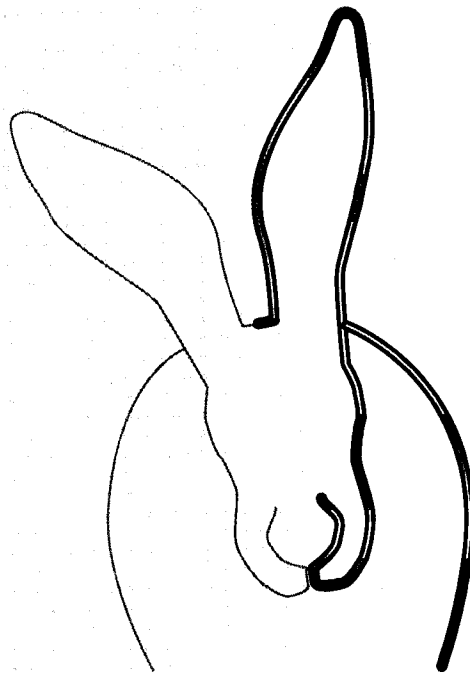


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Natural hybridization between the Iberian hare
(*Lepus granatensis*) and the brown hare (*L. europaeus*)
in northern Iberian Peninsula



Hélder Marques de Freitas

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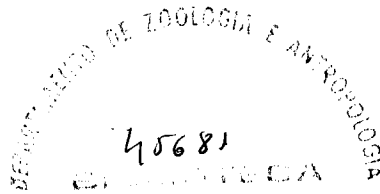
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Natural hybridization between the Iberian hare
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in northern Iberian Peninsula



*Dissertação de Mestrado em Biodiversidade e Recursos Genéticos
apresentada à Faculdade de Ciências da Universidade do Porto*

Hélder Marques de Freitas

Porto
2006

À memória do meu Avô

Many thanks to

I am thankful to several people that contributed in various ways to this work, and I would like to express here my gratitude.

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Resumo

A hibridação interespecífica é um fenómeno há muito conhecido que se mantém no topo do interesse em trabalhos de evolução, constituindo um importante modelo de estudo para a compreensão de fenómenos fundamentais como, por exemplo, a especiação. As oscilações climáticas são reconhecidas como tendo sido um importante catalisador para a ocorrência de zonas de contacto. Na Europa, as regiões localizadas a sul (as Penínsulas Ibérica, Itálica e os Balcãs) funcionaram como reservatórios de biodiversidade durante as épocas menos favoráveis à ocorrência de vida no resto da Europa e, à medida que as condições melhoraram, tornaram-se uma fonte para a recolonização do continente europeu. A colonização para norte a partir destas penínsulas promoveu o contacto entre espécies e, em alguns casos, conduziu à formação de zonas híbridas.

O norte da Península Ibérica é uma área onde estão descritas zonas de contacto secundário para diversos organismos. Entre estes, encontram-se duas espécies de lebre: a lebre ibérica (*Lepus granatensis*), que representa um endemismo da Península Ibérica, e a lebre europeia (*L. europaeus*), que ocupa o nordeste da Península Ibérica, bem como uma grande extensão do restante continente europeu. Embora as rotas de expansão pós-glacial de lebre europeia não sejam bem conhecidas, admite-se que a colonização para Noroeste do continente europeu tenha tido origem nos Balcãs. Assim, a zona de contacto secundário com a lebre ibérica ter-se-á formado recentemente aquando da chegada da lebre europeia à Península Ibérica, encontrando aí a já presente lebre ibérica. Não obstante a existência de dados que comprovam o cruzamento entre lebre variável (*L. timidus*) e lebre europeia, a hibridação entre esta e a lebre ibérica nunca foi demonstrada, apesar da existência de uma extensa zona de contacto entre estas duas espécies no norte da Península Ibérica. Investigar se as duas espécies se cruzam com sucesso foi o principal mote deste trabalho. Para tal, foram escolhidos marcadores nucleares neutros (microsatélites) e o gene mitocondrial do citocromo *b*. Os microsatélites utilizados provaram ser suficientemente informativos para distinguirem as espécies em questão e permitiram a detecção de quatro indivíduos de lebre ibérica com sinais de lebre europeia ao nível do genoma nuclear. O contrário também ocorre, tendo-se encontrado três indivíduos de lebre europeia com introgressão nuclear de lebre

ibérica, embora as provas neste caso sejam menos robustas. Quanto ao DNA mitocondrial, a análise a uma escala fina dum grande número de indivíduos das duas espécies permitiu confirmar a já descrita forte introgressão de DNA mitocondrial de lebre variável nas duas espécies de lebre da Península Ibérica estudadas, e ainda detectar um considerável nível de introgressão mitocondrial de lebre ibérica para lebre europeia ($\approx 33\%$). Em conjunto, os dados dos microssatélites e do DNA mitocondrial demonstraram a existência de hibridação entre lebre ibérica e europeia. Os diferentes padrões de introgressão observados ao nível do DNA nuclear e mitocondrial poderão estar associados a diferenças comportamentais entre as espécies e mesmo entre os sexos, podendo também ser explicados por hipóteses demográficas.

Os padrões de diversidade genética obtidos parecem corroborar o cenário de uma colonização Este-Oeste por parte da lebre europeia, uma vez que é detectada uma diminuição na diversidade genética das populações nessa direcção. Quanto à lebre ibérica, os baixos valores de *Fst* observados parecem suportar a hipótese da ocorrência de um fluxo génico elevado entre as populações, num cenário de panmixia. As populações de lebre ibérica poderão ter-se mantido estáveis ao longo do tempo, sem sofrerem reduções demográficas severas nem regressões para sul durante os períodos glaciais.

Quer a lebre ibérica, quer a lebre europeia, são muito adaptáveis às condições do habitat mas existem diferenças nos seus requisitos ecológicos que são confirmadas pelos dados obtidos. O uso integrado de dados genéticos e ecológicos permitiu verificar que a lebre europeia demonstra uma preferência por áreas relativamente mais húmidas e frias em comparação com a lebre ibérica. No ecótono estudado, uma clara transição climatológica define os limites de distribuição de ambas as espécies e é acompanhada pelos dados do genoma nuclear. Este resultado poderá indicar um papel selectivo do habitat na distribuição das espécies, o que torna este sistema muito interessante para uma futura modelação recorrendo a Sistemas de Informação Geográfica. Por outro lado, o padrão distinto encontrado para o DNA mitocondrial deverá ser estudado em maior detalhe, nomeadamente a hipótese de selecção pelo frio para a mitocôndria da lebre variável.

Este trabalho apresenta dados novos no que diz respeito à lebre ibérica e à lebre europeia, quer no que diz respeito à sua interacção, quer no que diz respeito à sua relação com o habitat. A mistura de genoma nuclear e uma nova introgressão mitocondrial em lebre europeia (no norte da Península Ibérica, a lebre europeia apresenta o seu DNA mitocondrial nativo e ainda duas linhagens introgridas), detectadas neste trabalho, poderão servir de base para estudos mais detalhados e para o desenvolvimento de novas hipóteses de trabalho. O género *Lepus* continua a mostrar ser um desafio para os estudos de evolução, apresentando-se como um excelente modelo de trabalho.

Summary

Interspecific hybridization is a long known phenomenon, and is still a matter of great interest, constituting an important model for the understanding of fundamental phenomena such as speciation. Climate oscillations were an important factor for the occurrence of contact zones. In Europe, southern regions as the Iberian, Italian and Balkans Peninsulas were biodiversity sinks during less favourable periods, and became sources of life when conditions improved, allowing the recolonization of the European continent. The northwards colonization routes promoted species contact and, sometimes, hybrid zones were established.

The north of Iberian Peninsula is an area where contact zones are described for several organisms. Among these, there are two species of hares: the Iberian hare (*Lepus granatensis*), which is an endemism of Iberian Peninsula, and the brown hare (*L. europaeus*), that inhabits the northeastern part of Iberia, as well as the majority of the remaining European territory. Although post-glacial expansion routes of brown hare are not well known, it is thought that the Northwest colonization of the European continent was originated in the Balkans. Thus, the secondary contact with the Iberian hare probably occurred recently, when brown hare arrived to the Iberian Peninsula. Although it is known that the brown hare naturally hybridizes with the mountain hare (*L. timidus*), hybridization between Iberian and brown hare has not been demonstrated, despite the existence of a long contact zone between both species in northern Iberian Peninsula. To investigate if successful interbreeding between Iberian and brown hare occurs was the main aim of this work. For that, neutral nuclear markers (microsatellites) and the mitochondrial gene cytochrome *b* were used. The chosen microsatellites set proved to be informative and to distinguish species in a frequency base, allowing the detection of brown hare alleles introgressed in four Iberian hare individuals. The converse also occurs, with three brown hares displaying Iberian hare nuclear introgression, although the evidences for these are weaker. As for the mitochondrial DNA, a thin scale analysis of large samples of both Iberian and brown hare enabled to confirm the previously described strong introgression from mountain hare haplotypes into the two iberian species of hares studied, and allowed the detection of a considerable amount ($\approx 33\%$) of introgression from Iberian hare into brown hare. Together, microsatellites and

mitochondrial DNA data strongly support the existence of hybridization between the two taxa. The discordance of introgression patterns between nuclear and mitochondrial markers may be explained by behavioural differences between species and between sexes or demographic hypothesis.

From the obtained data of genetic diversity, the scenario of East-West colonization by the brown hare seems plausible, given the decrease in samples genetic diversity along that axis. As for the Iberian hare, low values of pairwise F_{st} point to a high gene flow between samples and the existence of a metapopulation, fitting a panmixia scenario. Iberian hare populations may have been stable throughout time, without severe demographic decreases or population regression to south during glacial periods.

Iberian and brown hare are very adaptable to habitat conditions, but differences in ecological requirements exist and are confirmed by the data obtained. The integration of genetical and ecological data allowed to verify that the brown hare displays a preference for relatively wetter and colder areas in comparison with Iberian hare. In the studied ecotone, a sharp climatological transition marks species distribution limit and is accompanied by the nuclear genome data. This result may indicate a selective role of the habitat in species distribution, which is a very interesting system for future GIS modelling. The distinct pattern found for mitochondrial DNA should also be further investigated, namely the hypothesis of selection for the cold of the mountain hare mitochondria.

This work brings new knowledge about Iberian and brown hare, the interaction between them and the interaction of both species with the habitat. Nuclear genome exchanges and another mitochondrial introgression into brown hare (in northern Iberia, this species displays its native mtDNA and another two introgressed lineages) were found and may constitute the basis for more detailed works with new working hypothesis. The genus *Lepus* continuously proves to be a challenge to evolutionary studies and much may still be learnt with it.

I. General introduction

A long way has gone since all the organisms were seen in the light of creationism, with species regarded as static entities with no evolutionary history and no correlation between them. With the work of geneticists as R.A. Fisher, J.B.S. Haldane and S. Wright, the Synthetic Theory of Evolution was born around 1930, fusing Darwin's Theory of Natural Selection with Mendel's Theory of Heredity. With the establishment of these keystone concepts of evolution, a whole new world of ideas and experiments were based on them. Species were no more simple objects for description, ordering and collection, but were now dynamic units, with common features and possibly shared evolutionary paths. Speciation left the pure taxonomical field and started to integrate ecology, etology, physiology and genetics, for example. Nonetheless, the achieved broadness of knowledge did not prevent the birth of controversies. Species concepts, for instance, have been debated thoroughly (for a review, see Hey *et al.*, 2003) but no unifying concept has been attained. Even so, the Biological Species Concept (BSC; Dobzhansky, 1937; Mayr, 1942) might have passed the test of time (Ridley, 1996) and is upon it that most studies of speciation are based. This means that speciation works are, in fact, an analysis of reproductive isolation (Coyne & Orr, 1998; Turelli *et al.*, 2001), since the BSC advocates that "species are groups of interbreeding natural populations that are reproductively isolated from other such groups". However, species limits are not so well defined in natural populations, and hybridization is certainly a challenging phenomenon to the BSC, since it blurs species boundaries.

1) The occurrence of natural hybridization

Natural hybridization has for long puzzled evolutionary biologists. Several definitions of hybridization have been applied in scientific literature, but all of them relate to the levels of divergence between the individuals that undergo hybridization (Arnold, 1997). This author defines natural hybridization as a phenomenon that "involves successful

matings in nature between individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters”, where successful matings stands for the production of some viable F1 progeny that possess some level of fertility, even if both the number of offspring and their fertility level is reduced. This last scenario (reduced number of offspring and level of fertility) led several authors to minimize the importance of natural hybridization as a evolutionarily process, but as noted by Barton & Gale (1993), “if even a small proportion of hybrids reproduce, all the individuals in the vicinity of the hybrid zone eventually carry introgressed alleles in some of their genes”. Moreover, the occurrence of infrequent events of hybridization may lead to new evolutionary lineages (Arnold, 1997), which stresses the need to account for natural hybridization when studies of speciation or population genetics are performed. This idea is strengthened by the fact that natural hybridization occurs in a wide variety of organisms and has indeed been found to be surprisingly more common than previously thought (Hewitt, 1988; Mallet, 2005). Among plants, natural hybridization seems frequent: Grant (1981) suggested that the majority of plant species were hybrids; Knobloch (1972) listed 23675 possible hybrids among angiosperm species (although including artificial hybrids); Arnold (1994) considered that at least 50% of the angiosperms could have a hybrid origin; and from a review of five biosystematic floras, Ellstrand *et al.* (1996) calculate that there could be 27500 hybrid combinations among 250000 described plant species. So, even if estimates of natural hybridization incidence in plants vary greatly (Otto & Whitton, 2000) and even if these phenomena are taxonomically unevenly distributed (only 16% to 34% of plant families and 6% to 16% plant genera have one or more reported hybrid – Rieseberg, 1997), natural hybridization seems to be of tremendous importance as a source of variability and biodiversity.

As for animal taxa, “hybrid speciation” is not as common as seen in plants (Turelli *et al.*, 2001; Otto & Whitton, 2000). This may stem from the fact that polyploidy, apparently the major characteristic driving to this kind of speciation is much rarer in animals (Otto & Whitton, 2000). Nevertheless, natural hybridization among animals seems to be abundant (Mallet, 2005), as attested by several works. Species of North American freshwater fish (Hubbs, 1955) or birds (Mayr & Short,

1970) are for long known to hybridize in nature, and data from several other taxa is accumulating. Concerning mammals, the deer (Carr *et al.*, 1986), the house mice (Boursot *et al.*, 1993), the bear (Taberlet *et al.*, 1994), the pocket gopher (Ruedi *et al.*, 1997), the hedgehog (Santucci *et al.*, 1998) or wild and domestic cats (Pierpaoli *et al.*, 2003) are just some examples of the existence of natural hybridization.

Hybridization has also a geographic component. Species have a natural range of distribution and, in some areas, transitions to another forms occur. These contact areas define species limits where hybridization may happen, giving rise to hybrid zones. Presently, a hybrid zone is commonly accepted as an area of interaction of genetically distinct groups of individuals, where some admixed offspring might occur, still being possible to find parental or pure individuals outside of that same area (Harrison, 1990). Several models exist that predict the evolution of hybrid zones, where selection and/or dispersal play major roles (Arnold, 1997). Among them, the Mosaic model (Rand & Harrison, 1989) and the Tension zone model (Barton, 1979; Barton & Hewitt, 1985) are the most commonly invoked conceptual frameworks to understand and describe hybrid zones. Nevertheless, the Tension zone model, object of intensive theoretical work (Barton & Hewitt, 1985; Barton & Gale, 1993; Piálek & Barton, 1997), became predominant and has been applied throughout the years (e.g. Szymura & Barton, 1991; Phillips *et al.*, 2004; Bozikova *et al.*, 2005).

2) The origin of hybrid zones and the influence of climate

The origin of hybrid zones is a major issue in evolutionary biology, with primary or secondary contact as hypotheses. A hybrid zone of primary origin requires the establishment of gradual clinal variation in a continuous distribution of populations, with the maintenance of their distribution subsequent to differentiation. On the other hand, a steep cline will arise from the contact between two allopatrically differentiated populations, defining a hybrid zone of secondary intergradation (Hewitt, 1988; Harrison, 1990). If some, as Mayr (1942), only considered allopatric speciation and secondary contact as cause of hybrid zones, it has been theoretically demonstrated that

speciation in continuous populations may occur (Slatkin, 1973; Endler, 1977). The study of the marine snail *Littorina saxatilis* in Galicia, Spain (Rólan-Alvarez *et al.*, 1997) or Sweden (Panova *et al.*, 2006) and the Nicaraguan crater lake cichlid fish (Barluenga *et al.*, 2006) are some of the few examples supporting hybrid zones of primary origin found in animals, while accumulating evidence favour secondary contact as the path driving to the majority of the known hybrid zones (Hewitt, 2001).

Although other factors might be involved, climate has been of main importance for the establishment of hybrid zones. Coherent explanations for genetic patterns and species distributions come from palaeoclimatic studies (Hewitt, 2000), that unravelled a series of major ice ages during the Quaternary period of the geological time (from 2,4 million years to present), characterized by the advancement and recession of ice sheets. These events occurred at a global scale but were felt differently across the globe due to regional differences in landform, ocean currents and latitude (Hewitt, 2000). In the northern hemisphere, the impact of glaciations was so profound that almost all the fauna and flora north of the equator was affected. Virtually no phylogeographic work of northern hemisphere organisms ignores the effect of glaciations in the present day distribution of species, with consequences in their genetic pool due to repeated expansions into new habitats accompanying climate amelioration, and recessions to glacial refugia when conditions were less favourable (Taberlet *et al.*, 1998; Hewitt, 2000).

Europe has been mostly covered with ice during glaciations. However, a privileged southern position of the Iberian, Italian and Balkanic Peninsulas rendered these geographic areas reasonable conditions for the maintenance of biodiversity during ice ages (Figure 1) and, from there, species of plants and animals recolonized Europe when conditions improved (Taberlet *et al.*, 1998). The colonization patterns evidenced by many species reflect the southerly position they occupied during unfavourable conditions, and the subsequent expansion to north, east or west they followed. Interestingly, several species display coincident colonization routes, despite the existence of differences in ecological requirements and dispersal.

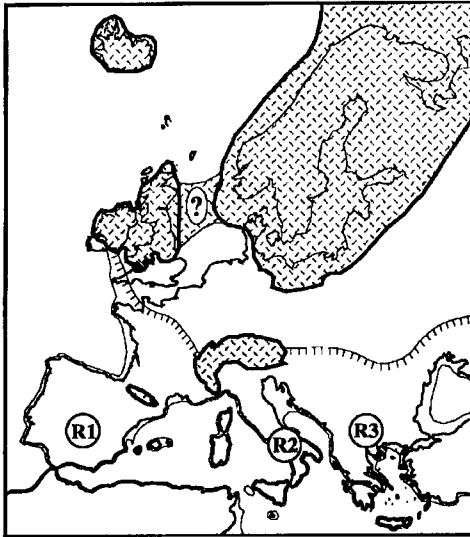


Figure 1: Extension of ice sheets in Europe (in blue) during the last cold period, 20000-18000 years ago. The scaled line indicates the southern limit of the permafrost. R1, R2 and R3 correspond to the Iberian, Italian and Balkans refugia areas, respectively. Adapted from Taberlet *et al.*, 1998.

As Europe was recolonized, closely related species that were trapped by ice sheets in separate geographic areas became into contact, and contact zones were created. The coincidence of species dispersal and, mainly, the existence of natural barriers to dispersion (e.g. mountain chains, rivers) led to the overlapping of hybrid zones of several organisms in the same area, which might still be seen. Three of the consensually considered strips of hybridization in Europe are placed in the exit of each of the referred southern peninsulas (Figure 2; Hewitt, 2001).

Figure 2: General position of well-known hybrid zones placed in the exit of southern refugia, adapted from Taberlet *et al.*, 1998 and Hewitt, 2000.



These hybrid zones harbour a wide number of species and may be seen as suture zones, described by Charles Remington (1968) as regions where many hybrid zones from different species are located. Focusing on the Iberian Peninsula, its northern area around the Pyrenean foothills displays a pattern that is consistent with this scenario. Even if the Pyrenees were less of a barrier than the Alps to the northwards expansion of species (Hewitt, 2001), they were probably enough to delay the exit of species from the Iberian Peninsula and provided the opportunity for the contact with expanding species from Eastern Europe. This has reportedly happened with species such as the alder (*Alnus glutinosa*; King & Ferris, 1998), the crested newt (*Triturus cristatus*; Wallis & Artzen, 1989) or the meadow grasshopper (*Chorthippus parallelus*; Szymura *et al.*, 1996), among others. From this evidence, it is reasonable to think that more species following similar colonization routes established contact zones, where some degree of admixture may occur. This is the case of two hare species, *Lepus granatensis* and *Lepus europaeus*, in Northern Iberian Peninsula.

3) Taxonomy of hares

Hares belong to the order LAGOMORPHA. This order was recognized in 1912, when a review by Gridley separated lagomorphs from rodents (order RODENTIA), to which they were previously allocated. The distinction is based in some morphological characters, as the presence of a second set of incisors teeth (named peg) behind the upper front incisors in lagomorphs. An elongated rostrum of the skull (Flux & Angermann, 1990) and the presence of a leporine lip are other characteristic anatomical traits of LAGOMORPHA in comparison with RODENTIA.

Within LAGOMORPHA, two families are currently recognized, OCHOTONIDAE and LEPORIDAE (Figure 3). While the former is a monotypic family harbouring the genus *Ochotona* (pikas), the latter contains eleven genera, divided in true rabbits (ten of the eleven genera) and true hares (genus *Lepus*).

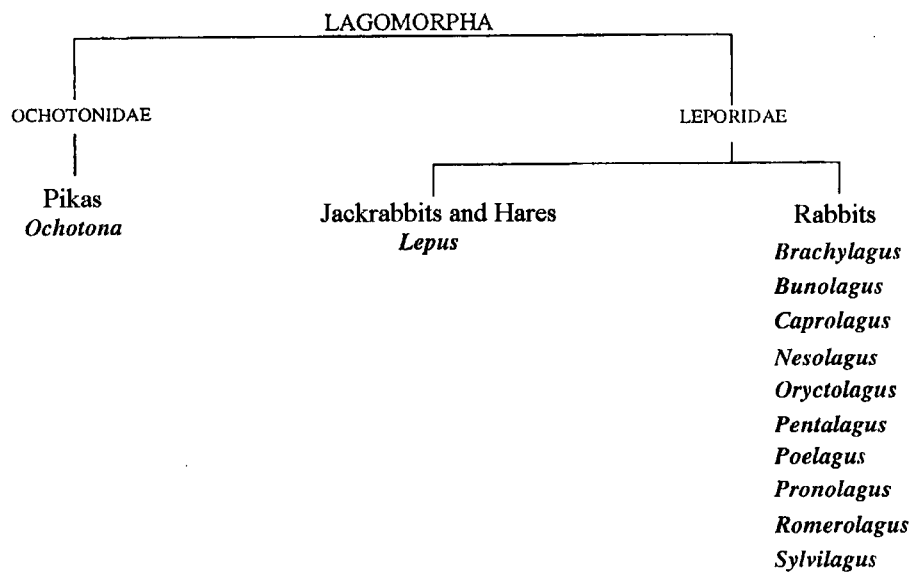


Figure 3: Families and genera of the order LAGOMORPHA (adapted from Flux & Angermann, 1990).

The species of the family LEPORIDAE have long hind legs and large movable ears, being adapted to quick movement and flight from danger, as well as large eyes, suited to their crepuscular and nocturnal habits (Flux & Angermann, 1990). The genus *Lepus* comprises jackrabbits and hares, with some controversy in the number of species. According to Flux & Angermann (1990), this monophyletic genus has 29 species, but depending on the authors, the number varies between 18 and more than 30. A more recent survey by Wilson & Reeder (2005) considers the existence of 33 hare species. The genus *Lepus* is very homogeneous in cytogenetic characteristics, with every species displaying $2n=48$ chromosomes and identical G-banded karyotype (Robinson & Matthee, 2005). Indeed, the level of variability in cytogenetic markers is generally very low in the LEPORIDAE, suggesting a fast expansion of this family. Within LAGOMORPHA, the most exclusive characteristic of hares is the fact that their young are born fully furred, with their eyes open and ready to move within minutes (Corbet, 1983). Comparing with rabbits, this feature leads to a difference in behaviour, since rabbits build nests or elaborate warrens in order to protect their young while hares will only use a shallow depression (Flux & Angermann, 1990).

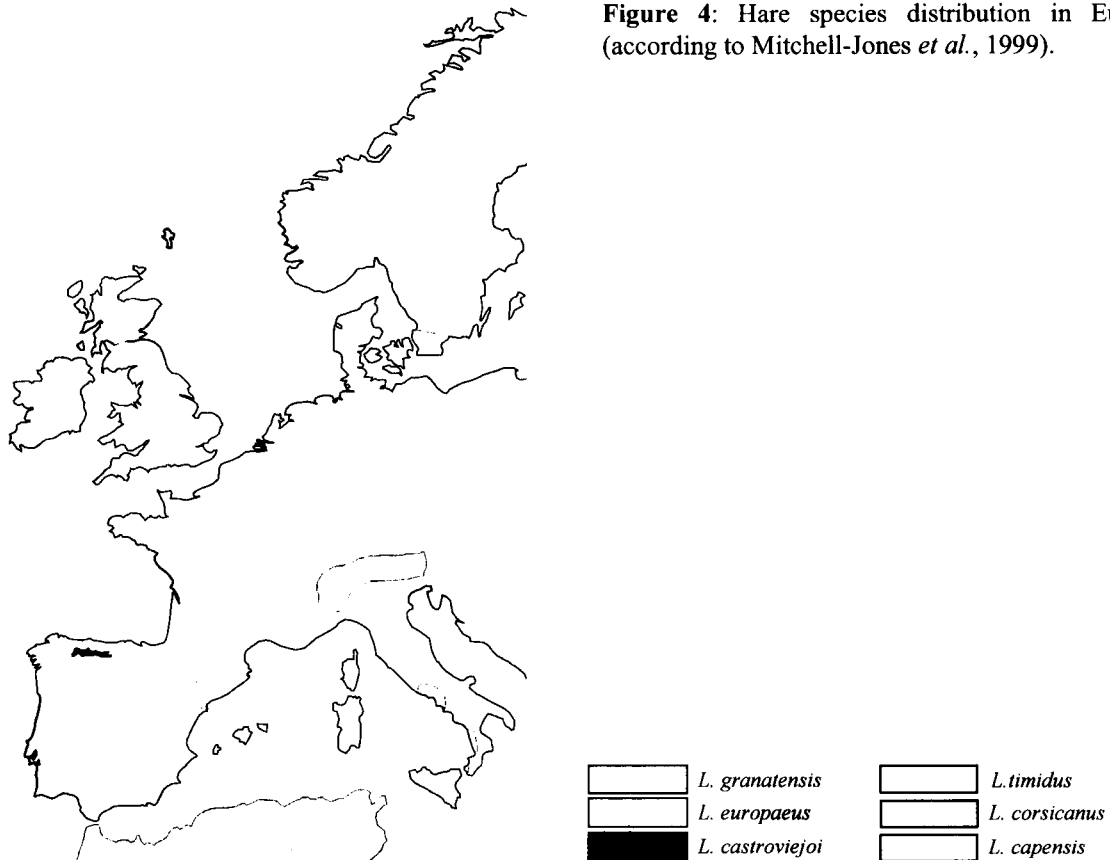
4) Hares in the world

Lepus is a widely distributed genus. In fact, hares are the lagomorphs with the most widespread natural distribution, occurring in North and Central America, Europe, Africa and Asia (Flux & Angermann, 1990). In general, hares are open country grazers, and so have benefited from habitat changes caused by traditional agriculture. Hare species fit Bergmann's rule, which states that animals tend to be larger in colder regions, i.e. in higher latitudes, presumably for reasons of thermoregulation. Another latitude-related characteristic is the fur coloration. The alpine or northern species of hares change from a darker colour to white in the winter, while the rest of the species have relatively similar "agouti" colorations in various shades of brown on the back and white or pale buff below (Flux & Angermann, 1990).

Hares constitute a fundamental ecological resource, since they are an essential food supply for several carnivores (e.g. the red fox – Schmidt *et al.*, 2004), and some species are important game animals in North America and Europe. In fact, mainly in some European countries as France, Italy, Germany, Austria, Poland, Sweden, Spain and Portugal, hares are a highly valuable game species, constituting an important economic resource.

5) Hares in Europe and in the Iberian Peninsula

Five *Lepus* species can be naturally found in the European continent (Figure 4). *Lepus europaeus* (the brown hare) and *Lepus timidus* (the mountain hare) are the most widely distributed species of the genus in Europe. The former inhabits most of the continent, from the Iberian Peninsula to Russia, while the latter occupies Northern Europe and has isolated populations in Ireland, Scotland and in the Alps (Flux & Angermann, 1990). With smaller distributions we can find the Italian hare, *Lepus corsicanus*, inhabiting Southern Italy, the Iberian hare, *Lepus granatensis*, in most of the Iberian Peninsula, and the broom hare, *Lepus castroviejoi*, restricted to the Cantabrian Mountains in Spain (Figure 4).



Therefore, the Iberian Peninsula harbours three of the five European hare species, strengthening its position as a biodiversity sink. A long contact zone between the Iberian and brown hare delimits both species distribution in northern Iberia, running from the Cantabrian Mountains to the Mediterranean coast (Palacios & Meijide, 1979).

6) Hybridization in hares

According to Angermann (1983), no morphological or karyological barriers exist between hare species that could prevent natural hybridization in contact areas where conspecific partners are rare. In agreement with this observation, similar chromosome number and karyotype are found across the genus *Lepus* (Robinson & Mathee, 2005)

and, despite well defined morphological differences between some species (Angerbjörn & Flux, 1995; Palacios, 1983; Palacios, 1989), a general trend of regional variation and phenotypic plasticity may approximate species appearance (Flux & Angerman in Flux & Angermann, 1990).

In captivity, mountain hare females spontaneously cross with brown hare males, whereas the converse must be performed by artificial insemination (Gustavsson & Sundt, 1965), producing fertile offspring (Gustavsson, 1971; Schröder *et al.*, 1987). Using species-specific immunoglobulin markers, Schröder *et al.* (1987) failed to detect natural hybridization in Finish wild populations of mountain and brown hare, but conclusive evidence for its occurrence in Sweden has been documented by Thulin *et al.* (1997). These authors described that mountain hare mtDNA is passing to brown hare individuals by introgressive hybridization, despite mtDNA sequence divergence between species of about 8%. In total, about 10% of the Swedish brown hares analysed by Thulin *et al.* (1997) have mountain hare mtDNA, even though presently sympatric and allopatric populations display significantly different values of introgression ($\approx 10,4\%$ vs. $0,6\%$, respectively) (Thulin & Tegelström, 2002). The unidirectionality of the mtDNA transfer in Sweden is only contradicted by one individual (Thulin *et al.*, 2006a) but this must be a rare situation, since none had been previously found in a sample of 671 individuals of both species (Thulin *et al.*, 1997). However, in Russia, as opposed to the Scandinavian scenario, only introgression of brown hare mtDNA into mountain hare has been reported (Thulin *et al.*, 2006b), although in a small scale. Changes in hybridization direction are possible (e.g. Bozikova *et al.*, 2005), and in the Russia-Sweden case, the authors argue that hybridization is frequency-dependent (Thulin *et al.*, 2006b). Another well documented hybridization event was described by Alves *et al.* (2003) and Melo-Ferreira *et al.* (2005) in the Iberian Peninsula. In this situation, hybridization occurred in the past, presumably when the mountain hare was present in Iberia, from where it is supposedly absent since the end of the last ice age. Introgressive hybridization led to the passage of mountain hare mtDNA to the Iberian, brown and broom hare from northern Iberian Peninsula (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005). In fact, some hare populations in that region display complete replacement of the present species mtDNA by mountain hare mitochondrial genome, configuring a

scenario of massive introgression and fixation (Melo-Ferreira *et al.*, 2005). Interestingly, in the Iberian Peninsula, the mountain hare mtDNA signal is currently strong, even though the long term absence of mountain hare.

Although the brown and the mountain hare may be separated since the late Pleistocene (Slimen *et al.*, 2005), most of the introgression evidences among *Lepus* concern this pair of species. Beside the described case of present (Thulin *et al.*, 1997) or past (Alves *et al.*, 2003) transfer of mountain hare mtDNA into brown hare, there is also data supporting the bilateral introgression between these two species of hares in natural populations of Switzerland, at the level of allozymes and mtDNA (Suchentrunk *et al.*, unpubl. data). On the other hand, hybridization has been discarded between brown and Italian hare, as well as between mountain and Italian hare in Italy, where mtDNA control region and cytochrome *b* analysis clearly allocated the three species individuals to distinct clusters (Pierpaoli *et al.*, 1999). These authors argue for the absence of either present or past interspecific gene flow and hypothesize completely independent evolutionary paths for Italian, brown and mountain hares. Recently, Estonba *et al.* (2006) surveyed the three hare species from Iberian Peninsula with microsatellites markers and found no evidence of hybridization. In this work, some individuals, namely two brown hares from Bulgaria that cluster with the broom hares, are interpreted as mislocalizations by chance, since no recent contact is reasonable due to the long geographic distance between Iberian and Balkan Peninsulas. The microsatellites markers allow these authors to separate all the species at the population level, which is interpreted as compelling evidence for the existence of a barrier to neutral gene flow between them. In contradiction, preliminary data of Melo-Ferreira *et al.* (2004) points to some degree of hybridization between Iberian and brown hare, shown by the analysis of the nuclear marker locus *e*. Also, one hybrid individual was obtained in captivity resulting from the cross of an Iberian hare male with a brown hare female (P.C. Alves, unpubl. data), although it is not known if the offspring is fertile. It does, however, show that species barrier separating Iberian from brown hare is not sufficiently strong to maintain total separation of species.

7) Ecological requirements of Iberian hare and brown hare

With few exceptions, hares are very flexible and adapt easily to habitat conditions. In general, the preferred habitat of hares is open country with shrubs, bushes or rocks for protection from predators (Flux & Angermann, 1990). Agricultural fields and wood patches constitute a good territory for hares (Homolka, 1985), with arable areas with intermediate-intensity farming displaying high demographic densities (Smith *et al.*, 2005). Presently, changes in agriculture techniques, the increased mechanization, use of pesticides and habitat fragmentation are negatively correlated with hare population densities (Dingerkus & Montgomery, 2002; Schmidt *et al.*, 2004; Smith *et al.*, 2005), as well as the extensive and monoculture farming (Smith *et al.*, 2005).

The Iberian hare is well adapted to extremely variable habitats, occurring in areas ranging from less than 300mm to more than 2000mm of annual rainfall (optimum between 300mm and 600mm), and from 3°C in January to 26°C in August (Palacios & Meijide, 1979). This species inhabits areas at the sea level or as high as about 2000m of altitude. It occupies humid mountain areas with Atlantic type climate and agricultural fields of rye, wheat or cork in northern Iberia; open areas of agricultural landscapes of wheat, barley or alfalfa in continental Iberia; and dry, Mediterranean regions with olive groves or vineyards in the south of the Iberian Peninsula (Alves & Niethammer, 2003). The brown hare in its Iberian range is also very plastic to ecological conditions, as it occurs in areas from the sea level up to 1700m of altitude, ranging from about 400mm to 2000mm (optimum between 1000mm and 1500mm) and with temperatures oscillating between 0°C in January and 18°C in August (Palacios & Meijide, 1979). It inhabits zones of agricultural exploitation as vineyards, almond groves and carob groves, as well as humid zones near the Cantabrian Mountains with woods of *Quercus robur*, *Q. pyrenaica* and *Fagus sylvatica*, for example. It also occurs in high altitude areas in the Pyrenees, where pine forests of *Pinus unciata* with underbrush of *Rhododendron ferrugineum* and *Vaccinium myrtillus* are present (Palacios & Meijide, 1979). Some other variables as predation (Schmidt *et al.*, 2004), field size (Smith *et al.*, 2005) or hunting (Marboutin *et al.*, 2003) were assessed for the impact in brown hare populations, but implications seem to vary regionally.

8) Objectives and thesis organization

The hare species from Iberian Peninsula have been studied mainly in the scope of phylogenetic or phylogeographic works and taxonomic classifications (Pérez-Suarez *et al.*, 1994; Pierpaoli *et al.*, 1999; Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005; Estonba *et al.*, 2006). However, other phenomena with potential evolutionary importance, such as hybridization, may occur and this has been an overlooked question. Also, the advancement in molecular and statistical techniques provides new tools for the intersection of genetics and ecology (i.e., landscape genetics), which may prove valuable to gain new insights into species dynamics. Here, both these guidelines – hybridization and landscape genetics – were followed to study the Iberian and brown hare in the Iberian Peninsula. Given this, this thesis has two main topics:

- 1) The assessment of potential admixture between Iberian and brown hare
- 2) The genetic characterization of a contact zone between Iberian and brown hare and the correlation between genetic patterns, species distribution and ecological variables.

The question of hybridization between Iberian and brown hare was tackled with the use of microsatellites. The first task was to assemble a set of microsatellites powerful enough to distinguish Iberian, brown and also mountain hare, since this species molecular signature is still present in the Iberian Peninsula. These molecular markers were then used to answer some defined objectives, namely:

- 1) Verify if there is any evidence for nuclear introgression of mountain hare into brown and Iberian hare
- 2) Investigate the putative admixture between Iberian and brown hare
- 3) Describe the patterns of introgression of microsatellites markers
- 4) Verify the effectiveness of the hybrid zone (as indicated by the existence of post-F1 hybrids).

All the efforts made in order to accomplish these objectives are described in Chapter II of this thesis.

To investigate the second main topic of this work, both nuclear and mitochondrial markers were used. Although mtDNA introgression from mountain hare into both Iberian and brown hare was already known, a bigger sampling effort can bring new knowledge to the subject and several objectives were pursued:

- 1) Search introgression between Iberian and brown hare at the level of mtDNA
- 2) Verify the concordance of nuclear and mitochondrial markers
- 3) Search for genetic discontinuities and correlate them with environmental ecotones
- 4) Correlate species distribution with ecological variables.

This work is described in Chapter III and consisted in a fine-scale analysis of a small study area (around 60x50 km) in Northern Iberia, using geo-referenced individuals,.

Both Chapter II and Chapter III are presented in scientific article format, and all the results obtained in both chapters are integrated and discussed in Chapter IV, the General Discussion. Chapters I and IV (General Introduction and General Discussion) are structured in numbered Sections and sub-Sections. The Conclusions are presented in Chapter V and the References used in the General Introduction and in the General Discussion are presented in Chapter VI.

Paper 1

Evidence of natural hybridization between *Lepus granatensis* and *L. europaeus* in northern Iberian Peninsula

Abstract

Hybrid zones are a challenging subject in evolutionary biology. Climatic oscillations provided good opportunities for allopatric speciation of organisms and for posterior secondary contact, which is apparently the most common way of establishment of hybrid zones. In the north of the Iberian Peninsula, two species of hares, *Lepus granatensis* and *L. europaeus*, are in contact but hybridization has not been demonstrated. Here, with a set of six microsatellites we aimed to assess the interaction between both species and also to verify if the massive introgression of *L. timidus* mitochondrial DNA in Iberian Peninsula hare populations was paralleled by the nuclear genome. Our results discard a nuclear presence of *L. timidus* in Iberia and support the existence of natural hybridization between *L. granatensis* and *L. europaeus*. Four individuals of the former species and three of the latter show evidences of admixture between both species, although introgression from *L. europaeus* into *L. granatensis* is more strongly supported. The reciprocity of hybridization, as well as the level of admixture or the hybrid zone structure should be reassessed. Nevertheless, Iberian and brown hare do hybridize in northern Spain and may now be included in the list of organisms contributing to the suture zone that exists in that region.

Introduction

Hybrid zones have for long been object of interest. Their study congregates knowledge from multiple fields such as taxonomy, genetics, ethology or palaeoclimatology, which renders it an intrinsic importance, but also an extra difficulty. Presently, a hybrid zone is commonly accepted as an area of interaction between genetically distinct groups of individuals, where some admixed offspring might occur, still being possible to find parental or pure individuals outside of that zone (Harrison, 1990). A secondary contact after allopatric divergence of evolutionary units is apparently the most parsimonious explanation for the origin of many contact zones (see Hewitt 2001). In the European Continent, which experienced several glacial and inter-glacial periods, the dynamics of ice sheets provided good opportunities for the occurrence of speciation, colonization, secondary contacts and hybridization phenomena (Hewitt, 2000; Taberlet *et al.*, 1998). Therefore, the distribution of its fauna and flora has been shaped through time by these macroscale events coupled with a peculiar geographic context.

In Southern Europe, the Iberian, Italian and the Balkanic peninsulas constituted biodiversity sinks, as they were the major ice age refugial areas. So, these peninsulas were the source of Europe recolonization by several organisms (Hewitt, 2001; Taberlet *et al.*, 1998) in a northwards post-glacial expansion which led to secondary contacts between recently diverged taxa. However, in Iberian and Italian peninsulas the presence of mountain chains (Pyrenees and Alps, respectively) strongly opposed species northwards expansion (Hewitt, 1999). Nonetheless, after the last glacial maximum, several contact zones have been generated (Hewitt, 1988; Hewitt, 2001). Major biogeographic barriers as mountain chains and congruent postglacial biotic range expansions (Hewitt, 1999; Hewitt, 2001; Swenson & Howard, 2004) led to the coincidence of various organisms contact zones, eventually fitting Remington's concept (1968) of a suture zone. In Europe, four such zones are usually recognized (Taberlet *et al.*, 1998): one corresponds to the Alpine barrier; another is located near the border between France and Germany; the third one occurs in Scandinavia; and the fourth is located in north-eastern Iberian Peninsula, in the Pyrenean foothills (Figure 1). In the latter suture zone, organisms such as the meadow grasshopper *Chorthippus parallelus*

(Cooper *et al.*, 1995), the alder *Alnus glutinosa* (King & Ferris, 1998) and the crested newt *Triturus cristatus* (Arntzen & Wallis, 1991), established hybrid zones after post-glacial range expansions.



Figure 1: Location of the four suture zones known in Europe (adapted from Taberlet *et al.*, 1998): A) the Alps, B) France-Germany border, C) Scandinavia and D) northern Iberian Peninsula.

The brown hare (*Lepus europaeus*), widespread in Europe, and the Iberian hare (*Lepus granatensis*), endemic to the Iberian Peninsula, also form a contact zone in northern Spain. This contact area runs from the Mediterranean Sea to Asturias, but the question of hybridization is still an open issue. Within genus *Lepus* (LEPORIDAE; LAGOMORPHA), some cases of hybridization have already been described. In Sweden, unidirectional introgression of the native mountain hare (*Lepus timidus*) mtDNA into the introduced brown hare was documented (Thulin *et al.*, 1997; Thulin *et al.*, 2006a). Recently, hybridization between the same two species has been shown in Russia, although the mtDNA introgression occurs in the opposite direction, *L. europaeus* to *L. timidus* (Thulin *et al.*, 2006b), and in the Alps where it is apparently bidirectional (Suchentrunk *et al.*, unpublished data). Also, although the mountain hare is presently

absent from Iberia, ancient massive introgression of its mtDNA into *L. europaeus*, *L. granatensis* and even into the relictual population of broom hare (*L. castroviejoi*), restricted to the Cantabrian Mountain (Palacios & Meijide, 1979), has been described (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005). However, despite the existence of several contact zones between other pairs of hare species, hybridization has been discarded: for example, no evidence for it was found between brown and Italian hare (*Lepus corsicanus*) or mountain and Italian hare (Pierpaoli *et al.*, 1999) in Italy, or between Iberian and brown hare (Estonba *et al.*, 2006) in the Iberian Peninsula.

Here, we present a detailed study of the contact zone between Iberian and brown hare in the Iberian Peninsula. Given our framework, an approach with nuclear markers that differentiate the brown, Iberian and mountain hare was needed. For that, we use microsatellites, which are nuclear markers with a high rate of mutation, leading to good discriminatory power in short time events. Using a set of six microsatellites markers, we prove that Iberian and brown hare hybridize in north-eastern Iberian Peninsula and draw plausible scenarios concerning the species dynamics.

Materials and Methods

Sampling

Ear tissue was collected from a total of 342 hare individuals (194 *L. granatensis*, 109 *L. europaeus* and 39 *L. timidus*) from several localities in Europe (Figure 2 and Table 1). Species identification in the field was done on the basis of distinctive morphological characters (Palacios, 1989). Near the contact zone between *L. granatensis* and *L. europaeus* in Northern Iberia, three samples of Iberian hare and three of brown hare were obtained. Parental samples of Iberian hare were collected from broadly distributed localities within Iberian Peninsula, while parental individuals of brown hare were taken from Central Europe sampling localities. Also, two sampling sites were surveyed for the mountain hare, where one (Italian Alps) represents the present southernmost distribution of the species.

| Species | Sample code | Location | <i>n</i> |
|-----------------------|-------------|-------------------------|-------------|
| <i>L. granatensis</i> | Alj | Aljustrel, Portugal | 19 |
| | Gm | Granada, Spain | 20 |
| | TC | Tierra de Campos, Spain | 11 |
| | Sor | Soria, Spain | 13 |
| | Alc | Alcañiz, Spain | 16 |
| | Ala1 | Álava, Spain | 46 |
| | Nav1 | Navarra, Spain | 28 |
| | Zar | Zaragoza, Spain | 41 |
| | | | Total = 194 |
| <i>L. europaeus</i> | Ala2 | Álava, Spain | 51 |
| | Nav2 | Navarra, Spain | 7 |
| | Jac | Jaca, Spain | 11 |
| | S Ger | Southern Germany | 8 |
| | N Ger | Northern Germany | 14 |
| | Aus | Eastern Austria | 18 |
| | | | Total = 109 |
| <i>L. timidus</i> | Alp | Italian Alps | 20 |
| | Swe | Sweden | 19 |
| | | | Total = 39 |

Table 1: Sampling localities surveyed and number of individuals analysed per sample (*n*).

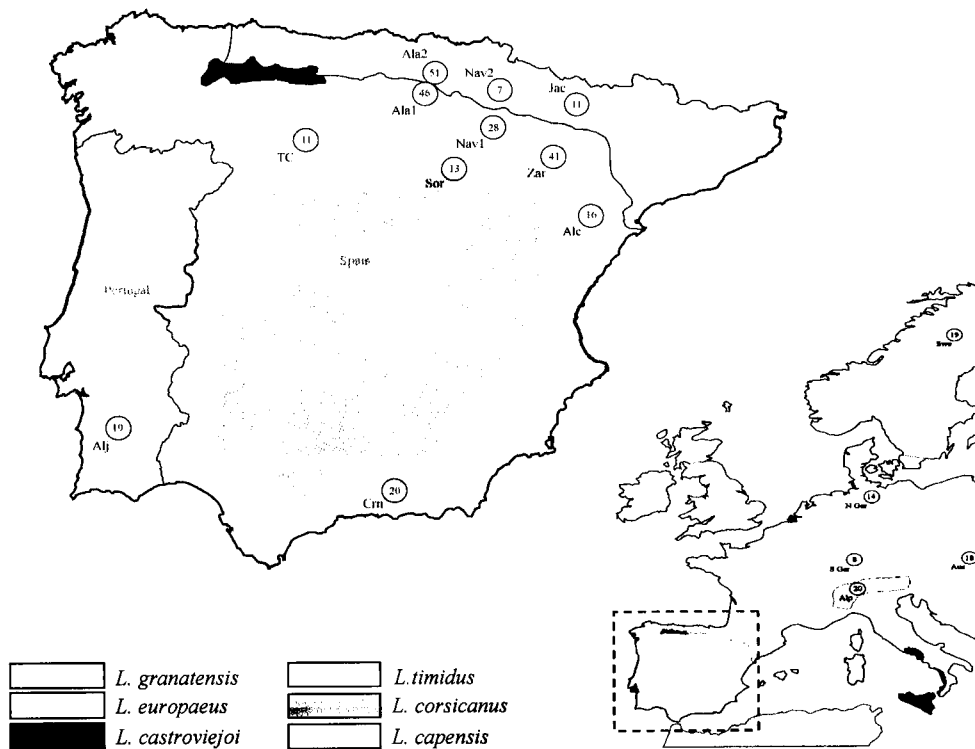


Figure 2: European hare species distribution (according to Mitchell-Jones *et al.*, 1999) and sampling localities. In the right bottom corner, sampling sites of central and Northern Europe are indicated. The dashed line box encloses the Iberian Peninsula, shown in detail on top. The number of individuals sampled in each locality (indicated inside circles) and sample codes are depicted in Table 1.

Microsatellites typing

Total genomic DNA was extracted from ear tissue using a standard high-salt protocol (Sambrook *et al.*, 1989). A set of 6 microsatellites *loci* was chosen from previously published works, based on their polymorphism and allele size (Table 2). The *loci* Sat2, Sat8, Sat12 and INRACCDDv358 were originally developed for the European rabbit (*Oryctolagus cuniculus*), also a member of LEPORIDAE, while Lsa2 was specifically developed for hares and Sol30Le was adapted for hares from a rabbit-specific *locus*. All the used *loci* consist in dinucleotide repeat motifs except Sat12, which is a tetranucleotide microsatellites *locus*. The microsatellites were PCR-amplified in 15 μ L reaction volumes, with 1x reaction buffer, 4 pmol of each primer, 1 pM of each dNTP, 1.5 mM MgCl₂ and 0.2 units of *Taq* polymerase. For each individual, 0.25 ng of genomic DNA was used per reaction. The thermal cycles consisted in an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 30s at 94°C, 30s at 40-58°C and 30s at 72°C. A final 15min elongation step at 72°C was used. The microsatellites typing was achieved using fluorescently labelled primers (Table 2) and an ABI 3100 automated sequencer (Applied Biosystems). In order to validate the allele calling results, which was done in GeneMapper Software v3.7 (Applied Biosystems), control individuals were included in every run and 15% of the dataset, chosen with a pseudo random number generator, was repeated.

Table 2: Microsatellite markers identification (*Locus* ID and Reference), number of alleles found (Nr.Alleles), size range, fluorescent label of the primers (Label) and annealing temperature used for PCR amplification.

| <i>Locus</i> ID | Reference | Nr.Alleles | Size Range | Label | Annealing T |
|-----------------|-------------------------------------|------------|------------|-------|-------------|
| Sat2 | Mougel <i>et al.</i> , 1997 | 20 | 222-266 | HEX | 44°C |
| Sat8 | Mougel <i>et al.</i> , 1997 | 7 | 87-101 | NED | 58°C |
| Sat12 | Mougel <i>et al.</i> , 1997 | 9 | 104-136 | HEX | 44°C |
| INRACCDDv358 | Chantry-Darmon <i>et al.</i> , 2005 | 5 | 252-270 | NED | 54°C |
| Lsa2 | Kryger <i>et al.</i> , 2002 | 11 | 237-257 | NED | 48°C |
| Sol30Le | Fickel <i>et al.</i> , 1999 | 21 | 156-256 | HEX | 56°C |

Data analyses

Standard measures of genetic diversity as the total number of alleles per sample, mean number of alleles per *locus* (MNA), expected (H_e) and observed (H_o) heterozygosity were assessed using the program GENETIX version 4.05 (Belkhir *et al.*, 2004). Linkage disequilibrium (LD) between pairs of *loci* and Hardy-Weinberg Equilibrium (HWE) over all *loci* across all samples were estimated using GENEPOP version 3.4 (Raymond & Rousset, 1995). The probability tests were based on a Markov Chain Monte Carlo (MCMC) simulation (Guo & Thompson, 1992), using GENEPOP default parameter values and the standard Bonferroni correction for multiple test was applied. F_{st} was calculated using the GENEPOP software to measure the differentiation between species and between pairs of samples. While the latter was calculated over all the studied samples, the former was calculated over the parental samples grouped by species.

A non-parametric 2 dimension Factorial Correspondence Analysis (FCA), as provided by GENETIX, and a model-based Bayesian method, as implemented in STRUCTURE (Pritchard *et al.*, 2000), were applied to the dataset. Five replicate runs of STRUCTURE, consisting in 500000 steps after a burnin period of 100000, were performed for each K value, from K=2 to K=7. No prior information about the origin of the specimens was used. Individuals were closely inspected for the possibility of admixture when the likelihood of belonging to one species was lower than 80%.

The Iberian hare and brown hare samples were also analysed with the software NEWHYBRIDS (Anderson & Thomson, 2002), using the same number of replicate runs and steps as previously used in STRUCTURE. NEWHYBRIDS, as STRUCTURE, is a model-based Bayesian software but fundamental differences stem between both models: using multilocus genotype data, STRUCTURE infers population structure and assigns individuals to populations, while NEWHYBRIDS assigns individuals to one of parentals and various classes of hybrids (F1's, F2's and various backcrosses). In the NEWHYBRIDS analysis, prior information was used to define parental samples.

Results

A total of 73 alleles were found, ranging from 5 to 21 alleles per microsatellites *locus*. No significant linkage or Hardy-Weinberg disequilibria were found. Although none of the *loci* was completely diagnostic, a frequency-based species distinction was possible (Figure 3). In fact, an almost complete concordance of microsatellites species assignment with previous morphological identification was achieved (one possible misidentification in 342 specimens – 99,7%).

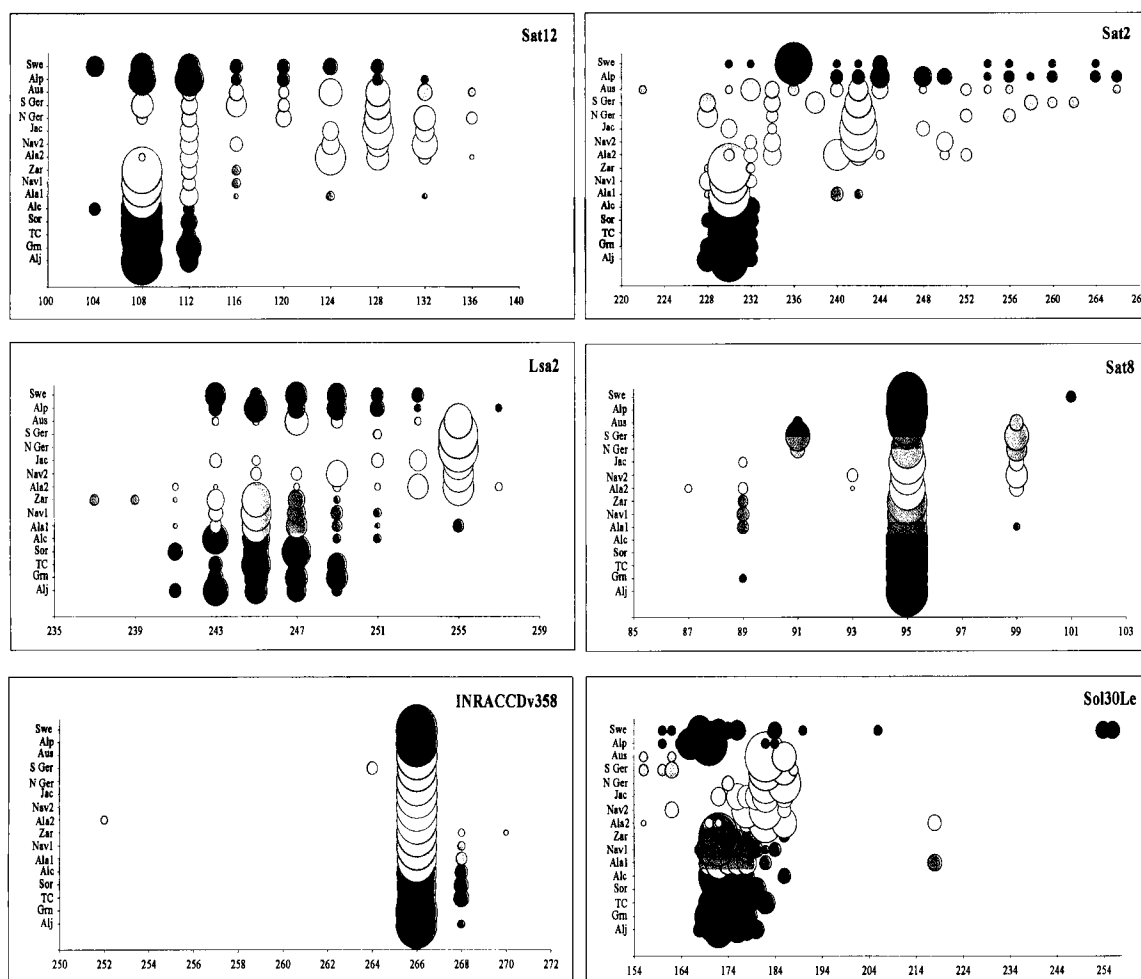


Figure 3: Allelic frequencies found in each sample, identified in the Y-axis. Allele sizes (in bp) are shown in the X-axis. In dark blue, parental samples of Iberian hare; in light blue, Iberian hare samples from the contact zone; in dark yellow, brown hare parental samples; in light yellow, brown hare samples from the contact zone; in brown, mountain hare samples.

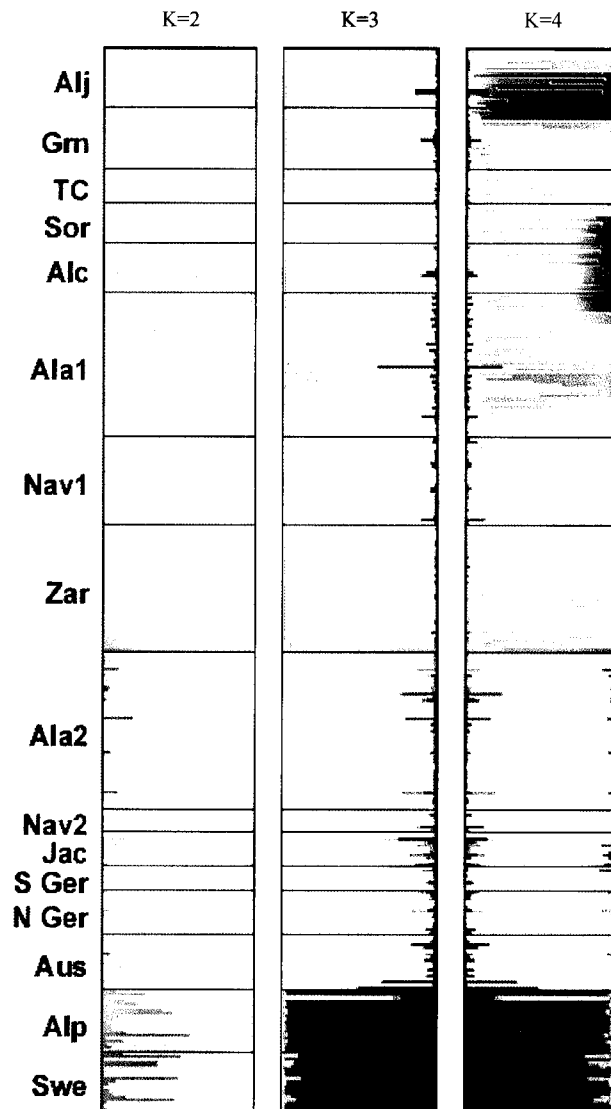
In general, the MNA, as well as the He and the Ho, are lower in the Iberian hare than in brown or mountain hare, which display similar values (Table 3). The MNA tends to increase in the samples from the contact zone when compared with the parental samples of the respective species, being the samples from Álava (either *L. granatensis* or *L. europaeus*) the ones that display a most conspicuous increment. The Ho also displays a slight increase, while He suffers no modifications. However, when individuals with suspected alien alleles (which only occur in the contact zone sampling localities) were removed from the dataset, the recalculated values of MNA, He and Ho fitted the species range of values or were even lower (see MNA in Alav2, Nav2 and Jac).

Table 3: Genetic diversity parameters as measured by expected heterozygosity (He), observed heterozygosity (Ho) and mean number of alleles per *locus* (MNA) found in each sample. For the contact zone samples, recalculated values without foreign alleles are also displayed (*Recalculated*). The box encloses the contact zone samples.

| Sample | Species | He | | Ho | | MNA | |
|--------|---------|-------|---------------------|-------|---------------------|-------|---------------------|
| | | Total | <i>Recalculated</i> | Total | <i>Recalculated</i> | Total | <i>Recalculated</i> |
| Alj | Lg | 0,39 | – | 0,25 | – | 3,33 | – |
| Grn | Lg | 0,39 | – | 0,23 | – | 2,67 | – |
| TC | Lg | 0,36 | – | 0,25 | – | 3 | – |
| Sor | Lg | 0,37 | – | 0,28 | – | 2,83 | – |
| Alc | Lg | 0,32 | – | 0,24 | – | 3 | – |
| Ala1 | Lg | 0,41 | 0,33 | 0,38 | 0,27 | 4,67 | 3,33 |
| Nav1 | Lg | 0,38 | 0,38 | 0,3 | 0,29 | 3,83 | 3,5 |
| Zar | Lg | 0,32 | 0,29 | 0,25 | 0,24 | 4,33 | 3,83 |
| Ala2 | Le | 0,54 | 0,52 | 0,45 | 0,44 | 6,17 | 5 |
| NAv2 | Le | 0,55 | 0,47 | 0,5 | 0,44 | 3,67 | 2,5 |
| Jac | Le | 0,49 | 0,53 | 0,53 | 0,5 | 3,83 | 2,17 |
| S Ger | Le | 0,45 | – | 0,45 | – | 3,33 | – |
| N Ger | Le | 0,54 | – | 0,46 | – | 4,5 | – |
| Aus | Le | 0,53 | – | 0,51 | – | 5,83 | – |
| Alp | Lt | 0,51 | – | 0,4 | – | 5,67 | – |
| Swe | Lt | 0,53 | – | 0,52 | – | 6,5 | – |

The F_{st} values estimated over the parental samples indicated high levels of differentiation between species ($F_{st(Lg-Le)} = 0,353$; $F_{st(Lg-Lt)} = 0,205$; $F_{st(Le-Lt)} = 0,208$). Accordingly, the three species are supported by the best result of STRUCTURE when $K=3$ (Figure 4) and could be depicted as separate clusters in FCA (Figure 5). The $K=3$ value of STRUCTURE analysis was chosen since the largest log probability jump occurs from $K=2$ to $K=3$ and, for the analysed values of $K>3$, we could only find the substructuring of the Iberian hare (see $K=4$ in Figure 4). Moreover, $K=3$ fits with the biological meaning of this work, since three recognized species were under study.

Figure 4: STRUCTURE results for $K=2$, $K=3$ and $K=4$. Sample codes identification can be depicted in Table 1. In $K=3$, blue represents the Iberian hare, light brown the brown hare and dark brown the mountain hare.



The tight cluster formed in FCA showed that the Iberian hare is the less diverse species. On the other hand, the brown and the mountain hare plots displayed a bigger dispersion. In agreement, the pairwise F_{st} analysis (Table 3) showed very low differentiation between Iberian hare samples (mean 0,030; range 0 – 0,079), while brown hare (mean 0,086; range 0,021 – 0,145) and mountain hare (0,101) showed higher degrees of divergence between species' samples.

Table 3: Pairwise F_{st} between each pair of samples analysed. Samples identification is shown in Table 1. In bold are the intraspecific F_{st} values.

| | Alj | Grn | TC | Sor | Alc | Ala1 | Nav1 | Zar | Ala2 | Nav2 | Jac | S Ger | N Ger | Aus | Alp |
|-------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|-------|--------------|--------------|--------------|--------------|--------------|-------|--------------|
| Grn | 0,031 | | | | | | | | | | | | | | |
| TC | 0,013 | 0,030 | | | | | | | | | | | | | |
| Sor | 0,007 | 0,047 | 0,000 | | | | | | | | | | | | |
| Alc | 0,015 | 0,103 | 0,026 | 0,046 | | | | | | | | | | | |
| Ala1 | 0,031 | 0,027 | 0,000 | 0,012 | 0,054 | | | | | | | | | | |
| Nav1 | 0,021 | 0,026 | 0,000 | 0,024 | 0,050 | 0,010 | | | | | | | | | |
| Zar | 0,044 | 0,079 | 0,031 | 0,039 | 0,012 | 0,033 | 0,030 | | | | | | | | |
| Ala2 | 0,305 | 0,302 | 0,322 | 0,312 | 0,351 | 0,309 | 0,331 | 0,376 | | | | | | | |
| Nav2 | 0,345 | 0,335 | 0,370 | 0,367 | 0,423 | 0,346 | 0,371 | 0,451 | 0,075 | | | | | | |
| Jac | 0,328 | 0,338 | 0,364 | 0,352 | 0,395 | 0,334 | 0,367 | 0,421 | 0,083 | 0,021 | | | | | |
| S Ger | 0,407 | 0,408 | 0,444 | 0,434 | 0,480 | 0,401 | 0,433 | 0,494 | 0,103 | 0,049 | 0,061 | | | | |
| N Ger | 0,382 | 0,386 | 0,396 | 0,392 | 0,436 | 0,382 | 0,402 | 0,462 | 0,145 | 0,091 | 0,131 | 0,026 | | | |
| Aus | 0,313 | 0,309 | 0,335 | 0,324 | 0,378 | 0,323 | 0,347 | 0,407 | 0,044 | 0,063 | 0,097 | 0,092 | 0,128 | | |
| Alp | 0,194 | 0,164 | 0,225 | 0,241 | 0,278 | 0,214 | 0,207 | 0,298 | 0,216 | 0,220 | 0,234 | 0,300 | 0,303 | 0,203 | |
| Swe | 0,210 | 0,216 | 0,261 | 0,250 | 0,281 | 0,252 | 0,259 | 0,320 | 0,212 | 0,230 | 0,233 | 0,297 | 0,296 | 0,190 | 0,101 |

The FCA allowed the depiction of five individuals in an intermediate position between the Iberian and the brown hare cluster, namely Ala1 40, Ala1 46, Ala1 48, Ala1 49 and Ala1 51. As for the analysis with the software STRUCTURE, 12 individuals fall below the defined threshold of 80% to acceptance of mixed individual ancestry. Four of these cases (Ala2 31, Jac 4, Aus 21 and Aus 26), morphologically identified as brown hares, assigned mostly to this species but had more than 20% of their genome attributed to the mountain hare; one specimen (Alp 832) previously identified as a mountain hare

was mostly assigned to the brown hare cluster, although more than 20% of its genome was assigned to the mountain hare species; and one individual (Ala1 48) assigned with similar probability to each population when $K=2, 3$ or 4 and randomly for higher K values and, for this reason, was excluded from further analyses. The extant six individuals (Ala1 40, Ala1 46, Ala1 49, Ala1 51, Ala1 53 and Ala2 101) were jointly attributed to the Iberian and brown hare cluster by the Bayesian analysis.

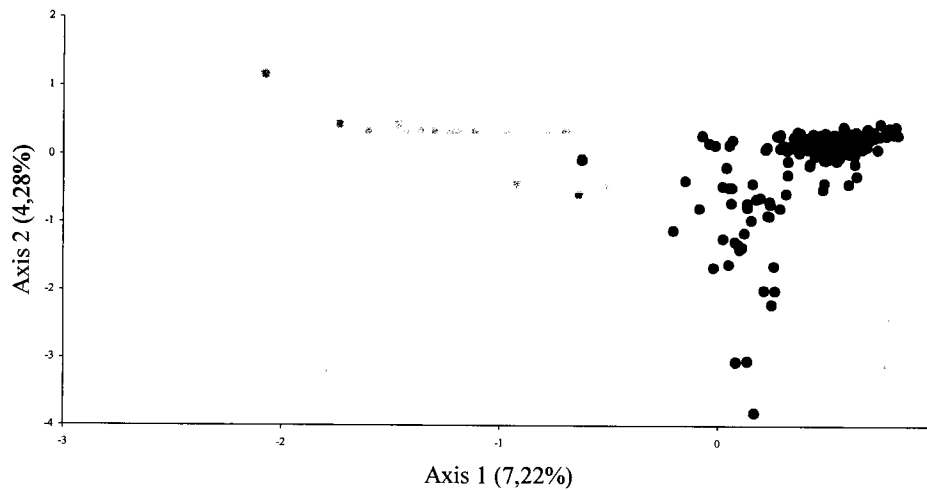


Figure 5: FCA individuals plot. Yellow dots represent brown hares, blue dots represent Iberian hares and brown dots represent mountain hares. In green are displayed individuals morphologically identified as Iberian hares which have signs of admixture with brown hare.

Given the absence of a mountain hare signal in general and, particularly, in Iberian hare, the mountain hare samples were removed for the analysis with the software NEWHYBRIDS. To ensure that no signs of the mountain hare were contained in the analysis, those individuals with some amount of potential admixture with *L. timidus* were also excluded. NEWHYBRIDS results identify Ala1 40, Ala1 46, Ala1 49, Ala1 51, Ala2 56, Ala2 101 and Jac 12 as having high probabilities of being post-F1 hybrids.

Discussion

The ancient massive introgression of the mountain hare mtDNA into the Iberian hare and the brown hare (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005) complicates the assessment of possible hybridization between the latter two species. The same event of hybridization could have left traces on the nuclear genome of *L. granatensis* and *L. europaeus*, although the available allozyme and molecular data do not provide evidence of differentiation in the nuclear background between the introgressed and non-introgressed populations of *L. granatensis* (Alves & Ferrand, 1999; Alves, 2002; Alves *et al.* 2003). However, the genomic and geographic coverage of these data were limited, forcing us to account for the possible contribution of *L. timidus* for the extant nuclear gene pool of the Iberian species. The highly polymorphic neutral nuclear markers used in this work allowed a clear distinction of the three studied species. *Fst* estimates over the parental samples proved that species differentiation was high and FCA and STRUCTURE both showed a good resolution, even including the contact zone samples. Although there was a constant presence of some background noise in STRUCTURE's analysis, no strong signs of mountain hare nuclear introgression were found. There were, however, four brown hare individuals (Ala2 31, Jac 4, Aus 21 and Aus 26) that displayed considerable posterior probabilities of assignment to the mountain hare species. The admixture found in the two Austrian individuals (Aus 21 and Aus26), together with the mountain hare individual Alp 832 from the Italian Alps, may support the occurrence of present hybridization between brown hares from Central Europe and mountain hares from the Alps as shown by Suchentrunk *et al.* (unpubl. data). The distance that separates Aus 21 and Aus 26 from the Alps, however, may indicate that these individuals are the evidence of past hybridization events that occurred in Eastern Austria, but such hypothesis must be reassessed. The brown and the mountain hare, as previously shown in Sweden (Thulin *et al.*, 1997; Thulin *et al.*, 2006a) and Russia (Thulin *et al.*, 2006b), are able to intercross and produce fertile offspring, leading to introgression events. It should be noticed that Alp 832, although caught in the mountain hare habitat and morphologically identified as a mountain hare, had its genome mainly assigned to the brown hare cluster. Therefore, it constitutes the only individual with discordant

identification between morphology and microsatellites among the 342 analysed. The mountain hare signal is also detected in two brown hare specimens from the Iberian Peninsula (Ala2 31 and Jac 4). This may result from the referred possible hybridization events in Central Europe between *L. europaeus* and *L. timidus* coupled with a long distance gene flow in the brown hare, as described by Suchentrunk *et al.* (2000), or could constitute the remnant signal of the ancient hybridization between *L. timidus* and the Iberian species of hare, as shown to occur with mtDNA (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005). Although the absence of the same introgression signal in *L. granatensis* and the short amount of time needed to erase nuclear introgression in the presence of backcrosses (Roca *et al.*, 2005) may argue against the latter hypothesis, different population size (larger in Iberian hare) when hybridization occurred could explain the detected pattern. Another possible explanation for these results is the low number of markers used and the lack of diagnostic *loci*, leading to a problematic assignment of some particular multilocus genotypes, even if our analyses showed a complete differentiation between species using this microsatellites set.

In the analysis with the software STRUCTURE, the homogeneity of most samples of brown hare and, specially, of Iberian hare is clear (Figure 4), which is in conformity with Estonba *et al.* (2006), who recognize a compact cluster for the latter species individuals in the NJ analysis. However, the Iberian hare sample of Álava shows clear evidence of admixture. The mixed ancestry of four *L. granatensis* specimens from Álava is supported by intersecting the three analysis softwares used. The presence of this evidence only in one sample of Iberian hare can point to a unidirectional introgression, with the invasion of brown hare genome into Iberian hare. The demographic dynamics of the species can explain this unidirectionality. The Iberian hare attains higher population densities than the brown hare (Ibón Telletxea, unpubl. data) and is progressively replacing it in northern Iberia. This difference in demography between the species increases the probability that hybrids backcross with Iberian hare in the contact zone (density-dependent hybridization; Wirtz, 1999). However, this hypothesis of unidirectional introgression is, in this particular case, contradicted by the MNA. An increase in the MNA per *locus* is seen in the contact zone samples of both species, and not only in the Iberian hare, as would be expected in such a scenario. The most sensible

increment was detected in either species samples of Álava, where robust evidence of admixture is found. This suggests that both Iberian and brown hare gene pools are incorporating foreign alleles (i.e., from the other species). The transferred alleles behave as rare alleles in the new background, and that justifies the conspicuous increase of MNA values but not of heterozygosities. Also, one brown hare individual was identified by both Bayesian softwares as hybrid (Ala2 101) and two more (Ala2 56 and Jac 12) were identified by NEWHYBRIDS. These results suggest that hybridization may be bidirectional and may be detected in other populations than Álava. Nevertheless, the signal of admixture is stronger in Iberian hare and, in this work, in the Álava sample. If hybridization happens in low levels, as the data suggests, and given the number of frequent private alleles of brown hare is higher than the Iberian hare ones, it is expected that one way introgression from brown to Iberian hare is easier to detect. The low frequency of hybridization could also mean that the admixed specimens exist only in a narrow area where the two species meet and hybridize. Since this is the first work clearly arguing for hybridization between Iberian and brown hare, the exact geographical location and the width of the hybrid zone between these two species is not known. Álava is not only our largest sample but also the most exhaustive sampling site of the contact zone, which could explain why only there robust evidence for the existence of hybrids were found. An experimental work using transects across the contact zone could help solving this question. Nevertheless, the increase in the MNA per *locus* even in samples where admixture is not detected (in Iberian hare samples of Navarra and Zaragoza and brown hare samples of Navarra and Jaca) may indicate an import and posterior spread of foreign alleles within each of the species. This scenario demands the existence of backcrosses, which would at the same time render this hybrid zone as an effective one, given the occurrence of fertile mixed individuals. As seen by the results obtained from the NEWHYBRIDS analysis, all the admixed individuals were assigned to post-F1 generations of hybrids, fitting these expectations. NEWHYBRIDS was run incorporating prior knowledge about parental samples since these were not only morphologically identified but strongly corroborated by STRUCTURE. In this way, using priors for the parental samples enhanced the discriminatory power of the software. Recently, Estonba *et al.* (2006), using a set of microsatellites markers as well, concluded that there is no

admixture between Iberian and brown hare. However, given the low level of hybridization found here and the apparent narrow distribution of this event, it is understandable that a considerably smaller and sparser sampling work did not show any signs of genetic admixture.

Although mainly focusing on the existence of a hybrid zone between Iberian hare and brown hare, our results can help to clarify some aspects of the past dynamics of these two species. Apparently, the contact zone between *L. granatensis* and *L. europaeus* resulted from a secondary contact of previously differentiated and allopatric species. The Iberian hare has presumably been present in the Iberian Peninsula for quite some time, since it is endemic to this region. However, during the glacial periods it could have been pushed to lower latitudes and experienced a recent northwards post glacial expansion as documented for other species, such as the wild rabbit (Branco *et al.*, 2002). As for the brown hare, even though it is still a matter of debate, several data argue for a refugial area in the Balkans region and subsequent expansion to west during the post-glacial period, arriving recently to the Iberian Peninsula (Pierpaoli *et al.*, 1999; Kasapidis *et al.*, 2005). These historical scenarios could lead to reduction of genetic diversity from South to North in the Iberian hare within Iberia and in brown hare from East to West across Europe, sharpened by the crossing of the Pyrenean mountains. However, the detected values of genetic diversity, even when calculated without foreign alleles, do not conform to this expectation. Instead, Iberian hare samples of northern Spain display similar (MNA and H_o) or even higher values (H_e) of genetic diversity parameters than the southernmost samples. This result, coupled with the very low pairwise F_{st} within this species, argues for a historical stable metapopulation of Iberian hare with high gene flow, as also suggested by allozymes data (Alves, 2002). Concerning the brown hare, comparing with the Balkans population (Estonba *et al.*, 2006), the MNA decreases in a westwards direction (Balkans samples > Central Europe samples > Northern Iberia samples), fitting the hypothesized colonization route. As for the heterozygosities, however, higher values are attained by the Balkans population, whereas the Central European samples are similar to Northern Iberian ones. Such pattern could be explained by several colonizations of northern Spain by brown hares with different genetic pools, establishing a good subsample of the Central European genetic stock. The microsatellites

mutation rate and size-constraint may also account for the homogenization of genetic diversity values. Together, these results join the body of evidence for a Balkan origin of the European colonization by brown hare (Kasapidis *et al.*, 2005), as opposed to the possibility of the existence of an Iberian glacial refugium of this species.

Conclusion

The morphological identification of specimens was almost completely supported by molecular data (99,7%), suggesting that these three hare species maintain enough distinctive physical traits even when occurring in parapatry. In spite of the absence of diagnostic *loci* and the possible homogenising effect of homoplasmy caused by the high mutation rate of microsatellites combined with an allele size constraint phenomenon, we have provided unequivocal evidence of ongoing hybridization between *L. granatensis* and *L. europaeus* in Northern Iberian Peninsula. This hybridization seems rather rare and restricted to a narrow area of contact between the species, which has made it previously undetected. This might also result from the very conservative conditions adopted in this work to consider individuals as hybrids or to a low resolution power of the used microsatellites set. The directionality of the introgression, its true level or the exact location and width of the hybrid zone, are questions that need to be reassessed with the use of high resolution tools such as diagnostic single nucleotide polymorphisms coupled with thin scale sampling of the hybrid zone. Some demographic scenarios were also tackled, with genetic diversity parameters arguing for the Balkans origin of Western Europe colonization by the brown hare and for a stable Iberian hare metapopulation with high gene flow within Iberian Peninsula. The Iberian and brown hare seem to be contributing to the old Remington's concept of a suture zone, in this case located in north-eastern Iberian Peninsula.

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Paper 2

Finescale analysis of a hybrid zone between Iberian and brown hare: integration of genetical and ecological data

Abstract

A hybrid zone is an area of more or less pronounced transitions between states. The widespread phenomenon of hybridization uncovers its important role in speciation events. Distinct characteristics define hybrid zones, and even hybrid zones between the same pair of species may differ from place to place, raising the question of what importance has the environment and ecological conditions. In the genus *Lepus*, a hybrid zone exists in northern Spain between *Lepus granatensis* and *L. europaeus*. Using geographical information of sampled individuals, a small area was screened for six microsatellites locus and cytochrome *b* mitochondrial DNA gene. Also, topographical and meteorological conditions were integrated in the analysis, providing some insight into both species ecological requirements. Microsatellites results were found to fit previous morphological species identification, whereas mitochondrial DNA displayed distinct patterns. A unidirectional introgression of Iberian hare mtDNA into seventeen brown hare individuals was detected, strengthening the evidence that support the existence of a hybrid zone between both species. The Iberian and brown hare distribution in the study area (as indicated by morphological and microsatellites data) was found to be significantly associated with some ecological variables, in contrast with the distribution of the different mitochondrial lineages. In this work, strong evidence of hybridization using mtDNA was found and a first approach to landscape genetics in the area was performed.

Introduction

In order for a hybrid zone to be established and noticed, at least two evolutionary units must come into contact. Also, these units must be to some extent genetically differentiated, or else they would simply immediately merge into a homogeneous group. Although the way by which differentiation is achieved might be discussed (Harrison, 1990; Barton & Hewitt, 1985), what seems clear is that in every hybrid zone some kind of discontinuity is detected. Transitions in morphology, behaviour or molecular information (Harrison, 1990), among others, are characteristic of these contact zones, which have been modelled into the cline theory (Barton, 1979; Barton & Hewitt, 1985). The maintenance and degree of concordance of clines is a result of several variables such as the level of reproductive isolation, selection or environmental factors (Barton & Hewitt, 1985; Hewitt, 1988; Harrison, 1990). Also, these transition zones define barriers to gene flow, where sexual selection (McDonald *et al.*, 2001), prezygotic (Servedio, 2001) or postzygotic isolation (Szymura & Barton, 1991) may account for it. However, these barriers appear to be more porous than previously thought (Rundle *et al.*, 2001) as evidence of genetic exchange between species of several kinds of organisms accumulates. Indeed, hybridization seems to be a widespread phenomenon probably with an important role in the process of speciation and acquisition of genetic diversity by increasing the “field of recombination” (Harrison, 1990).

The study of hybrid zones has been based in various characters and markers, as morphology, behaviour, allozymes, mitochondrial DNA, microsatellites and non-neutral nuclear markers, and although all have been found to overcome the genetic barrier at some extent, discordances have been depicted in the speed, patterns and amount of introgression in a “foreign” background. None of these parameters can be seen as independent, and neither can be their causes. The degree of reproductive isolation of genetically distinct organisms is certainly important for the amount of hybridization, but ecological requirements have a significant role as well. Patterns of introgression will characterize the genetic architecture of the barrier. As for the speed of introgression, alleles with even a small selective advantage will penetrate relatively quickly if compared with neutral alleles, even though phenomena as linkage disequilibrium,

hitchhiking and background selection can blur this association (Barton & Hewitt, 1985; Piałék & Barton, 1997).

A clear discordance in introgression characteristics has long been found between nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) (Powell, 1983; Tegelström, 1987). Moreover, it seems to be a common feature: fruit flies (Ballard *et al.*, 2002), cichlid fishes (Rognon & Guyomard, 2003), rodents (Božíková *et al.*, 2005; Ruedi *et al.*, 1997), newts (Babik *et al.*, 2003) or songbirds (Rohwer *et al.*, 2001) are some examples where discordant patterns of introgression have been detected, where mtDNA introgression is not accompanied by or at least is more extensive than nDNA.

In Northern Iberian Peninsula, two species of hare meet in a long contact zone (Figure 1). The Europe-spread *Lepus europaeus* (the brown hare) and the Iberian Peninsula endemics *Lepus granatensis* (the Iberian hare) hybridize to some extent in that area, as evidenced by microsatellites markers (Chapter II) and preliminary results in locus *e* (Melo-Ferreira *et al.*, 2004), but still maintain distinctive gene pools at this nuclear neutral markers level. The scenario for mtDNA, however, is expected to be complicated by the described picture of massive ancient introgression in both brown and Iberian hare by the mountain hare (*Lepus timidus*) mitochondrial genome (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005). In fact, Melo-Ferreira *et al.* (2005) report the complete replacement of the native brown hare mtDNA by the mountain hare one in some hare populations of Northern Iberia, namely in the region of Alava, where this study is focused (Figure 1).

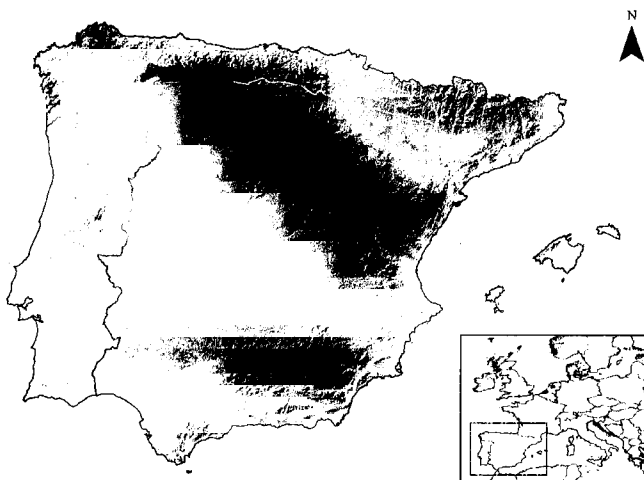


Figure 1: Iberian and brown hare populations from a small study area (red square) in the province of Álava, Spain were surveyed for microsatellites and *cytb*. The study area is located in northern Iberian Peninsula and comprises the contact zone between *L. granatensis* and *L. europaeus* (yellow line).

We surveyed Álava's hare populations of brown and Iberian hare for six nuclear microsatellites and mitochondrial DNA *cyt b* gene, and geo-referenced all the individuals. We expect to check for the concordance between both types of markers, hypothesizing the influence of ecological conditions, since the Cantabrian Mountain defines an ecotone which divides both species distribution as identified in a morphological basis. Given that our study area and experimental design are inadequate for a cline work, we used the recently developed software GENELAND (Guillot *et al.*, 2005) to integrate genetical and spatial information. Also, in a geographic information system (GIS) framework, we overlap several ecological data to draw some ideas about species dynamics. Although the ecological requirements for the brown and Iberian hare are not fully understood (Flux & Angermann, 1990), their allocation in this area of abrupt transition in habitat conditions suggests some influence of the habitat in species distribution. Our results reveal an unusual case of a species (brown hare) that harbours three different mitochondrial genomes and may be a first approach to the modelation of brown and Iberian hare species distribution in Iberia, leading to a finescale plot of the hybrid zone between them.

Materials and Methods

Study area

The study area is located in the province of Álava, in the Spanish Basque Country, and comprises around 60x50 km (Figure 1 and Figure 2). The Cantabrian Mountain represents an ecotone, dividing this region in two main areas in a north-south axis. Both areas differ in climatic conditions, leading to habitat heterogeneity. Hare species, identified in a morphological basis, mimic the habitat transition, with brown hare occupying the northern zone whereas Iberian hare inhabits the southern one.

Sampling

Ear tissue samples were obtained during the official hunting seasons between 2000 and 2005 and stored in 70% alcohol. Forty five specimens of Iberian hare and 51 of brown hare were collected. For all individuals coordinates were taken in WGS84/UTM coordinate system.

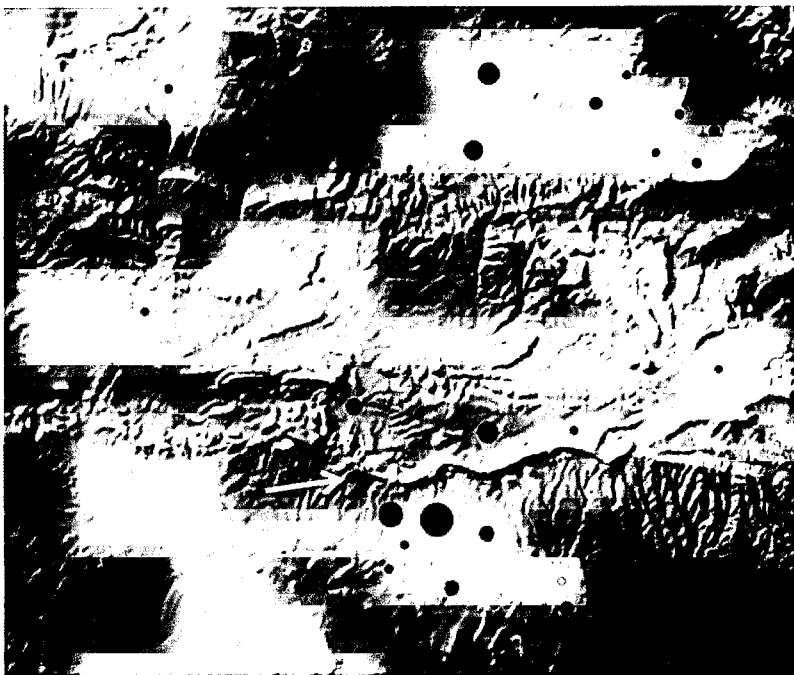


Figure 2: Study area (red box in Figure 1) with sampling coordinates. The size of dots is proportional to the number of individuals collected. The Cantabrian Mountain is indicated by a yellow arrow.

Microsatellites and mtDNA typing

Total genomic DNA was extracted from ear tissue using a standard high-salt protocol (Sambrook *et al.*, 1989). Every individual was typed for the six microsatellites set used in Chapter II following the described conditions. As for mtDNA, a 669bp fragment of *cyt b* was screened with a PCR-RFLP approach as described by Melo-Ferreira *et al.* (2005).

Ecological data

Using geographic information system (GIS) software, several parameters for the study area were obtained. From WorldClim (Hijmans *et al.*, 2005), we use data of precipitation (measured in millimetres), maximum temperature and minimum temperature (measured in °C). All data was averaged to annual mean values. From a Digital Elevation Model, elevation (measured in meters), slope (measured in degrees relative to horizontal) and aspect (measured in degrees relative to north in clockwise direction) were calculated.

Analysis

Data was analysed in a mixed genetical and spatial framework. This was done using the software GENELAND (Guillot *et al.*, 2005), which tries to detect population structure using geo-referenced individual multilocus genetic data. Microsatellites and mtDNA data were analysed separately and results were compared. Assuming that mtDNA is recombination-free, individual haplotypes were coded as completely species diagnostic genotypes to fit GENELAND analysis. All the ecological parameters were integrated in a GIS software (ArcMap9, ESRI, 2004) and their values pulled for each individual coordinate. Significance analyses were performed by a Mann-Whitney U test for two different groupings of individual coordinates. First, coordinates were separated by species, i.e., coordinates where brown hare occurs were tested against coordinates where Iberian hare is found. Second, coordinates were separated by the mtDNA haplotype found, being one group constituted by the mountain hare-like haplotype and the other by non-mountain hare-like haplotype.

Results

The microsatellites analysis clearly identified all the individuals with at least 96% of posterior probability of assignment to brown or Iberian hare species.

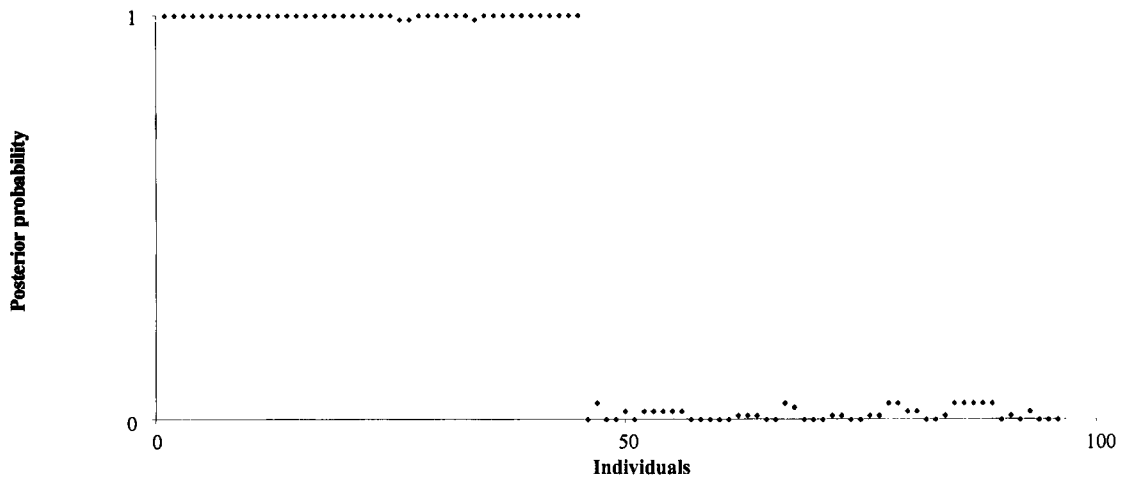


Figure 3: Posterior probability of individual assignment to Iberian hare using microsatellites data and GENELAND software. A clear identification of Iberian hares (value 1) and brown hares (value 0) was obtained.

This result was concordant with the previous morphological identification of all the specimens. There was no evidence of hybridization detected in this analysis. Moreover, the transition in microsatellites genotypes accurately mimics the location of the ecotone in the study area. Consequently, it fits the transition in habitat conditions and is concordant with the species distribution (Figure 4A and 4C). However, the scenario is very different for the mitochondrial DNA. With this marker, three groups were detected, each corresponding to the native haplotype of brown, Iberian or mountain hare (Figure 4B). The geographic distribution of each of the haplotypes in the study area is not concordant with the microsatellites, habitat conditions or species distribution. Instead, mountain hare haplotypes are widespread in the study area and occur both in Iberian and brown hares (31% and 63%, respectively), as identified by morphological traits and microsatellites; Iberian hare haplotypes can be found in 69% of Iberian hare individuals and in 33% of brown hares; and the native brown hare mtDNA haplotype occurs in only 4% of brown hare specimens, from the same coordinate.

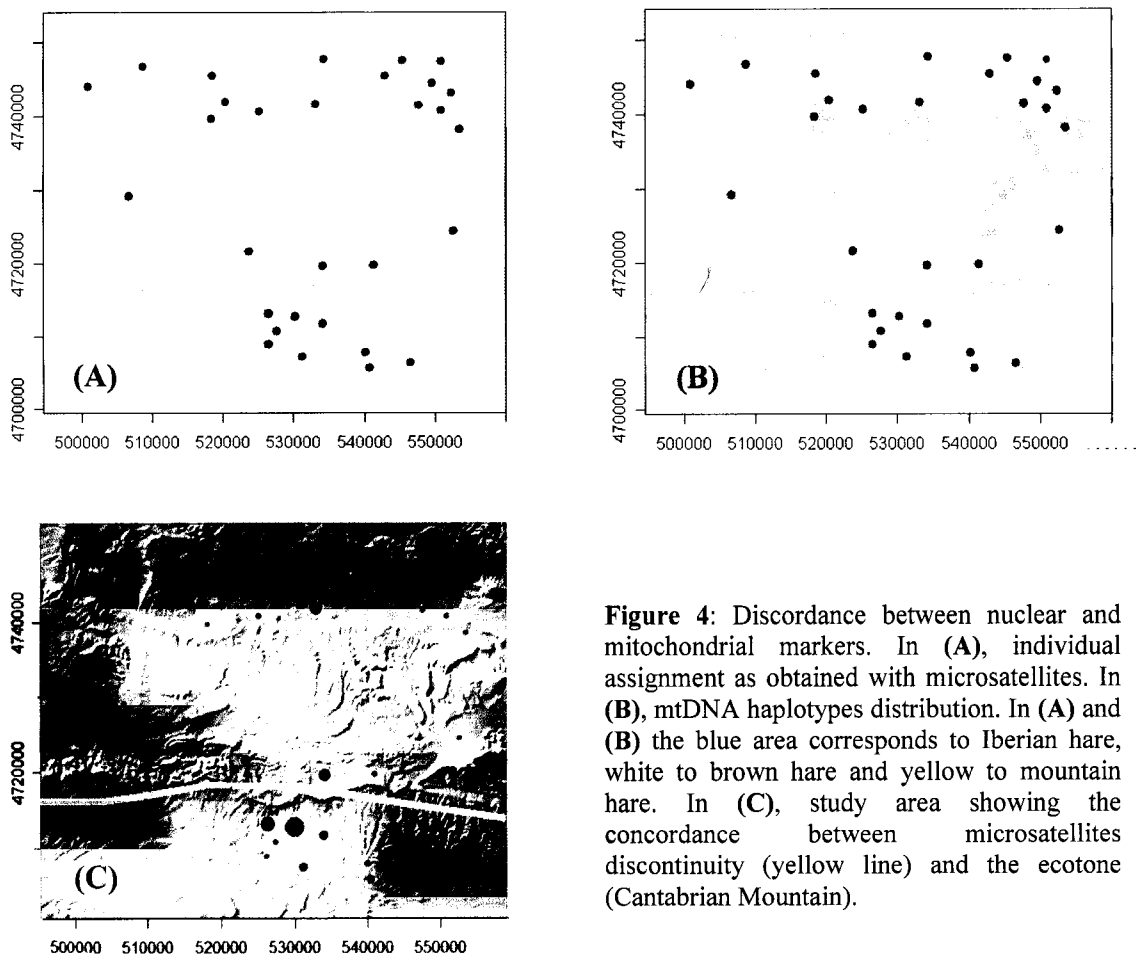


Figure 4: Discordance between nuclear and mitochondrial markers. In (A), individual assignment as obtained with microsatellites. In (B), mtDNA haplotypes distribution. In (A) and (B) the blue area corresponds to Iberian hare, white to brown hare and yellow to mountain hare. In (C), study area showing the concordance between microsatellites discontinuity (yellow line) and the ecotone (Cantabrian Mountain).

As for the ecological parameters, significant differences can be found between the brown and Iberian hare coordinates for all the studied parameters except for the slope. In fact, the detected differences between the northern and southern regions of the study area are highly significant, with p -values equal or lower than 0,054 (Table 1). The coordinates of brown hare show higher values of precipitation (Prec), lower minimum temperature (Tmin) and maximum temperature (Tmax), higher elevation and are placed in colder places, since their mean Aspect is $\sim 238^\circ$ (southeast), in contrast with the south-facing Iberian hare coordinates ($\sim 181^\circ$). In general, the habitat to the north of the Cantabrian Mountain is cooler and rainier, while to the south the habitat is hotter and drier. When we compare points where the mountain hare mtDNA haplotype occurs with

points where we find non-mountain hare haplotypes, significant differences are found in T_{min}, Prec, Slope and Aspect. The *p*-values for these significantly different parameters range between 0,002 and 0,035. In this comparison, T_{min}, Prec and Aspect display higher values in mountain hare haplotype coordinates.

| Variable | <i>p</i> -value Lg vs Le | <i>p</i> -value Lt haplo vs non-Lt haplo |
|------------------|--------------------------|--|
| T _{max} | 0,000 | 0,332 |
| T _{min} | 0,054 | 0,002 |
| Prec | 0,000 | 0,035 |
| Elevation | 0,000 | 0,181 |
| Slope | 0,635 | 0,015 |
| Aspect | 0,000 | 0,008 |

Table 1: Mann-Whitney U test *p*-values for each variable compared between *L. granatensis* and *L. europaeus* coordinates (*p*-value Lg vs Le) and compared between coordinates where *L. timidus* haplotype occurs and coordinates where non-*L. timidus* haplotypes are found (*p*-value Lt haplo vs non-Lt haplo) .

Discussion

The microsatellites analysis for the hare population from Álava was totally concordant with the previous identification, in a morphological basis, of the species to which every individual belongs. Although a hybrid zone between Iberian and brown hare is present in this region, as shown in Chapter II, morphological differences between both species are maintained and allow an accurate identification. The low amount of current hybridization may justify this, since the hybrid individuals included in the analysis are the result of some number of generations of backcross (Chapter II). These hybrid individuals are not detected in the present work, what may stem from the difference in sampling between Chapter II and Chapter III and from the absence of parental populations in the latter. Also, the presence of alien alleles in both species (Chapter II) may contribute to mask admixed individuals, especially when using non-diagnostic loci. Nevertheless, the microsatellites markers used have enough information to clearly separate Iberian from brown hare. In contrast with microsatellites information,

mitochondrial DNA presents a different scenario. The finding of mountain hare haplotypes in both Iberian and brown hares was expected, given the reported massive ancient introgression that occurs in the north of Spain (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005). However, Melo-Ferreira and colleagues showed that the brown hare population from Álava was fixed for the mountain hare haplotype, which is not corroborated by this work. Our sample is more than twice the used in Melo-Ferreira *et al.* work (51 vs. 24) and that may account for the discrepancies. First, in our analysis native brown hare mtDNA haplotypes are found, although in low quantity (~ 4%). Second, we detected the presence of Iberian hare haplotypes in brown hare individuals. This unidirectional introgression of mtDNA from Iberian to brown hare strongly supports the hybridization scenario for this pair of species, strengthening the findings of Chapter II. The mtDNA introgressed brown hares represent 33% of the brown hare sample. This is a more than three-fold higher amount of introgression than the one found from mountain to brown hare in Sweden (Thulin *et al.*, 1997), five-fold higher than the one from brown to mountain hare found in Russia (Thulin *et al.*, 2006a) and about ten times higher than the reported introgression from brown to mountain hare in Sweden (Thulin *et al.*, 2006b).

Another interesting issue is the discordance between the mitochondrial and nuclear genome introgression patterns. Both markers have very distinct and fundamental differences. The mtDNA shows a uniparental (maternal) inheritance, haploid nature, lack of recombination (although this has been challenged by several authors, see Rokas *et al.*, 2003 for a review) and a smaller effective population size (1/4 of the nuclear genome), while nDNA is the complete opposite of these features. So, one might also expect discrepant behaviour of both markers in natural populations, which indeed happens. Commonly, mtDNA and nuclear markers are discordant in their patterns, and this may well be considered a broadly distributed dynamics, since it is found in a variety of organisms which display a multitude of dissimilarities in mating behaviour, sexual selection, dispersal or ecological requirements, for example. Cichlid fishes from West Africa (Rognon & Guyomard, 2003) show a strongly supported separation of two species with allozymes data, but an almost complete replacement of one species mtDNA for the other species mitochondrial genome. This has also been

found in other species of fish, as the arctic char and the lake trout (Wilson and Bernatchez, 1998) or the bull trout and the Dolly Varden (Redenbach & Taylor, 2002). In newts, even though some nuclear introgression is detected, mtDNA introgression is much more pronounced and bidirectional, even if a 14% excess of one of the species is seen (Babik *et al.*, 2003). Another noteworthy work was performed in commensal mice, where two transects (one in Czech Republic and one in Bavaria) were analysed (Bozikova *et al.*, 2005). The authors of this last work found that mtDNA clines fell into the range of the allozymes but not with that of X markers. An interesting feature of this study is that the mtDNA introgression occurs from one species to the other, but the direction changes between the Czech and the Bavarian transects. The differential introgression of mtDNA and nDNA has been reported for several other organisms, from insects to reptiles, or molluscs to mammals (for a list, see Chan & Levin, 2005). In the present case, microsatellites reflect the present species distribution with weak signs of hybridization (Chapter II), while mtDNA might represent a long-lasting introgression from Iberian to brown hare. Due to its transmission pattern, mtDNA signal will be kept long after the nuclear genome is erased (Roca *et al.*, 2005). The direction of the mtDNA transfer is concordant with the principle enounced by Grant & Grant (1997), which states that when two species meet and hybridize, it is expected that males from the bigger species (here, the brown hare) mate with females from the smaller (Iberian hare).

As mentioned before, the genetic discontinuity found for the microsatellites is coincident with a topographical barrier found in the field, the Cantabrian Mountain (Figure 4A and 4C). With steep slopes and considerable elevation, this mountain poses a very hard obstacle for some animals, as hares, to overcome. Moreover, the Cantabrian Mountain not only rises as a physical barrier, as it interferes in the climatic conditions of the habitat. The significant differences in precipitation, minimum and maximum temperature, aspect and elevation between the coordinates of brown and Iberian hares (as identified morphologically and confirmed with microsatellites) are concordant with a broad scale description of habitat preferences for both species (Palacios & Meijide, 1979). In fact, the brown hare is apparently better adapted to colder and wetter places: it occurs in areas with higher precipitation, with lower minimum and maximum temperature, with higher elevation and in colder hills (higher values of aspect). As for

the comparison between coordinates registering the occurrence of mountain hare mtDNA haplotype and coordinates with non-mountain hare haplotypes, it aimed to check if the mountain hare haplotypes were preferably distributed in colder places, which would be a suggestion for the positive selection of the “cold-mitochondria” (Melo-Ferreira *et al.*, 2005). This trend was verified by the values of precipitation and aspect (higher values for mountain hare haplotypes) but contradicted by the mean value of minimum temperature, which was higher for the mountain hare haplotypes coordinates. So, no clear tendency can be inferred by these analyses. A bigger and more homogeneous sampling of the study field, as well as thinner information of climatic variables could be helpful to solve this question, since it appears to be a valuable approach to the problem.

Conclusion

The widespread phenomenon of natural hybridization also occurs among *Lepus* species. It has been proved to happen by the first time in Sweden by Thulin *et al.* (1997) and, more recently, by Alves *et al.* (2003) in the Iberian Peninsula. However, in both studies mountain hare seems to be involved in the genetic exchange. Here, robust evidence for mtDNA unidirectional change from Iberian to brown hare was found in a considerable amount (~33%). At the species level, the brown hare in northern Iberia attains a peculiar situation, since it displays three different mitochondrial genomes with no apparent decrease in fitness. Cytonuclear disequilibria studies should be performed to clarify this question. Another interesting issue is the discordance between mtDNA and nDNA patterns. The latter follows the habitat transition set by the Cantabrian Mountain, whereas the former presents a more complex scenario.

This work might be the first step in the landscape genetics of Iberian and brown hare. Overlapping the species distribution and microsatellites data with ecological variables, models can be designed to predict the correct location of the hybrid zone along northern Spain. A transect study should also be performed across the hybrid zone

to characterize it in a quantitative way (width, cline steepness and cline concordance), but the first indication of markers discordance in these species is set. Also, Y and X chromosome markers would be a natural development of such work, in order to assemble for these species the histories of uni (maternal and paternal) and biparentally inherited genomes.

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IV. General Discussion

Natural hybridization is an evolutionary phenomenon that contributes to the dynamics of natural populations of organisms. Common to plants and animals, it should not be seen as a failure to the reproductive isolation (Arnold, 1997). However, the degree of differentiation between organisms is correlated to their ability to hybridize, although the degree of this dependence is arguable. Climate oscillations, with the advance and retreat of ice sheets, are a recognized fundamental process to promote divergence between organisms in allopatric refugia and posterior secondary contacts, giving rise to several contact or hybrid zones. Obviously, these events of population expansion and retraction involve several stochastic (e.g., genetic drift) and/or selective processes (e.g., adaptation to new unoccupied habitats along colonization routes), which leave a genetic signature and shape the genetic patterns we see today.

In this work, the assessment of natural hybridization between two species of hare, the iberian hare (*L. granatensis*) and the brown hare (*L. europaeus*), in northern Iberian Peninsula was the main objective. Despite the existence of a long contact zone between both species in northern Iberia, hybridization between Iberian and brown hare has not been described and has even been discarded (Estonba *et al.*, 2006). Here, a more detailed analysis of the potential admixture between these species was attained by the use of a larger sampling and by the use of new analytical methodologies. Both nuclear and mitochondrial markers were used to obtain a more complete scenario of Iberian and brown hare histories and interaction. The genetic structure of both species was also described and used to infer possible phylogeographic scenarios that fit species history. This is discussed in Section 1 of the General Discussion. Then, natural hybridization between the analysed species of hares, as inferred from microsatellites and cytochrome *b* mitochondrial gene, is discussed in Sections 2 and 3, as well as the comparison between both markers patterns. The integration of genetic and ecological data, and the role of habitat in species distribution, are discussed in Section 3. A critic to the application of different methodologies is presented in Section 4 and, finally, several possibilities to expand this work in the future are presented in Section 5.

1) Genetic structure

In this work, three hare species had to be clearly distinguished at the molecular level. A set of six microsatellites, comprising four originally developed for the wild-rabbit, one specifically developed for African hares and one adapted for the brown hare, proved to be polymorphic and to separate the three studied hare species, although in a frequency base (see Chapter II). The high amount of homoplasy could constitute a problem, as happens in the Iberian Peninsula wild-rabbit where microsatellites do not distinguish between two well differentiated lineages (Queney *et al.*, 2001). This, however, does not seem to be the case in hares. In fact, both the Factorial Correspondence Analysis (FCA) and the Bayesian STRUCTURE analyses displayed each species as a different cluster, which is corroborated by the high F_{st} estimates between species ($F_{st(Lg-Le)} = 0,353$; $F_{st(Lg-Lt)} = 0,205$; $F_{st(Le-Lt)} = 0,208$). This resolution power allowed to exclude the possible introgression of nuclear alleles from *L. timidus* as was reported for the mtDNA (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005) and focus in the hybridization problem between the Iberian and the brown hare, the main purpose of this work. In fact, two brown hare individuals from the Iberian Peninsula display some signs of admixture with mountain hare. We may hypothesize that this results from an ancient hybridization event with mountain hare in the Iberian Peninsula but the long term absence of this latter species from the region (Sesé, 1994; Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005), along with the fast erasing rate of nuclear markers (Roca *et al.*, 2005) and the non-existence of such signal in the Iberian hare (Chapter II), argue against such hypothesis. Another possible explanation would be the import of mountain hare alleles through present hybridization events and dispersal within brown hare. Suchentrunk *et al.* (unpubl. data) described present hybridization events between both species in Central Europe, which may be supported by the detection, in this work (Chapter II), of two Austrian (Aus 21 and LE Aus 26) and one Italian Alps (Alp 832) specimens with admixture signs. Also, long distance gene dispersal within brown hare is described (Suchentrunk *et al.*, 2000) and, together, both phenomena could explain the presence of mountain hare signal in Iberia. Finally, we must not eliminate the possibility of lack of resolution of the markers for some particular multilocus genotypes. The increase in markers numbers should be enough to eradicate this problem.

In more general terms, microsatellites reveal very interesting patterns. The Iberian hare species displays lower values of genetic diversity, perhaps reflecting small historical population size given its confinement to the Iberian Peninsula or the origin of the entire current metapopulation from a small refugial population. On the other hand, brown and mountain hare display higher, similar genetic diversity values. The analysed samples of this latter species represent two very different contexts: one (Sweden) was collected within the supposedly continuous arctic habitat of the species, while the other (Italian Alps) represents the relictual southernmost distribution of *L. timidus*. However, excepting the locus Sol30, no substantial differences between both samples are found. More than one wave of colonization of the Alps by mountain hare, as suggested by Melo-Ferreira *et al.* (in press) could have provided a representative sample of the original genetic pool, overcoming the reduction in genetic diversity usually detected in such situations. Regarding the brown hare, northern Iberia and Central Europe samples display similar values of diversity (heterozygosities and mean number of alleles per locus (MNA)). However, removing the putative foreign alleles (i.e., alleles that only occur in Iberian hare parental samples) that occur in the Spanish samples leads to a considerable decrease of their MNA values. Together with the genetic diversity analysis performed by Estonba *et al.* (2006) in Bulgarian *L. europaeus*, a westwards decrease in genetic diversity parameters can be observed, especially the MNA. The Iberian hare also shows an increment in MNA values of the contact zone samples, while heterozygosities remains stable among all samples. These results argue for a stable metapopulation of Iberian hare with high gene flow between subpopulations within Iberia, in agreement with the allozyme data described by Alves (2002), and for a westwards colonization of Europe by the brown hare, with origin in a Balkans glacial refugium, in concordance with Suchentrunk *et al.* (2003) and Kasapidis *et al.* (2005). A recent post-glacial contact between both species apparently provided the conditions for the occurrence of hybridization, which is supported by the high values of MNA in the contact zone samples.

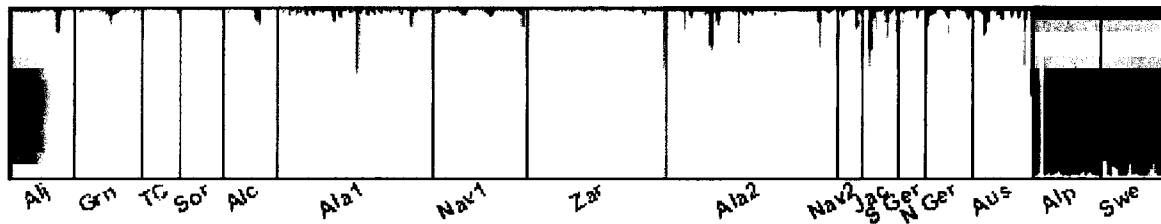
2) Natural hybridization in hares

In hares, natural hybridization has been an overlooked subject. It has been mostly approached as a secondary issue in phylogenetic and phylogeography studies (Pérez-Suarez *et al.*, 1994; Pierpaoli *et al.*, 1999; Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005; Estonba *et al.*, 2006) and not as a main question with potentially evolutionary importance. Within the genus *Lepus*, only the contact zones between the brown and mountain hare in Sweden (Thulin *et al.*, 1997; Thulin *et al.*, 2006a), Russia (Thulin *et al.*, 2006b) and Switzerland (Suchentrunk *et al.*, unpubl. data) have been screened for the existence of hybridization, which was found to occur. Together, these studies used mtDNA, microsatellites and allozymes to search for admixture signs. The use of diverse types of markers allows answering to distinct questions, since their mode of transmission, selective pressures and intrinsic dynamics is different. In hybrid zones, discordant introgression patterns of the markers are often detected, especially if uniparental and biparentally inherited markers are compared (Chan & Levin, 2005). In this work, the question of hybridization between the Iberian and brown hare in northern Iberian Peninsula was first approached with microsatellites (Chapter II). The use of mtDNA for this purpose was prevented by the massive ancient introgression of the mountain hare mitochondrial genome into both Iberian and brown hare, previously described by Alves *et al.* (2003) and Melo-Ferreira *et al.* (2005). Nonetheless, mtDNA was used to check for admixture between Iberian and brown hare in a large thin scale sample (Chapter III).

2.1) Patterns revealed by microsatellites

The microsatellites set used in this work was similar in size (six) to the one used to distinguish the three hare species from Iberia (brown, broom and Iberian hare) by Estonba *et al.* (2006). Three of the microsatellites were common to both studies. Both sets of microsatellites allowed a clear distinction between the Iberian and the brown hare, and the one used in this thesis also identified the mountain hare as a separate cluster (see Section 1 and Figure 1).

Figure 1: Identification of the three species by STRUCTURE. In blue are the Iberian hare samples; in light brown the brown hare samples; in dark brown, the mountain hare samples.



Hybridization between Iberian and brown hare was discarded by Estonba *et al.* (2006) but, using a larger and more exhaustive sampling of the contact zone, robust evidence for it was detected in the sample of Álava (Chapter II). Four specimens, previously attributed to Iberian hares in a morphological basis, were identified by the three used softwares as intermediate between both species. NEWHYBRIDS gives them high probabilities of representing backcrosses with Iberian hare, which could justify their morphological identification. If we use less restrictive conditions (i.e., not demanding the intersection of all softwares) to accept potential hybrids, three more individuals from Álava (one Iberian hare and two brown hares) and one from Jaca may be considered as hybrids. These results point to a bidirectional introgression of alleles, which is in agreement with the MNA values, that display and increase in all the contact zone samples, either from Iberian or brown hare. Asymmetry in introgression may, however, occur. Demographic reasons such as differences in density between species in the contact zone may justify it (density-dependent hybridization; Wirtz, 1999), as well as distinct behaviours (competition for females, for example, is only described for brown hare; Flux & Angermann, 1990). Another bias in this study is the genetic diversity of species. Brown hare has a wider range of frequent alleles, and a higher amount of those are private to brown hare species, in comparison with the Iberian hare. This leads to an easier detection of introgression from brown to Iberian hare, but does not exclude the converse. More markers should be used in the future to account for this problem. From our data, Álava seems to be the region with higher amount of hybridization. This can however be merely due to a sampling bias. The sample of Álava is the largest of the work and was collected exactly in the transition between both species, while the other

samples are more distantly located from the contact zone. Therefore, and since we expect a reduction in the hybridization signal with the increasing distance from the center of the hybrid zone, the results may simply be interpreted as a resolution problem and not a real pattern. Increasing samples and sampling closer to the hybrid zone should contribute for clarifying this issue.

2.2) Patterns revealed by mtDNA

At the mtDNA level, introgression of mountain hare native genome was expected in the northern Iberia populations, but not in hare populations outside Iberia or in southern populations within Iberia (Alves *et al.*, 2003; Melo-Ferreira *et al.*, 2005). This pattern was confirmed in this work, since no mtDNA introgression was found in Northern Germany, Southern Germany, Austria, Aljustrel or Granada samples (data not shown). However, new knowledge was obtained from the analysis of an increased sample of Álava (Chapter III), in comparison with the mentioned studies, since it allowed to detect brown hare individuals with native brown hare mtDNA haplotype and, also, brown hare specimens with introgressed Iberian hare mtDNA. None Iberian hare individual with brown hare mtDNA was found, configuring a unidirectional transfer of mtDNA from Iberian to brown hare (Figure 2).



Figure 2: MtDNA distribution in the study area. Blue corresponds to Iberian hare haplotypes; white corresponds to brown hare haplotypes; light brown corresponds to mountain hare haplotypes.

Apparently, brown hare is a species that easily captures the mtDNA from related taxa. In Sweden, unidirectional introgression from mountain to brown hare has been reported for the mtDNA (Thulin *et al.*, 1997) and now a similar unidirectional transfer from Iberian to brown hare was detected in Iberian Peninsula. The extent to which this occurs in the analysed sample from northern Iberia (~33%) is more than three-fold higher than the one found in Sweden (~10%). The opposite situation (brown hare mtDNA introgressing into other species) was detected in Sweden (Thulin *et al.*, 2006a) and Russia (Thulin *et al.*, 2006b), but this seems to occur to a much smaller extent (3,3%, 6,5%, respectively). In Switzerland (Suchentrunk *et al.*, unpubl. data), bidirectional introgression of mtDNA was found to occur, also in low amount (~6%). Thus, in the Iberian Peninsula, the brown hare harbours three different mitochondrial genomes and, curiously, its native mtDNA is not predominant (Melo-Ferreira *et al.*, 2005).

2.3) Discordance between mtDNA and microsatellites

Nuclear and mitochondrial genomes have fundamental distinct characteristics, and are differently used in population genetics studies according to them and to the proposed objectives. Mitochondrial DNA has played and continues to play an important role in population genetics and phylogeography studies (Moritz *et al.*, 1987; Ballard & Rand, 2005). The ease of getting new sequence data for a set of orthologous genes and the availability of mtDNA sequences for a wide variety of species, in part due to the absence of recombination or selection of mtDNA (although studies exist opposing to this strictly neutral role of mtDNA –Nachman, 1998; Rokas *et al.*, 2003; Bazin *et al.*, 2006), justifies the broad use of this marker. However, extreme caution must be used when inferring population history or species relatedness solely based in mtDNA (see Alves *et al.*, 2006). It is known that the history of a gene might not be the history of the species (Maddison, 1997). MtDNA is a maternally inherited genome, with none or low levels of recombination. Therefore, it behaves as a single locus, which may bias analysis. However, phylogenies based only in mtDNA are still being published, sometimes with seriously flawed conclusions (e.g. Wu *et al.*, 2005). Another potentially biasing feature of mtDNA is its high capability of jumping species barrier and introgress

into related species (Chan & Levin, 2005). On the other hand, the diploid, biparently inherited nuclear DNA (nDNA) presents a more complex dynamics. Portions of the nDNA may, for example, be under strong direct selection, may be selected by hitchhiking (Maynard-Smith & Haigh, 1974) or background selection (Charlesworth, 1994), may suffer recombination, duplications, gene conversion or may be neutral. Again, the history of a gene or a neutral portion of the nDNA might not be, and often is not, the history of the species and that is the reason why biologists started to simultaneously combine several markers and locus in their works.

In this thesis, microsatellites and mtDNA from the hybridizing hare population of Álava are found to display discordant patterns (Figure 3 of this Chapter). Such result was expected since a high level of introgression from the mountain hare is described, both for the Iberian and brown hare of this area. In fact, the latter species revealed a total replacement of its mtDNA by the mountain hare type (Melo-Ferreira *et al.*, 2005). However, as shown in Chapter III, native brown hare and introgressed Iberian hare mtDNA were for the first time detected in the brown hare population from Álava, which displays around 63% of mountain hare mtDNA, 33% of Iberian hare mtDNA and 4% of native brown hare mtDNA. Therefore, the homogenizing mountain hare mtDNA is still heavily present in Álava (63% in brown hare and 31% in Iberian hare) but it is now possible to detect a unidirectional transfer of mtDNA from Iberian to brown hare, which is not accompanied by microsatellites.

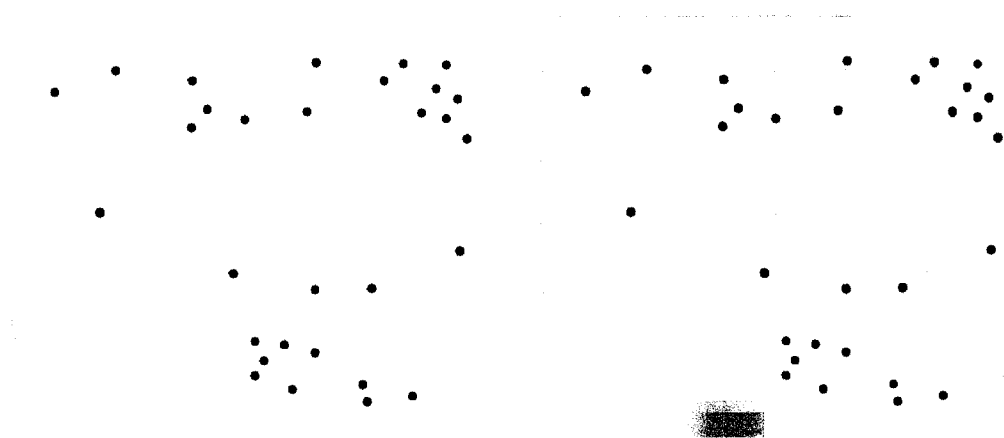


Figure 3: Distribution of microsatellites genotypes (right) and mtDNA haplotypes (left) in the study area, showing discordant patterns. Blue corresponds to Iberian hare, white to brown hare and light brown to mountain hare.

With these markers, unidirectional introgression is seen in the opposite direction (brown hare alleles in four Iberian hares) when using more conservative conditions to detect hybrids. With more relaxed conditions, another Iberian hare and two brown hares are classified as hybrids. Together with some diversity parameters that increase in the contact zone samples of both species, bidirectional hybridization may be hypothesized. The mtDNA pattern demands that Iberian hare females cross with brown hare males and that the progeny backcross with brown hare. The converse (brown hare females with Iberian hare males) is not excluded, but was not detected by the performed analysis. Also, the backcross of hybrid individuals with Iberian hares must occur, as supported by the detection of nuclear brown hare alleles' introgression into the Iberian hare. To account for this mtDNA and microsatellites pattern, density-dependent hybridization (Wirtz, 1999) can play a role, since Iberian hares seem to outnumber brown hares in the region (Ibón Telletxea, unpubl. data). The density fluctuations suffered by hares due to epidemics or hunting may also influence hybridization, since they can be quite severe and so move the density trough. Also, Grant & Grant (1997) principle (when two species meet and hybridize, crosses are usually between the females of the smaller one with males of the larger) may be acting in hares, since Iberian hare individuals are smaller than brown hares.

Discordant patterns between mtDNA and nDNA are commonly found in several organisms. For example, cichlid fishes from West Africa (Rognon & Guyomard, 2003) show a strongly supported separation of two species with allozymes data, but an almost complete replacement of one species mtDNA for the other species mitochondrial genome occurs. This has also been found in other species of fish, as the artic char and the lake trout (Wilson and Bernatchez, 1998) or the bull trout and the Dolly Varden (Redenbach & Taylor, 2002). In newts, even though some nuclear introgression is detected, mtDNA introgression is much more pronounced and bidirectional, even if a 14% excess of one of the species is seen (Babik *et al.*, 2003). Another noteworthy work was performed in commensal mice, where two transects (one in Czech Republic and one in Bavaria) were analysed (Bozikova *et al.*, 2005). The authors of this work found that mtDNA clines fell into the range of the allozymes but not with that of X markers. An interesting feature of this study is that the mtDNA introgression occurs from one

species to the other, but the direction changes between the Czech and the Bavarian transects. The hybrid zone between Iberian and brown hare in northern Spain is, thus, another case of discordance between markers, where the introgression of a third species mtDNA adds to the complexity of the pattern.

3) Association of genetic and ecological data

Explaining observed spatial genetic patterns with landscape variables led to the emergence of landscape genetics (Manel *et al.*, 2003). Its development and use was enhanced by the improvement of molecular genetics and statistical techniques, as well as the increase of computation power. The association between organisms distribution and geography is for long known and constitutes the object of study of biogeography (Crisci, 2001), whereas more recently, evolutionary biologists focused in genealogical lineages distribution in space, constituting phylogeography (Avice, 2000). However, landscape genetics uses typically thinner scale sampling than biogeography or phylogeography and aims to provide insight about the interaction between landscape features and microevolutionary processes (e.g. gene flow, genetic drift, selection; Manel *et al.*, 2003). The ability of unravelling population substructure and cryptic genetic boundaries are two of the main advantages of landscape genetics. In fact, the detection of genetic discontinuities and their correlation with environmental features (e.g., mountains, rivers, roads, gradient of humidity, and deforested areas) are two key steps of this discipline (Manel *et al.*, 2003).

In this thesis, a clear genetic discontinuity was detected in the area of Álava for microsatellites, distinguishing two classes (Chapter III). The transition between the two units perfectly fitted the Cantabrian Mountain, which divides the study area (Figure 4). Species distribution, as identified in a morphological basis, was concordant with microsatellites markers, whereas mtDNA displayed a more chaotic distribution with no correlation with the environmental feature, due to introgression (see Chapter III and Section 2 of this Chapter).

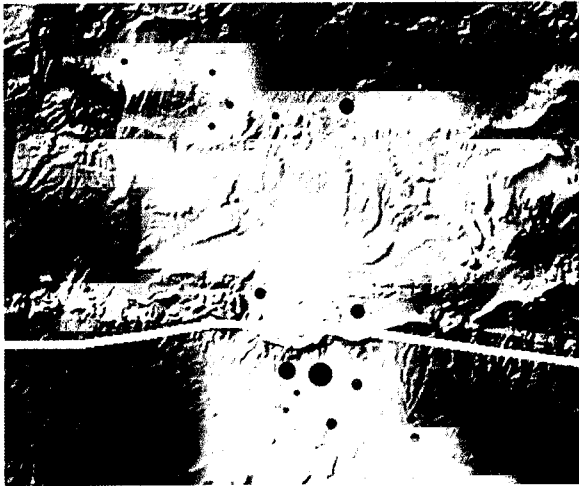


Figure 4: Species distribution fits microsatellites transition (yellow line). To north of this line occurs brown hare, whereas to the south occurs Iberian hare. The microsatellites transition fits the ecotone found in the area (Cantabrian Mountain). The size of dots is proportional to the number of individuals collected in each coordinate.

The occurrence of species transition in such a sharp ecotone suggests different specific ecological requirements. In fact, the brown hare individuals, located to the north of the mountain, occur in significantly rainier and colder spots than the Iberian hares, located to the south of the mountain (Chapter III). These results are in agreement with Palacios & Meijide (1979). One must also consider another possibility: if the hybrid zone between Iberian and brown hare is, in fact, a tension zone, it will tend to get trapped in a density trough (Hewitt, 1988). The Cantabrian Mountain may promote such a decrease in densities, and so the hybrid zone position may be a result of this phenomenon and not of an active selection of the habitat by the individuals. Also investigated was the possibility that individuals harbouring the mtDNA native of the mountain hare would preferentially occur in colder places within the study area, since this mitochondrial genome has been suggested to confer advantages in cold areas (Melo-Ferreira *et al.*, 2005). However, no evidence was found supporting this hypothesis (Chapter III). On the other hand, the result presented here may be biased by sampling. The number of individuals sampled is probably low for such study and individuals were collected in a small study area (60x50 km). The resolution used is probably inadequate to test this hypothesis and so, no conclusions can be drawn about selection for the cold of mountain hare mtDNA type.

4) The use of new analysis methodologies

The large number of programs presently available provides researchers the ability to tackle several questions and issues previously untreatable. However, the choice of how to approach a problem may be a delicate theme, since it is necessary to understand the capacity and limitations of each software to correctly apply it and, mainly, to interpret the results.

In Chapter II, the well known and widely used individual assignment Bayesian software STRUCTURE (Pritchard *et al.*, 2000) was used together with the FCA analysis of GENETIX (Belkhir *et al.*, 2004) and the more recent NEWHYBRIDS software (Anderson & Thompson, 2003). Although none of these analyses methods is new, their joint use is a novelty and is justified by the following reasoning: FCA is a canonical analysis that allowed to project individuals in microsatellites allele frequency space with no model assumption. On the other hand, STRUCTURE implements a model-based clustering method for inferring population structure using genotype data consisting of unlinked markers. This program probabilistically assigns individuals in the sample to a population or jointly to two or more populations if its genotype is found to be admixed. Finally, NEWHYBRIDS is also a model-based software that aims to compute the posterior distribution that individuals in a sample fall into different parental or hybrid categories. The objective of using these three programs was i) to apply very conservative conditions to accept the admixed ancestry of individuals and ii) to have more resolution of the interaction between the Iberian and brown hare. The first objective was achieved by only accepting as admixed those individuals identified as intermediate by the three programs; the second objective was attained since hybrid individuals were identified and also attributed to post-F1 generations of hybrids. This is very important since it means that at least some F1 individuals are fertile and reproduce. Such information cannot be confidently obtained from STRUCTURE's output, given the model underlying this software, which justifies the use of NEWHYBRIDS. The methodology used in Chapter II was, thus, very restrictive but allowed to find robust evidence of hybridization between Iberian and brown hare, which had been previously discarded (see Chapter II and Section 2 of this Chapter).

In Chapter III, the recently developed program GENELAND (Guillot *et al.*, 2005a; Guillot *et al.*, 2005b) was applied. It consists in a Bayesian model implemented in a Markov chain Monte Carlo scheme that allows inference of the location of genetic discontinuities from individual geo-referenced multilocus genotypes, without *a priori* knowledge on populational units and limits (Guillot *et al.*, 2005a). GENELAND was used to detect genetic discontinuities for microsatellites and for mtDNA in a small study area in Álava comprising the hybrid zone, or at least part of it. Both markers revealed very different patterns (see Chapter III and sub-Section 2.3 of this Chapter). These results were integrated with a GIS approach and a perfect concordance between microsatellites transition and a landscape ecotone (the Cantabrian Mountain) was found. Extending the GIS analyses, environmental (precipitation, minimum and maximum annual temperature) and habitat conditions (elevation, slope and aspect) were compared between both sides of the transition area to search for habitat preferences of both species of hares. The use of software as GENELAND is a good tool for landscape genetics and a valuable first approach to GIS modelling.

The results presented in this thesis using the mentioned softwares argue for the validity of their integrated use, taking advantage of each program potential. The assumptions underlying each of them must be taken into account in order to draw correct inferences and to have confident results. Together, the interaction between Iberian and brown hare, as well as some aspects of their ecology, were tackled during this work, which must of course be further investigated.

5) Future perspectives

This work might redefine the knowledge about Iberian and brown hare interaction and give rise to new perspectives for studying it. For the first time, evidence was found to support hybridization between these two species. Nevertheless, several questions remain to be answered. The amount of hybridization, its directionality, the centre and width of the hybrid zone, the penetrance of introgressed alleles or, more generally, the type of hybrid zone are still open issues. A well defined experimental design for a transect study of the area is needed to answer such questions.

The number of markers should be increased to have more resolution power. Microsatellites proved to be useful and increasing the number of microsatellites used could render good results. Given their high mutation rate, recent genetic novelties could be found and studied to infer some demographic parameters as dispersal. The locus Sol30 presents a possible new allele (allele 218), which is only present in the samples of Álava of both species. Sequencing this allele is important to understand the mechanism by which it arose. Other rare, clustered alleles could prove valuable for microscale demographic studies. The use of other types of markers would also help to complement and add knowledge to what is known. Typing Y chromosome markers would input the parental history to the scenario and diagnostic SNP's could refine the analyses and clarify some hypothesis.

Increasingly important information to be taken is the geographic localization of the captured individuals. A larger sampling effort in the hybrid zone and the use of adequate resolution information layers may lead to a good modelling and extrapolation of species habitat preferences. Such information would be useful not only for an academic motif but also for conservation reasons. Finally, sampling all the northern Iberia area where mountain hare mtDNA introgression occurs and using a landscape genetics approach may lead to exciting results concerning the hypothesis of selection for the cold of this mtDNA type.

Conclusions

The work presented in this thesis contributes to a better knowledge about the genus *Lepus* by analysing a particular situation, the interaction between Iberian and brown hare in northern Iberia. It may also prove important for the opening of new perspectives and new working hypothesis in that context, since hybridization between both species had been previously discarded. Some major conclusions of the performed work are:

- i) The use of a microsatellites set that distinguishes Iberian, brown and mountain hare allowed to discard a massive introgression of mountain hare nuclear DNA into both Iberian and brown hare, as described for mitochondrial DNA (mtDNA). The shallow evidence for such phenomenon may stem from the used resolution power and so increasing the number of markers should clarify this situation.
- ii) Robust signs of admixture between Iberian and brown hare were detected, both at the microsatellites and mtDNA level. The direction of introgression is opposite for both type of markers, which can be explained by demographic processes, but reciprocal introgression is still a hypothesis that deserves to be further investigated. The occurrence of introgressive hybridization demands the existence of fertile admixed offspring, as supported by the detection of post-F1 hybrid individuals. In this way, the hybrid zone is effective and, in this case as in several others, F1 individuals are rarely observed.
- iii) As described for several other taxa, discordance between nuclear and mitochondrial DNA patterns was found. This stresses the need of using different kinds of markers to prevent the bias in the analyses introduced by differences in their dynamics, contributing to obtain a reliable history of the species.

- iv) The genetic diversity data obtained during this work joins the body of evidence for the colonization of Western Europe by brown hare with origin in the Balkans, having recently arrived to the Iberian Peninsula. On the other hand, Iberian hare may fit a south to north colonization scenario within Iberian Peninsula, but a stable metapopulation with high gene flow between populations within this region is also plausible.

- v) The genetic discontinuity found at the level of microsatellites mimics species distribution, as identified in a morphological basis. It also perfectly fits the ecotone found in the study area, pointing in the direction of the important role environment may play in species geographic allocation. In general terms, brown hare occurs in more humid and colder areas than Iberian hare, which should be extended and tested in different regions, namely in thin scale analyses and ecological modelling.

- vi) No correlation was found between the distribution of different mitochondrial types and ecological variables so, no evidence for the adaptation of mountain hare mtDNA to colder regions was obtained.

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