genetic differentiation and geographical structure within *M. daubentonii* in Europe. So far, the function and selection of the repeats in the mitochondrial D-loop of vesperilionine bats are unclear (Wilkinson and Chapman, 1991; Wilkinson et al., 1997). Wilkinson (1997) suggested that control region tandem repeats may provide signal redundancy and a primitive repair mechanism in the event of somatic mutations to two conserved protein-binding sequences in mtDNA. I found repeated fragments before R1 repeats (81 bp; Wilkinson et al., 1997) of *HVI* and *HVII* of D-loop in *M. daubentonii* sequences, with great variability and similarities corresponding to the geographical origin: samples from Central Italy had a greater number of repeated fragments than Iberian samples.

I found three distinct mitochondrial lineages across much of European species range (Fig. 7), one of which restricted to the Iberian Peninsula, while the others widespread across the study area. These lineages are consistent with the results obtained in previous studies (Mayer and von Helversen, 2001; Simões et al., 2007; Ngamprasertwong et al, 2008). Mayer and von Helversen (2001) highlighted four distinct clades using *ND1* mtDNA sequences within *M. daubentonii* throughout its European distribution, one of which was exclusively located in Spain. The Italian and Iberian lineages carried out in the present study correspond to the two distinct clades identified by Simões et al. (2007) as *M. d. daubentonii* and *M. d. nathalinae*; phylogenetic reconstructions shared some Cyt *b* sequences (Appendix 1) and the sequence divergence (2.5-3%) between the two groups is similar to the genetic distance (2.13%) resulted from my analysis. Although samples from the UK were missing in the present study, a previous research on *M. daubentonii* in Scotland (Ngamprasertwong et al, 2008) showed that the clade B emerged from phylogenetic analysis was equivalent to the *M. d. daubentonii* clade in the study of Simões et al. (2007). Therefore, the Italian lineage is probably also present in Scotland. The three distinct lineage distributions seem to overlap only in the North of the Iberian Peninsula (Fig. 4): this may be due to a small sample size or lack of samples for some geographical areas. For example, a previous research found three distinct clades within *M. daubentonii* in Germany (Mayer and von Helversen, 2001), while in the present study only a single sample from Germany was available, which was included in the Italian lineage.

Phylogeographical studies showed that, during the Pleistocene, the main glacial refugia were located in Italy, Iberia and the Balkans, which were the source populations of most European taxa in temperate zones (Taberlet et al., 1998; Hewitt, 1999). Three main routes of postglacial expansion were identified (Hewitt, 1999) and it was found that the relative importance of each glacial refugia has not been the same for all species (Taberlet et al., 1998). The rapid postglacial recolonization to the north of the species produced a latitudinal gradient in genetic diversity, characterized by southern richness and northern paucity or even a loss of diversity in populations sink due to founder effects (Hewitt 1996). In this study, genetic diversity found within the Iberian lineage and in the samples from the Italian and Balkan Peninsulas was greater than that of samples belonging to the same clades but originating from areas outside the peninsulas (Table 3): this suggests that for *M. daubentonii* the three peninsulas acted as major glacial refugia during the Pleistocene. In addition, the Fu's neutrality test (*Fs*) and the raggedness (*r*) indicated that the Balkan lineage experienced a rapid population expansion after the LGM, although the observed distribution in pair-wise differences was not fully unimodal (Fig. 14), probably because of the small sample size. While the mismatching distribution observed for the Italian lineage (Fig. 14) clearly showed that a past expansion event occurred, this was also confirmed by the star-like topology of the parsimony haplotype network (Fig. 13). The rapid expansion of the Italian lineage took off from the haplotype located in the north of Italy (Fig. 13; Appendix 1). On the contrary, the results obtained for the Iberian lineage and multimodal mismatching distribution (Table 5; Fig. 14) suggested constant growth process having a within broad diversity and deep structuring (Table 4; Fig. 9). Bayesian inference and parsimony network evidenced in the Iberian lineage that southern populations are quite distinct from those of the rest of the Peninsula and haplotypes from the same or nearby regions tend to be more similar than those from more geographically distant populations (Fig. 9; Fig. 12). Intermediate haplotype appeared absent within Iberian clade, as revealed in the parsimony network (Fig. 12) with no haplotype shared by subgroups, suggesting a possible pattern of *refugia-within-refugia* in Iberia as a consequence of the climatic cycles from the Pleistocene. During the postglacial recolonization of Central and Northern Europe, the mountain ranges (e.g. Alps, Pyrenees) presented significant barriers for many taxa (Taberlet et al., 1998; Schmitt, 2007). For *M. daubentonii* no barrier seems to occurred preventing expansions of the Italian and Balkan lineages, but no individual included into the Iberian lineage occurred outside the Iberian Peninsula. The Pyrenees may has been a geographic barrier for *M. d. nathalinae*, as already demonstrated for other species (Taberlet et al., 1998; Schmitt, 2007).

In summary, this study highlights the existence of a large genetic variability within *M. daubentonii* across its European range with clear differentiation into three main lineages. A highly structured lineage spread throughout the Iberian Peninsula – that, identified as *M. d. nathalinae* in previous research (Mayer and von Helversen, 2001; Simões et al., 2007). A lineage was found in Italy, France, Switzerland, Germany, Sweden and in the Central and Northern Iberia. A final lineage consisted of samples from Serbia, Montenegro, Greece, Netherlands and from the North of Spain and Portugal. The polymorphism analysis demonstrated that the Mediterranean Peninsulas (Italy, Iberia, Balkan) acted as glacial refugia for *M. daubentonii* and its European populations have originated from the postglacial Palaearctic expansions of the Italian and Balkan lineages, while the Iberian lineage did not cross the Pyrenees. However, strong conclusions about the postglacial expansion routes followed by *M. daubentonii* across Europe will require further investigations.

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3.6 Appendix

Appendix 1. List of taxa, localities, geographical coordinates and GenBank accession numbers (Cyt *b*, *HVI*, *HVII*) of the sequences of the samples used for the study. Countries abbreviations in ID paper: France (FR), Germany (GE), Greece (GR), Italy (IT), Montenegro (MN), Netherlands (NE), Portugal (PO), Serbia (SB), Spain (SP), Sweden (SD), Switzerland (SW).

ID	ID Paper	Province/district	Latitude	Longitude	GenBank Accession N°			References
					Cyt *b*	*HVI*	*HVII*	
YT1_Mda	FRCA01	St-Sauveur	42.87	-0.01				This paper
YT2_Mda	FRCA02	St-Sauveur	42.87	-0.01				This paper
20131007_13	FRHE01	Hérault	43,68	4,09				This paper
20131007_14	FRHE02	Hérault	43,68	4,09				This paper
20131007_15	FRHE03	Hérault	43,68	4,09				This paper
20131007_16	FRHE04	Hérault	43,68	4,09				This paper
AF376847	GEBA01	Bavaria	48.76	11.47	AF376847	-	-	Ruedi and Mayer, 2001
1807Mda45	GRMA01	Macedonia	40.67	23.16				This paper
MYDA06_12	ITAQ01	L'Aquila	41.78	13.83		KJ607010	KJ607073	This paper
MYDA07_12	ITAQ02	L'Aquila	41.78	13.83		KJ607011	KJ607073	This paper
MYDA13_12	ITAQ03	L'Aquila	41.77	14.03		-	KJ607074	This paper
MYDA14_12	ITAQ04	L'Aquila	41.77	14.03		KJ607013	-	This paper
MYDA46_12	ITAQ05	L'Aquila	41.77	14.09		KJ607014	KJ607075	This paper
MYDA50_12	ITAQ06	L'Aquila	41.77	14.09		KJ607012	KJ607076	This paper
4427	ITBG01	Bergamo	45.75	9.99				This paper
4428	ITBG02	Bergamo	45.75	9.99				This paper
4431	ITBS01	Brescia	45.58	10.35				This paper
4432	ITBS02	Brescia	45.58	10.35				This paper
TPP14_801	ITCN01	Cuneo	44.39	7.82				This paper
TPP14_802	ITCN02	Cuneo	44.39	7.82				This paper
TPP14_803	ITCN03	Cuneo	44.39	7.82				This paper
TPP14_804	ITCN04	Cuneo	44.39	7.82				This paper
TPP14_805	ITCN05	Cuneo	44.39	7.82				This paper
TPP14_806	ITCN06	Cuneo	44.39	7.82				This paper
TPP14_807	ITCN07	Cuneo	44.39	7.82				This paper
TPP14_808	ITCN08	Cuneo	44.39	7.82				This paper
5549	ITCO01	Como	45.08	9.23				This paper
5552	ITCO02	Como	45.08	9.23				This paper
5554	ITCO03	Como	45.83	9.20				This paper
5555	ITCO04	Como	45.83	9.20				This paper
MYDA66_12	ITIS01	Isernia	41.74	14.20		KJ607015	KJ607077	This paper
080818Mda1	ITPO01	Potenza	40.02	16.13	-	KJ607016	KJ607078	This paper
080818Mda2	ITPO02	Potenza	40.02	16.13		KJ607017	KJ607079	This paper

SM14_701	ITSM01	San Marino	43.95	12.40				This paper
SM14_702	ITSM02	San Marino	43.95	12.40				This paper
SM14_703	ITSM03	San Marino	43.95	12.40				This paper
SM14_704	ITSM04	San Marino	43.95	12.40				This paper
SM14_705	ITSM05	San Marino	43.95	12.40				This paper
SM14_706	ITSM06	San Marino	43.95	12.40				This paper
SM14_707	ITSM07	San Marino	43.95	12.40				This paper
4993	ITVA01	Varese	45.68	8.68				This paper
4994	ITVA02	Varese	45.68	8.68				This paper
733	MNBE01	Berane	42.83	19.90				This paper
Bat2012JB11	NEFT01	Fort Giesen	52.24	6.18		-	-	This paper
Bat2012JB98	NEHE01	Heerhugowaard	52.65	4.84		-	-	This paper
MD140318.1	NESC01	Schaarsbergen	52.03	5.86				This paper
MD140318.2	NESC02	Schaarsbergen	52.03	5.86		-	-	This paper
MD140318.4	NESC03	Schaarsbergen	52.03	5.86				This paper
MD140318.5	NESC04	Schaarsbergen	52.03	5.86				This paper
090726Mda1	POBO01	Boticas	41.68	-7.69		KJ607018	KJ607080	This paper
090726Mda3	POBO02	Boticas	41.68	-7.69				This paper
EU153111	POBO03	Boticas	41.84	-8.23	EU153111	-	-	Simões et al., 2007
100831Mda1	POBR01	Bragança	41.90	-6.73		KJ607019	KJ607081	This paper
100831Mda2	POBR02	Bragança	41.90	-6.73		KJ607020	KJ607082	This paper
EU153103	POBR03	Bragança	41.89	-6.85	EU153103	-	-	Simões et al., 2007
EU153104	POBR04	Bragança	41.89	-6.85	EU153104	-	-	Simões et al., 2007
EU153107	POBR05	Bragança	41.89	-6.85	EU153107	-	-	Simões et al., 2007
EU153108	POBR06	Bragança	41.89	-6.85	EU153108	-	-	Simões et al., 2007
EU153109	POBR07	Bragança	41.89	-6.85	EU153109	-	-	Simões et al., 2007
EU153110	POBR08	Bragança	41.89	-6.85	EU153110	-	-	Simões et al., 2007
EU153105	POEV01	Évora	38.58	-8.08	EU153105	-	-	Simões et al., 2007
EU153106	POEV02	Évora	38.58	-8.08	EU153106	-	-	Simões et al., 2007
EU153120	POMD01	Mogadouro	41.26	-6.64	EU153120	-	-	Simões et al., 2007
EU153121	POMD02	Mogadouro	41.26	-6.64	EU153121	-	-	Simões et al., 2007
EU153122	POMD03	Mogadouro	41.26	-6.64	EU153122	-	-	Simões et al., 2007
EU153123	POMD04	Mogadouro	41.26	-6.64	EU153123	-	-	Simões et al., 2007
EU153112	POME01	Mértola	37.68	-7.65	EU153112	-	-	Simões et al., 2007
EU153113	POME02	Mértola	37.68	-7.65	EU153113	-	-	Simões et al., 2007
EU153114	POME03	Mértola	37.68	-7.65	EU153114	-	-	Simões et al., 2007
EU153115	POME04	Mértola	37.68	-7.65	EU153115	-	-	Simões et al., 2007
EU153116	POME05	Mértola	37.68	-7.65	EU153116	-	-	Simões et al., 2007

EU153117	POME06	Mértola	37.68	-7.65	EU153117	-	-	Simões et al., 2007
EU153118	POME07	Mértola	37.68	-7.65	EU153118	-	-	Simões et al., 2007
EU153119	POME08	Mértola	37.68	-7.65	EU153119	-	-	Simões et al., 2007
EU153124	POPT01	Portalegre	39.30	-7.41	EU153124	-	-	Simões et al., 2007
EU153125	POPT02	Portalegre	39.30	-7.41	EU153125	-	-	Simões et al., 2007
620	SBBO01	Bor	44.02	21.96				This paper
621	SBBO02	Bor	44.02	21.96				This paper
24	SBDE01	Degurić	44.23	19.88				This paper
59	SBDE02	Degurić	44.23	19.88				This paper
191	SBDE03	Degurić	44.24	19.93				This paper
EU153102	SDBL01	Blentarp	55.58	13.59	EU153102	-	-	Simões et al., 2007
090610Mda1	SPAL01	Álava	42.97	-2.73		KJ607021	KJ607083	This paper
090629Mda1b	SPAN01	Andorra	42.56	1.60		KJ607022	KJ607084	This paper
080825Mda1	SPAV01	Ávila	40.28	-5.26		KJ607023	KJ607085	This paper
080825Mda7	SPAV02	Ávila	40.28	-5.26		KJ607024	KJ607086	This paper
080712Mda3	SPAV03	Ávila	40.28	-5.51		KJ607025	KJ607087	This paper
080814Mda	SPBA01	Baleares	39.97	4.24		KJ607026	KJ607088	This paper
080815Mda4	SPBA02	Baleares	39.97	4.24				This paper
080722Mda1	SPBU01	Burgos	42.66	-2.50				This paper
080722Mda2	SPBU02	Burgos	42.66	-2.50		KJ607027	KJ607089	This paper
060705Mda3	SPCA01	Cáceres	40.37	-6.08		KJ607028	KJ607090	This paper
060919Mda	SPCA02	Cáceres	40.34	-5.29		KJ607029	KJ607091	This paper
060926Mda1	SPCA03	Cáceres	40.34	-5.29		KJ607030	KJ607092	This paper
040726Mda2	SPCA04	Cáceres	40.30	-5.51		KJ607031	KJ607093	This paper
090623Mmys1	SPCB01	Cantabria	43.46	-3.55		KJ607032	KJ607094	This paper
AF376862	SPCD01	Ciudad Real	38.98	-3.91	AF376862	-	-	Ruedi and Mayer, 2001
090526Mda1	SPCR01	Córdoba	38.22	-4.27		KJ607033	KJ607095	This paper
080806Mda1	SPGI01	Girona	42.12	2.75				This paper
080703Mda1	SPGI02	Girona	42.27	2.57		KJ607034	KJ607096	This paper
2x19096Mda	SPGR01	Granada	37.79	-2.77	-	KJ607036	KJ607097	This paper
2x19094Mda	SPGR02	Granada	37.79	-2.77		KJ607037	KJ607098	This paper
090814Mda1	SPGU01	Guipúcoa	43.22	-1.91		KJ607038	KJ607099	This paper
090814Mda2	SPGU02	Guipúcoa	43.27	-1.99		KJ607039	KJ607100	This paper
P4Mda	SPHS01	Huesca	42.61	-0.36		KJ607035	KJ607101	This paper
J27Mda	SPHS02	Huesca	42.06	-0.08		KJ607040	KJ607102	This paper
110711Mda1	SPHS03	Huesca	42.58	-0.01		KJ607041	KJ607103	This paper
110825Mmys1	SPHS04	Huesca	42.55	0.08		KJ607042	KJ607104	This paper
020725Mda11	SPHU01	Huelva	37.64	-6.94		KJ607043	KJ607105	This paper
2x18883Mda	SPHU02	Huelva	37.64	-6.94		KJ607044	KJ607106	This paper
2x18874Mda	SPHU03	Huelva	37.64	-6.94		KJ607045	KJ607107	This paper

090423Mda2	SPLC01	La Coruña	43.37	-7.97		KJ607046	KJ607108	This paper
090423Mda3	SPLC02	La Coruña	43.37	-7.97		KJ607047	KJ607109	This paper
080502Mda2	SPLC03	La Coruña	43.58	-8.19				This paper
090825Mda2	SPLC04	La Coruña	43.37	-7.97		KJ607048	KJ607110	This paper
110526Mda1	SPLE01	León	42.83	-6.72				This paper
110526Mda2	SPLE02	León	42.83	-6.72		KJ607049	KJ607111	This paper
080728Mda1	SPLL01	Lieida	42.59	1.13				This paper
080728Mda2	SPLL02	Lieida	42.59	1.13				This paper
080711Mda1	SPLL03	Lieida	41.61	0.64		KJ607050	KJ607112	This paper
2x08727Mda	SPLR01	La Rioja	42.36	-2.69		KJ607051	KJ607113	This paper
2x21566Mda	SPLR02	La Rioja	42.36	-2.69		KJ607052	KJ607114	This paper
060831Mda	SPLR03	La Rioja	42.09	-2.60		KJ607053	KJ607115	This paper
070824Mda1	SPLR04	La Rioja	42.09	-2.60		KJ607054	KJ607116	This paper
2x21545	SPLR06	La Rioja	42.34	-3.00	-	KJ607055	KJ607117	This paper
070617Mda1	SPLU01	Lugo	42.59	-7.19		KJ607056	KJ607118	This paper
080625Mda9	SPLU02	Lugo	42.86	-7.32				This paper
080712Mda1a	SPLU03	Lugo	43.22	-7.29		KJ607057	KJ607119	This paper
070615Mda2	SPLU04	Lugo	42.60	-7.23		KJ607058	KJ607120	This paper
100828Mda1g	SPLU05	Lugo	42.86	-7.34		KJ607059	KJ607121	This paper
030713Mda1	SPMA01	Málaga	36.90	-4.11		KJ607060	KJ607122	This paper
070601Msp1	SPMA02	Málaga	36.71	-5.24		KJ607061	KJ607123	This paper
2x19127Mda	SPMA03	Málaga	36.61	-5.34		KJ607062	KJ607124	This paper
090624Mda1	SPNA01	Navarra	42.91	-2.04				This paper
102Mdau1	SPNA02	Navarra	42.98	-1.62		KJ607063	KJ607125	This paper
080920Mda1	SPNA04	Navarra	42.98	-1.62		-	-	This paper
DQ120896	SPNO01	Northern Iberia			DQ120896	-	-	Ibañez et al., 2006
100804Mda1g	SPOR01	Orense	42.35	-7.90		KJ607064	KJ607126	This paper
100728Mda2	SPOR02	Orense	41.89	-8.15				This paper
100728Mda3	SPOR03	Orense	41.89	-8.15		KJ607065	KJ607127	This paper
2x24114Mda	SPSE01	Segovia	40.89	-4.00				This paper
2x24120Mda	SPSE02	Segovia	40.89	-4.00		-	-	This paper
2x24138Mda	SPSE03	Segovia	40.89	-4.00		KJ607066	KJ607128	This paper
DQ120897	SPSU01	Southern Iberia			DQ120897	-	-	Ibañez et al., 2006
DQ120898	SPSU02	Southern Iberia			DQ120898	-	-	Ibañez et al., 2006
100621Mda1	SPSV01	Sevilla	37.92	-5.71		KJ607067	KJ607129	This paper
090618Mda1	SPTA01	Tarragona	41.38	1.08		KJ607068	KJ607130	This paper
090618PMda2	SPTA02	Tarragona	41.38	1.08	-	KJ607069	KJ607131	This paper
080708Mda1	SPTA03	Tarragona	41.28	0.75		KJ607070	KJ607132	This paper
080917Mda7	SPVI01	Vizcaya	43.07	-2.81		KJ607071	KJ607133	This paper
080917Mda3	SPVI02	Vizcaya	43.07	-2.81		KJ607072	KJ607134	This paper
080918Mda2	SPVI03	Vizcaya	43.30	-2.55		-	-	This paper

AF376843	SWGE00	Jura	47.33	7.14	AF376843	-	-	Ruedi and Mayer, 2001
1981Mda2	SWGE01	Genève	46.20	6.16				This paper
1958Mda18	SWGE02	Genève	46.25	6.12				This paper
1967Mda74	SWGE04	Genève	46.25	6.20				This paper
1805Mda58	SWLA01	Lausanne	46.51	6.63				This paper
1981Mda1	SWPO01	Corsier, Port	46.26	6.20				This paper
1808Mda13	SWVA01	Allaman, Vaud	46.47	6.40				This paper
1805Mda55	SWVA02	Dorigny, Vaud	46.51	6.58				This paper
1805Mda56	SWVA03	Ecublens, Vaud	46.52	6.56				This paper
1805Mda59	SWVA04	Préverenges, Vaud	46.51	6.52				This paper

4. CONCLUSIONS

My thesis analyzed the ecological and genetic variability of the riparian habitat specialist *M. daubentonii* respectively at small and large spatio-temporal scales.

The first part of my study offered a comprehensive picture of thermal and foraging ecology of male *M. daubentonii* living at different altitudes along a river basin that, despite its limited slope (ca. 300 m), leads bats to adopt completely different ecophysiological strategies. From the phylogeographic study a large genetic variability emerged within *M. daubentonii* across its European range with clear differentiation into three lineages, which originated from isolated populations in the three main Mediterranean refugia (Italian, Iberian and Balkan Peninsulas) during the Pleistocene Glacial Ages.

The management and conservation of ecosystems is a priority of the new millennium in landscapes which are being increasingly altered by human action. Chiroptera is among the most threatened mammal taxa in the world; for instance all Italian bat species feature in the I, II annexes of 92/43/EEC "Habitat Directive" and at least part of this endangerment is linked with the alteration of wetlands. Efficient management and conservation of *M. daubentonii* should consider the intraspecific behavioural, ecological and genetic variability emerged from my research. On a smaller scale, the population I examined only partially lies in the Abruzzo, Lazio and Molise National Park; nursery roosts and males mostly contributing to reproduction (i.e. to gene flow) are located outside the boundaries of the Park, so not subject to any specific protection. Therefore, priority is to include the buffer zones in the protected areas planning. On a large scale, this cannot overlook the importance of taxonomic subunits clearly defined, such as the Iberian lineage (*M. d. nathalinae*). Overall, the take-home message of my work is the need of taking spatial scales into account to achieve sound conservation planning.

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