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The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula

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Running title: The rise and fall of *Lepus timidus* in Iberia

Abstract

Populations of Iberian (*Lepus granatensis*), brown (*Lepus europaeus*) and broom (*Lepus castroviejoi*) hares in Northern Iberia harbour mitochondrial haplotypes from the mountain hare (*Lepus timidus*), a cold adapted species presently absent from the Peninsula. To understand the history of this massive past introgression, we sequenced a fragment of cytochrome *b* and the control region of mitochondrial DNA of *L. timidus* origin found in 378 specimens of these four species. Among 124 *L. timidus* from the Northern Palaearctic and the Alps we found substantial nucleotide diversity but little geographic differentiation. Based on the mismatch distribution, we propose this could result from an expansion at a time of temperature decrease favourable to this arctic species. The nucleotide diversity of *L. timidus* mtDNA found in Iberian *L. granatensis*, *L. europaeus* and *L. castroviejoi* (183, 70 and 1 specimens respectively) was of the same order as that in *L. timidus* over its range (1.9 vs. 2.3%), suggesting multiple hybridization events. The coalescence pattern of the introgressed lineage in *L. granatensis* indicates a recent demographic expansion which is compatible with a scenario of progressive replacement with hybridization of *L. timidus* by *L. granatensis* when temperatures started to rise and favour this temperate species. *L. europaeus* could have hybridized with *L. timidus* in Iberia or on its way to the Peninsula, and according to our data it could also have hybridized with introgressed *L. granatensis*.

Introduction

The climatic oscillations that characterized the Pleistocene imposed important range shifts on Palaearctic biota, and contributed decisively to shape their demographic history and genetic diversity (Avise *et al.* 1998). Cooling of the climate forced temperate species to retract into fragmented distribution ranges in Southern refugia, creating high levels of diversity and endemism in these areas (Hewitt 1996). In Europe the Balkans, Italy and the Iberian Peninsulas represent the major ice age refugia (Taberlet *et al.* 1999). Temperate biota normally show lower genetic diversity in the formerly glaciated regions, due to founder effects during their post-glacial expansion, unless their mobility was sufficient to ensure an admixture from the different refugia during the interglacials (Hewitt 1996; Cruzan & Templeton 2000). A different pattern could however prevail for arctic species. Generally, given the much colder climates during glacial periods and the extent of the arctic ice sheets, these species must have been pushed to lower latitudes. However, large areas of Northeast Asia are known to have remained deglaciated and are proposed as refugial areas (see Hewitt 2004). Still, these species are well adapted to cold conditions and some could have maintained large distribution areas during the ice ages across the steppe and tundra stretches that covered Europe. To many, the cooling of the climate could have represented periods of population expansion while the warmer stage may be a time of population reduction (see Hewitt 2001). Consequently, some regions must have been occupied by an alternation of arctic and temperate species as the climate oscillated. This probably set the conditions for temporal and moving overlaps of the ranges of these two types of species, competition between them, and eventually hybridization. The Iberian Peninsula seems to have been an arena for such a type of interplay between hare species.

The genus *Lepus* is presently represented in Iberia by three species, two of which are endemic: the broom hare, *Lepus castroviejoi*, restricted to the Cantabrian Mountains, and the Iberian hare, *Lepus granatensis*, which covers the whole Iberian Peninsula except the Northeast, along the Pyrenees, where the brown hare, *Lepus europaeus*, prevails. Mitochondrial DNA studies (Pérez-Suárez *et al.* 1994; Alves *et al*. 2003) have identified lineages that are specific to each of these species, but Alves *et al.* (2003) have also detected haplotypes inherited from the mountain hare, *Lepus timidus*, currently extinct from Iberia, in specimens of *L. granatensis* and *L. europaeus*. *L. timidus* is an arcto-alpine species with a wide range in the Northern part of the Palaearctic region, from the British islands to the Russian Far East, and some isolated populations in the Alps, Poland and Japan (Angerbjörn & Flux 1995). According to the fossil record it was the most common and most widely distributed hare species in Europe during the last glacial periods (Lopez-Martinez 1980). Upper Pleistocene fossil records of mountain hares have been found for instance in Central Europe, Southern France (Lopez-Martinez 1980), Northern Spain (Altuna 1970) and Ireland (Woodman *et al.* 1997). Recent molecular analyses demonstrated that mtDNA of *L. timidus* origin is widespread in the Iberian Peninsula (Melo-Ferreira *et al*. 2005). It predominates in *L. granatensis* populations from the North, but becomes rarer towards the South, where it is absent. Furthermore, it is almost fixed in Iberian *L. europaeus* and also present in *L. castroviejoi*. Even though mitochondrial introgression in contact zones is not uncommon (e.g. Ferris *et al*. 1983; Tegelström 1987; Arnold 1997; Ruedi *et al*. 1997; Goodman *et al*. 1999; Bachtrog *et al*. 2006), the geographic and taxonomic ranges of this introgression are unusual, and the donor species is now extinct form the concerned region.

In this work, we wanted to better understand the time scale and demographic processes characterizing the spectacular past invasion of the genomes of these three Iberian species. To do this, we studied mtDNA sequence variation in a sample of *L. timidus* spanning most of its present distribution area, and compared it with the diversity of the *L. timidus* haplotypes found in the Iberian species. Our results are compatible with the scenario of an expansion of *L. timidus* prior to the Eemian interglacial, followed by a retraction to the North at the end of the Pleistocene, accompanied by replacement with hybridization by the temperate species which, as they expanded, spread the traces of hybridization to the recolonized regions.

Materials and Methods

Samples and laboratory methods

A total of 378 individuals from four hare species from the Iberian Peninsula (*L. granatensis*, *L. europaeus* and *L. castroviejoi*) and Eurasia (*L. timidus*) was analysed (Table 1; Fig. 1). The Iberian specimens had previously been identified has having the mtDNA of *L. timidus* origin through a PCR-RFLP approach (Melo-Ferreira *et al.* 2005).

Total genomic DNA was extracted from liver or ear tissue using standard methods similar to those described in Sambrook *et al.* (1989). A portion of the mitochondrial cytochrome *b* (Cytb) was amplified using primers LCYF (Alves *et al.* 2003) and LCYTBR (Melo-Ferreira *et al.* 2005), the 5' terminal nucleotides of which correspond respectively to positions 14251 and 14919 of the reference *L. europaeus* mitochondrial genome (GenBank Accession No. AJ421471; Arnason *et al.* 2002). Additionally, a fragment of the mitochondrial control region (CR) was amplified using primers LCRSEQ (5'-CACCATCAGCACCCAAAG-3') and LepD2H (Pierpaoli *et al.* 1999) which start, respectively, at positions 15395 and 15947 of the reference mitochondrial genome. Both PCR products were sequenced (617 bp from the Cytb and 471 to 473 bp of the CR) using LCYF and LCRSEQ primers, respectively, following the ABI PRISM BigDye Terminator Cycle Sequencing 3.1 (Applied Biosystems) standard protocol.

Sequences analyses

The Cytb and CR sequences were visually inspected, aligned using CLUSTAL W (Thompson *et al.* 1994) and concatenated. MtDNA haplotypes were defined using NETWORK 4.1.0.9 (http://www.fluxus-technology.com/).

A Neighbor-Joining tree (using the TN93 distance; Tamura & Nei 1993) was reconstructed using MEGA 3.1 (Kumar *et al.* 2004; http://www.megasoftware.net) in order to detect any error in the former PCR-RFLP determination of the mitochondrial lineage (Melo-Ferreira *et al*. 2005). No ambiguities were detected (data not shown).

When analysing intraspecific sequence data, that normally have large sample size and low genetic distances between haplotypes, the results are better expressed using a network which allows for alternative connections and for extant ancestral haplotypes in the populations (Bandelt *et al.* 1999). Since the introgressed specimens in Iberia and the *L. timidus* specimens share the mtDNA lineage, these two datasets were analyzed jointly using NETWORK 4.1.0.9 and a Median-Joining network was computed (Bandelt *et al.* 1999).

The nucleotide diversity (π), $\theta_{(S)}$ computed from the number of segregating sites, haplotype diversity (h) and mismatch distributions were determined using ARLEQUIN 3.0 (Excoffier *et al.* 2005). The mismatch distributions were analysed according to the Sudden Expansion Model (Rogers & Harpending 1992). This model assumes that an initial population at equilibrium with $\theta = \theta_0$ grows rapidly to a new size with $\theta = \theta_1$, τ units of mutational time ago, where $\theta = N_e u$ and $\tau = 2ut$ (Ne = effective population size, u = mutation rate and t = time since the expansion in generations). Goodness-of-fit tests (Schneider & Excoffier 1999) of the observed to the expected distribution were computed. The confidence intervals for τ were obtained from 1000 bootstrap replicates. The conformation to a model of selective neutrality and population equilibrium by Tajima's *D* (Tajima 1989a) and Fu's *Fs* (Fu 1997) was tested with 5000 bootstrap replicates.

To further assess the demographic history of the analysed samples we determined the population growth parameter *g* using FLUCTUATE 1.4 (Kuhner *et al.* 1998), a coalescent-based method which takes into account the genealogical relationships among haplotypes. Positive values of *g* indicate population growth and negative values population reduction. We ran the program several times with different combinations of short and long chains to ensure consistency of the estimates. The final estimates were based on a run of 10 short chains of 1,000 steps followed by 10 long chains of 20,000 steps, sampling every 10 steps. The estimates of the growth parameter *g* are known to be biased upwards (Kuhner *et al.* 1998). Therefore, we followed the conservative method used by Lessa *et al.* (2003) and considered *g* to indicate population growth only if $g > 3(\mathrm{SD})$ and population decline if $g < -3(\mathrm{SD})$.

Population pairwise Φ_{ST} were calculated and tested for significance (10000 permutations; significance level 0.05). An analysis of molecular variance (AMOVA; 10000 permutations; Excoffier *et al.* 1992) was then computed to test for population structure in *L. timidus*, grouping the samples according to their geographic location (Northern Europe, Alps, Eastern Europe and Eastern Russia).

To obtain an estimate of interspecific divergence time in *Lepus*, Pierpaoli *et al.* (1999) proposed that a Cytb divergence rate of 4% per Myr, which corresponds to the basal splitting of the genus at 3 Myr, is in accordance with the palaeontological data that reports the first appearance of the genus at ≈ 2.5 million years ago (e.g. Lopez-Martinez 1980). In order to calibrate the rate of substitution in *L. timidus*, we calculated the average nucleotide TN93 distance between the two major lineages of *L. timidus* origin found in *L. granatensis*, for the Cytb fragment alone and for the concatenation of the Cytb and CR fragments. By simple proportionality, assuming that the rate of divergence for Cytb is 4% per Myr, we found that for the concatenated fragments the divergence rate is 15.8% per Myr.

Results

Sequence diversity

After concatenating the Cytb and CR fragments, (378 individuals; 1088 to 1090 bp) we identified 167 haplotypes defined by 270 polymorphic sites, of which 267 had substitutions and 5 contained insertions/deletions (Table 1; GenBank accession numbers: Cytb - ###-###; CR - ###-###;

haplotypes with frequency higher than 1 are shown in the appendix). The Cytb sequences appear to be of mitochondrial origin and not nuclear integrated copies, as the reading frame is intact and the third position base composition is typical (A 38.5%, C 32.3%, G 2.7% and T 26.5%) compared to the average in mammals (A 39%, C 36%, G 3% and T 21%; Johns and Avise 1998). A separate analysis of the Cytb and CR datasets did not show any phylogenetic incongruence (data not shown) suggesting that the CR fragment is also of mitochondrial origin.

The 124 *L. timidus* specimens harboured 90 distinct haplotypes. Sequence diversity was high (h = 0.991 ± 0.003; π = 0.023 ± 0.011; Table 2) and the haplotypes were evenly distributed, all having frequencies lower than 6%. Each of the major geographic regions that we defined separately displayed similarly high sequence diversity (Table 2).

Seventy-seven different mitochondrial haplotypes of *L. timidus* origin were found among the Iberian species: 67 in *L. granatensis*; 11 in *L. europaeus*; and 1 in *L. castroviejoi*. Two haplotypes (i9 and i66) were found both in *L. granatensis* and *L. europaeus*. The introgressed *L. granatensis* showed high sequence diversity (Table 2), with haplotypes evenly distributed in the sample, all having a frequency lower than 7%. Haplotype diversity (h = 0.978 ± 0.003) and nucleotide diversity (π = 0.018 ± 0.009) were high, suggesting that *L. timidus* mtDNA introgression in this species had multiple origins. The diversity among the haplotypes of *L. timidus* origin found in *L. europaeus* was also rather high (h = 0.820 ± 0.026; π = 0.017 ± 0.008; Table 2). In this species, two haplotypes, i09 and i72, occurring with a frequency of 26% and 30% respectively, are clearly predominant over the others.

Network analysis and population differentiation

The Median-Joining network split the introgressed haplotypes in the Iberian species in two well defined divergent haplogroups (average uncorrected p-distance = 0.030), which will be referred to as groups A and B (Fig. 2). No haplotype was shared between true *L. timidus* and the other species. Group A of introgressed haplotypes is found in the three Iberian species, and one haplotype is common to *L. granatensis* and *L. europaeus*. This group is not monophyletic, as the smallest clade in which it is included also comprises haplotypes form Eastern Russia, Northern Europe and the Alps. Group B of introgressed haplotypes is found in *L. granatensis* and *L. europaeus*, also with one haplotype shared between these species. The smallest monophyletic group including group B also comprises haplotypes of true *L. timidus* from the Alps and Northern Europe. The haplotypes from Northern Europe, Eastern Russia and the Alps were scattered throughout the network. However, many haplotypes from the Alps fell into two clusters closely related to the introgressed Iberian groups A and B, suggesting relatedness. The British Isles haplotypes form two well defined divergent clusters which correspond to the Irish and Scottish specimens.

The AMOVA showed that in *L. timidus* 7.5% of the variation is explained by differences among major geographic groups, 28.3% among populations within groups and 64.2% within sampled populations (Φ_{ST} = 0.36, Φ_{SC} = 0.31, Φ_{CT} = 0.07). Pairwise Φ_{ST} distances among the *L. timidus* populations range from 0 to 0.805. The Scottish and Italian populations show the higher levels of differentiation relative to the others. In general, the Northern European *L. timidus* populations are little differentiated from the Eastern Russia ones (Table 3). The introgressed Iberian and brown hare populations are well differentiated from the native *L. timidus* (Φ_{ST} from 0.822 to 0.859). The differentiation between the introgressed *L. granatensis* and *L. europaeus* is moderate (0.102).

Demographic analyses

The mismatch analysis of the sequences from true *L. timidus* showed a unimodal distribution of the number of pairwise differences that fitted the expectation under the Sudden Expansion Model (Fig. 3a). The main expansion event was estimated to have occurred at $\tau = 28.2$ (95% CI 22.4-31.2).

The *timidus*-like haplotypes in *L. granatensis* show a bimodal distribution of pairwise differences, rejecting, as expected, the Sudden Expansion Model (Fig. 3b). The observation of two clearly separated sublineages in this species suggests independent origins of the introgressed clades. The mismatch distribution for each lineage analysed separately is unimodal, not rejecting the expectation under the Sudden Expansion Model, showing that the group A main expansion event occurred at $\tau = 5.7$ (95% CI 3.0-14.0; Fig. 3c) while in group B it occurred at $\tau = 6.0$ (95% CI 3.4-13.6; Fig. 3d). In *L. europaeus*, the mismatch distribution shows three peaks at 0, 15, and 33 pairwise differences, rejecting the tested model (Fig. 3e). When analysing separately groups A and B (Figs. 3f and 3g respectively), we found that for the latter the rapid expansion model is not rejected, with an estimated $\tau = 6.0$ (95% CI 1.6-13.0). In *L. europaeus* group A however, it was not possible to perform the goodness-of-fit test, since the least square procedure to fit model distribution and observed distribution did not converge after 1800 steps.

Tajima's *D* values were negative in *L. granatensis* groups A and B, group A of *L. europaeus*, and in *L. timidus*, except for the analysis of the Alpine haplotypes (Table 2). However, none of the values was significantly different from zero ($p > 0.05$). Fu's *Fs* values were negative except in *L. europaeus* (both groups A and B) and the Alpine and Eastern European *L. timidus* (Table 2). This parameter was significant ($p < 0.02$) in *L. granatensis* group B, in *L. timidus* as a whole and in the Northern European sample. Negative values of these parameters can be due to selection, but also population expansion, bottleneck or heterogeneity of mutation rates (Tajima 1989b; Aris-Brosou & Excoffier 1996; Fu 1997). In fact, the *Fs* index is particularly sensitive to population expansion (Fu 1997; Ramos-Onsins & Rozas 2002), and thus at least in some cases, these results are concordant with those of the mismatch analysis.

The estimates of the growth parameter *g* show that both lineages in *L. granatensis* underwent a population growth, but this was not the case in *L. europaeus*. In true *L. timidus* the overall sample and the partitions indicate growth, except for the Alpine population (Table 2).

Discussion

L. timidus population history and genetic structure

Although our sample of *L. timidus* covers most of the species range, from the Atlantic to the Pacific and from Scandinavia and the British Isles to the Alps, little geographic structure of mtDNA variation is apparent on the haplotype network of Fig. 2. Only 7.5% of the molecular variance lies in differences between the major geographic regions, most of the variance (64.2%) being attributable to intra-population diversity. The Φ_{ST} value (0.36) found among populations covering such a large area is low when compared to that found in other mammals such as wolf (0.69; Vilà *et* *al.* 1999), roe deer (0.44; Randi *et al*. 2004) or brown hares (0.42; Kasapidis *et al.* 2005). Likewise, the pairwise Φ_{ST} values between some Northern European and Eastern Russian populations are generally low (for example Sweden and Finland vs. Amurskaya Territory and Kamchatka Peninsula; Table 3), indicating little differentiation. Although hares are mobile species, the relatively low differentiation over such large distances is unlikely to exclusively reflect ongoing gene flow, but rather suggests a common history of colonization. In fact, we have seen that Fu's *Fs* statistics, the growth parameter (Table 2) and the mismatch distribution (Fig. 3a) are compatible with an expansion of this species, that we have dated at 164 000 years BP (130 000-181 000 years BP, 95% CI), i.e. before the last interglacial (130 000 to 116 000 years BP; Kukla *et al.* 2002), in agreement with earlier more restricted studies (Waltari & Cook 2005), and with a previous estimate (135 000 BP; Pierpaoli *et al.* 1999). *L. timidus* being an arctic species, the glacial periods have logically affected it differently from the temperate species. It would appear logical that the expansion of this species occurred when temperatures were dropping, rather than during the warming of an interglacial period as is proposed for several arctic taxa (Hewitt 2001; Flagstad & Røed 2003; Dalén *et al.* 2005). As a result, during the last glacial period *L. timidus* could have maintained a large and continuous distribution south of the ice rim, and ice-age palaeontological remains of *L. timidus* have been found throughout Europe (e.g. Altuna 1970; Lopez-Martinez 1980; Woodman *et al.* 1997). Of course more recent expansions must also have occurred in the Northern Palaearctic that was covered with ice during the last glacial maximum. This would explain the low levels of allozyme and mitochondrial differentiation among European mountain hares found by Suchentrunk *et al.* (1999) and Ben Slimen *et al.* (2006) respectively. Moreover, Thulin *et al.* (1997a), given the close phylogenetic associations between Scandinavian and non-Scandinavian mtDNA haplotypes, suggested that recent colonization from multiple areas explains the origin of the Scandinavian mountain hares. On the other hand, fragmentation and shrinking of the species range during warmer times could have induced partial differentiation of isolated populations by drift, especially in enclaves such as mountain chains. We note that the Italian population (our largest sample from the Alps) is significantly differentiated from all other populations (Table 3), presumably as result of this effect. The Scottish population also appears significantly different from most others (Table 3). The Scottish haplotypes clearly appear separated from the others in the network from Fig. 2, except one (t30) that clusters with the Irish samples. However these sampled specimens were from the Isle of Mull, Western Scotland, where Irish hares have been introduced earlier (see Angerbjörn & Flux 1995). As has been observed before (Pierpaoli *et al.* 1999), the Irish haplotypes are more related to the continental ones than to those from Scotland.

Multiple L. timidus mtDNA introgression in Iberia

None of the *L. timidus* mtDNA haplotypes found in the Iberian Peninsula is found elsewhere. This translates into elevated pairwise Φ_{ST} between the introgressed *L. granatensis* and *L. europaeus* and the true *L. timidus* populations (Table 3). It is also striking that the nucleotide diversity among the *L. timidus* haplotypes in the Iberian Peninsula (17-19%) is comparable to that encountered over the whole range of the donor species, *L. timidus* (23%; Table 2). This high diversity mainly results from the introgressed haplotypes belonging to two divergent lineages (Fig. 2). These two observations together suggest that some of the variation seen in *L. granatensis* and *L. europaeus* pre-existed the introgression, which thus occurred through multiple hybridization events. They also suggest that some evolution occurred after the introgression, to produce the high differentiation from the donor populations. This rules out the possibility that the introgression in the Iberian Peninsula results from a single accidental hybridization, followed by an expansion of the introgressed haplotype. Evidence for single hybridization would have strengthened the idea that the introgression was driven by selection given its extraordinary extent over half of the Peninsula and three different species as shown by our previous study (Melo-Ferreira *et al.* 2005). Thus, in a sense, the great diversity of the introgressed haplotypes renders a test of the selection hypothesis more delicate, and we must attempt to reconstruct more precisely the history of the introgression.

Both in *L. granatensis* or in *L. europaeus*, the introgressed haplotypes belong to two groups (which we named A and B) that are closely related to the two major haplotype clusters found in the present Alpine population of *L. timidus* (Fig. 2). This indicates that the *L. timidus* population that bequeathed its mtDNA to the Iberian hares was related to the ones that retreated up the Alps when it became warmer, which makes geographical sense.

Most of the introgressed haplotypes found in *L. granatensis* fall into the two compact and well separated groups A and B, which would mean that at least two main waves of *L. timidus* hybridization occurred in Iberia. We can thus try to date each introgression wave by assuming that it was followed by a simple demographic expansion. Both *timidus*-like groups in *L. granatensis* show signs of an increase in population size, and the mismatch distributions are compatible with recent expansions at 33 000 years BP for group A (95% CI 17 000 - 81 000 years) and 35 000 years BP for group B (95% CI 20 000 - 79 000 years), a time when *L. timidus* presence in Iberia has been documented by fossil records (Sesé & Sevilla 1996). The maximum extent of the glaciers in the Pyrenees during the last glacial period occurred more than 30 000 years BP (García-Ruiz *et al.* 2003; Peña *et al.* 2004). A later advance coincides with the global last glacial maximum around 20,000 years BP but was less extensive than the previous one (García-Ruiz *et al.* 2003). Thus the sudden demographic expansion detected in the introgressed groups of *L. granatensis* could correspond to the date when *L. timidus* reached its southernmost extension in the Northern Iberian Peninsula, before it retreated and gave ground to *L. granatensis* as the latter expanded from its Southern refuge with the climate getting milder. Currat and Excoffier (2004) have simulated such situations of competitive replacement of one species by the expansion of another, and found that even rare hybridization events could suffice to initiate extensive introgression of the invading species by genes of the disappearing species. Hybridization is likely to occur mostly when the invading species is still rare, and experiences some difficulties in finding conspecific mating partners, thus eventually raising the introgressed haplotypes to relatively high frequencies on the invasion front. Subsequent demographic expansion of these initially rare colonisers could further amplify this effect, potentially driving the introgressed genes to high frequencies ahead of the invasion front. This expansion process is likely to leave a trace on the coalescent. This scenario appears plausible to explain the introgression in *L. granatensis*, in which we observe these two predicted patterns, high frequency of introgressed haplotypes and a star-like coalescent. The fact that the introgressed haplotypes do not form monophyletic groups but are intermingled with lineages found in other distant populations shows that several independent hybridizations have occurred on this front of replacement of *L. timidus* by *L. granatensis*.

Our data on *L. europaeus* seem to indicate a shared history of introgression with *L. granatensis* since representatives of the same lineages are found in both species. However, although it is quite clear that *L. granatensis* has always been in the Iberian Peninsula, to which it is endemic, the brown hare is thought to have arrived to Western Europe after the last glacial maximum, based on palaeontological and molecular data (Lopez-Martinez 1980; Pierpaoli *et al.* 1999). Did *L. europaeus* reach Iberia before *L. timidus* had disappeared, and replace it in the Pyrenean foothills, just as *L. granatensis* did further south? This is not certain. If alternatively we suppose that it arrived in Iberia after *L. timidus* went extinct there, then it must have hybridized with *L. timidus* before reaching

Iberia. This is conceivable since it must have cut across, or come close to, the range of *L. timidus* on its way. In Sweden, native *L. timidus* hybridize with introduced *L. europaeus* (Thulin *et al.* 1997b; Thulin & Tegelström 2002), and such crosses are also observed in captivity (Gustavsson & Sundt 1965). In both cases mating occurs only in the direction required to account for the observed introgression, i.e. *L. timidus* females with *L. europaeus* males. However, recently, reciprocal transfer of mtDNA between these two species was described in Russia (Thulin *et al*. 2006) and the Alps (Suchentrunk *et al.* unpublished data). *L. europaeus* could also have borrowed its alien mtDNA from *L. granatensis* after or during its arrival in Iberia, and after the extinction of *L. timidus*. Two introgressed haplotypes are shared by these two Iberian species and suggest exchanges between them. Recently Estonba *et al.* (2006), using microsatellites, could not find any sign of hybridization between *L. granatensis* and *L. europaeus*. However, a reduced number of specimens (19 *L. granatensis* and 39 *L. europaeus*) was analysed in this work and the contact area was not comprehensively sampled. Further, our preliminary data also using microsatellites (to be published elsewhere) clearly demonstrate ongoing hybridization between these species in the Pyrenean foothills. The introgressed haplotypes of group A found in *L. europaeus* are in fact quite close to those in *L. granatensis*. However some of those in group B are not, thus making it more doubtful that *L. granatensis* be the sole source of *L. timidus* haplotypes in *L. europaeus*.

The time-frame of the demographic events

The estimates of time-frame that we propose for the demographic events rely on a number of approximations. A first and strong assumption is that mtDNA diversity mostly reflects purely demographic processes. However, a recent meta-analysis of animal mtDNA variation (Bazin *et al.* 2006) has shown a lack of relationship between population size and nucleotide diversity for mtDNA, and given evidence that this is the result of recurrent selective sweeps on mtDNA, as predicted and modelled by Gillespie (2000, 2001). Our demographic inferences would clearly be invalidated if such events occurred in the recent history of *L. timidus*. A second approximation was to extrapolate by simple proportionality the rate of substitution of the Cytb, calibrated by Pierpaoli *et al.* (1999), to the CR. It is known that the CR has several mutational hotspots and thus mutations are more likely to be superimposed over log timescales (Sigurðardóttir *et al.* 2000). A third approximation was to take the rate of evolutionary substitution thus determined as an estimate of the mutation rate. It has been broadly observed that rate estimates obtained from population-level studies are generally higher that those obtained in phylogenetic (species-level) studies (Sigurðardóttir *et al.* 2000; Ho *et al.* 2005). Ho *et al.* (2005) show that the relationship between the age of calibration and the rate of change can be described by a vertically translated exponential decay curve, concluding that for timescales less than about 1-2 Myr the application of phylogenetic substitution rates lead to overestimate the divergence times. If we take, for example, the average p-distance between groups A and B in *L. granatensis*, 0.031, which using our rate means 196 000 years of divergence, and apply the correction suggested by Ho *et al.* (2005) both for CR and Cytb, we obtain a 2 to 3-fold decrease in the divergence times (85 000 and 62 000 years respectively). Of course this is just indicative of the potential quantitative effect of this phenomenon, since the correction proposed by Ho *et al.* (2005) is based on primate data, but this suggests that both the *L. timidus* demographic expansion and the introgression in Iberia could be more recent than we estimated. In Iberia some fossil records of *L. timidus* are as recent as 17 000 to 10 000 years BP (Altuna 1970; Sesé 2005). However, these data are scarce and there is great uncertainty in distinguishing *Lepus* species on the basis of palaeontological records (see Sesé 2005). The fossil record is much better for other arctic species such as the grouse (*Lagopus mutus*), and a comparison can help us reconstruct the history of *L. timidus* in Iberia. The rich grouse fossil record shows it was very abundant in the North of the Iberian Peninsula during the Upper Pleistocene and maintained populations there during the several glacial and interglacial periods (Tyrberg 1995). Interestingly, its present distribution worldwide is strikingly similar to that of *L. timidus,* the only major difference being that it is still present in Northern Iberia, in some parts of the Pyrenees. Therefore it is plausible that the contact and hybridization between *L. granatensis* and *L. timidus* remained until the Holocene.

Conclusion

We have clearly made significant progress in our understanding of the history of *L. timidus* and of the spectacular introgression of its mitochondria in the Iberian Peninsula in this study. The observed data are compatible with a scenario of competitive expansion and replacement of a cold adapted species by a better adapted species during a climatic change. The scenario is coherent in terms of geographical and time scales, at least in the case of *L. granatensis*. The extension of the same scenario to *L. europaeus* remains somewhat uncertain, but the fact that the phenomenon occurred in both species and to a certain extent also in *L. castroviejoi* (which we have not discussed in detail due to the limited sampling) should still invite us to consider the hypothesis that selection could have favoured this massive introgression. At the present time this idea appears difficult to test using solely the available data, because selection is expected to leave the same kind of trace on the coalescent as the demographic processes that we put forward and that appears plausible. If mtDNA introgression is neutral, one expects to observe the same consequences of these demographic processes on the coalescent of the aboriginal mtDNA lineages and the nuclear genes of the introgressed populations as was seen on the introgressed lineages. This will be the object of future work.

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Figure Legends

Fig. 1 – Species ranges of *L. granatensis*, *L. europaeus*, *L. castroviejoi* and *L. timidus* in Eurasia according to Flux & Angermann (1990) and Mitchell-Jones *et al.* (1999). Sample locations are shown (see also Table 1).

Fig. 2 – Median-Joining network of the haplotypes found in *L. timidus* and introgressed in the Iberian hare species. Branches are generally proportional to the number of differences between haplotypes. Dots on branches indicate the mutational steps when more than 1.

Fig. 3 – Observed (bars) and expected (solid lines) mismatch distributions of: a) *L. timidus* haplotypes; b) introgressed *L. granatensis* haplotypes; c) *L. granatensis* introgressed Group A haplotypes; d) *L. granatensis* introgressed Group B haplotypes; e) introgressed *L. europaeus* haplotypes; f) *L. europaeus* introgressed Group A haplotypes; g) *L. europaeus* introgressed Group B haplotypes. Values of the expansion parameters are shown when sudden population expansion assumption was not rejected.

Table 1: Sampled species, sample localities, their size (*n*) and the haplotypes detected in each locality.

Species	No.	Code	Locality	*n*	Haplotypes
	Iberian Peninsula				
gra	1	IBGRA	Iberian Peninsula	183	i1 to i67
eur	2	IBEUR	Iberian Peninsula	70	i9, i66, i68 to i76
cas	3	IBCAS	Cantabrian Mountains	1	i77
	Northern Europe				
tim	4	SWE	Sweden	20	t1 to t20
	5	NOR	Norway	3	t21 to t23
	6	FIN	Finland	6	t24 to t29
	7	SCO	Scotland	15	t30 to t36
	8	IRE	Ireland	3	t37 to t39
	Alps				
	9	FRA	France	3	t40 to t42
	10	SWI	Switzerland	3	t43, t44
	11	AUS	Austria	3	t45
	12	ITA	Italy	38	t40, t41, t46 to t63
	Eastern Europe				
	13	URA	Urals	3	t64 to t66
	14	RUS	Western Russia	1	t67
	Eastern Russia				
	15	AMU	Amurskaya territory	4	t68 to t71
	16	KAM	Kamchatka Peninsula	4	t72 to t74
	17	KOL	Kolyma river basin	7	t75 to t81
	18	MAG	Magdan city	5	t82 to t84
	19	PRI	Primorve territory	3	t85 to t87
	20	YAK	Yakutsk city	3	t88 to t90

gra: *L. granatensis*; eur: *L. europaeus*; cas: *L. castroviejoi*; tim: *L. timidus*

Table 2: Estimates of sequence diversity, neutrality tests and growth rate in native *L. timidus* and in *L. granatensis*, *L. europaeus* and *L. castroviejoi* with *L. timidus* mtDNA haplotypes.

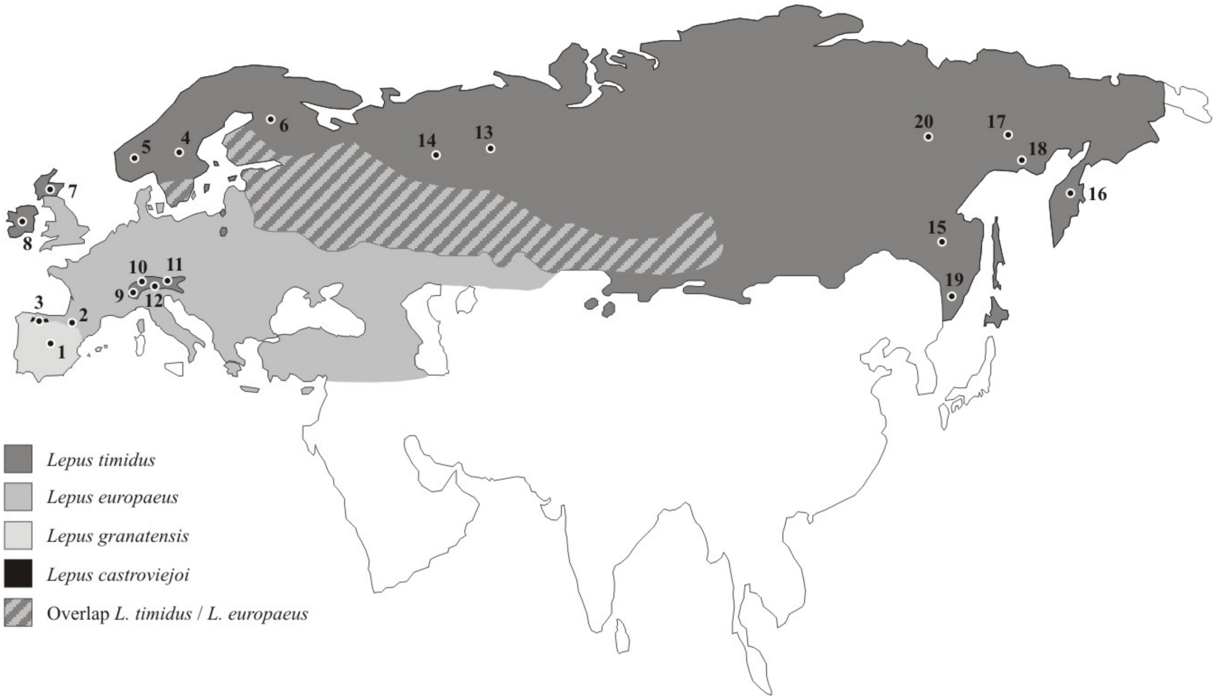
Group	ni	nh	h	π (%)	θ(s) per site (%)	Tajima's *D*	Fu's *Fs*	Growth rate
Iberian species								
gra, eur and cas	254	77	0.974 (0.003)	1.9 (0.9)	1.7 (0.4)	-	-	-
gra	183	67	0.978 (0.003)	1.8 (0.9)	1.7 (0.4)	-	-	-
eur	70	11	0.820 (0.026)	1.7 (0.8)	1.0 (0.3)	-	-	-
gra, lineage A	103	34	0.963 (0.006)	0.7 (0.4)	1.2 (0.3)	-1.43	-7.95	152.9 (50.8)†
gra, lineage B	80	33	0.946 (0.013)	0.6 (0.3)	1.0 (0.3)	-1.30	-12.07*	232.2 (52.3)†
eur, lineage A	37	4	0.673 (0.050)	0.1 (0.1)	0.1 (0.1)	-0.05	0.44	611.4 (1035.2)
eur, lineage B	33	7	0.587 (0.096)	0.6 (0.3)	0.5 (0.2)	0.31	4.71	-244.6 (108.9)
Native mountain hare								
Total	124	90	0.991 (0.003)	2.3 (1.1)	2.9 (0.7)	-0.70	-23.86*	203.5 (15.0)†
Northern Europe	47	39	0.987 (0.009)	2.0 (1.0)	2.5 (0.7)	-0.73	-11.29*	143.1 (22.0)†
Alps	47	24	0.955 (0.015)	1.9 (1.0)	1.6 (0.5)	0.70	0.82	23.4 (30.8)
Eastern Europe	4	4	1.000 (0.177)	1.6 (1.1)	1.6 (0.9)	-0.17	0.95	288.6 (65.1)†
Eastern Russia	26	23	0.991 (0.013)	2.1 (1.1)	2.3 (0.8)	-0.32	-4.35	236.2 (27.2)†

gra: *L. granatensis*; eur: *L. europaeus*; cas: *L. castroviejoi*; tim: *L. timidus*; ni = number of analysed individuals; nh = number of observed mtDNA haplotypes; h = haplotype diversity; π = nucleotide diversity; θ(s) , computed from the number of segregating sites (Tajima 1983). Standard deviations (SD) are shown in brackets. The significant values are indicated by an asterisk. † indicates $g >$ 3(SD).

Table 3: Pairwise Φ_{ST} values for the populations († indicates values not significantly different from zero). See Table 1 for population codes. Only populations with sample size >= 4 individuals are shown.

	SWE	FIN	SCO	ITA	AMU	KAM	KOL	MAG	IBPGRA
SWE									
FIN	0.052†								
SCO	0.337	0.312							
ITA	0.165	0.222	0.446						
AMU	0.094	-0.023†	0.307	0.232					
KAM	0.291	0.404	0.718	0.332	0.382†				
KOL	0.037†	0.020†	0.393	0.192	-0.024†	0.176†			
MAG	0.461	0.609	0.805	0.505	0.640	0.610	0.377		
IBPGRA	0.822	0.827	0.842	0.826	0.827	0.834	0.823	0.843	
IBPEUR	0.830	0.841	0.862	0.835	0.841	0.848	0.834	0.859	0.102

Fig. 1:


5
4
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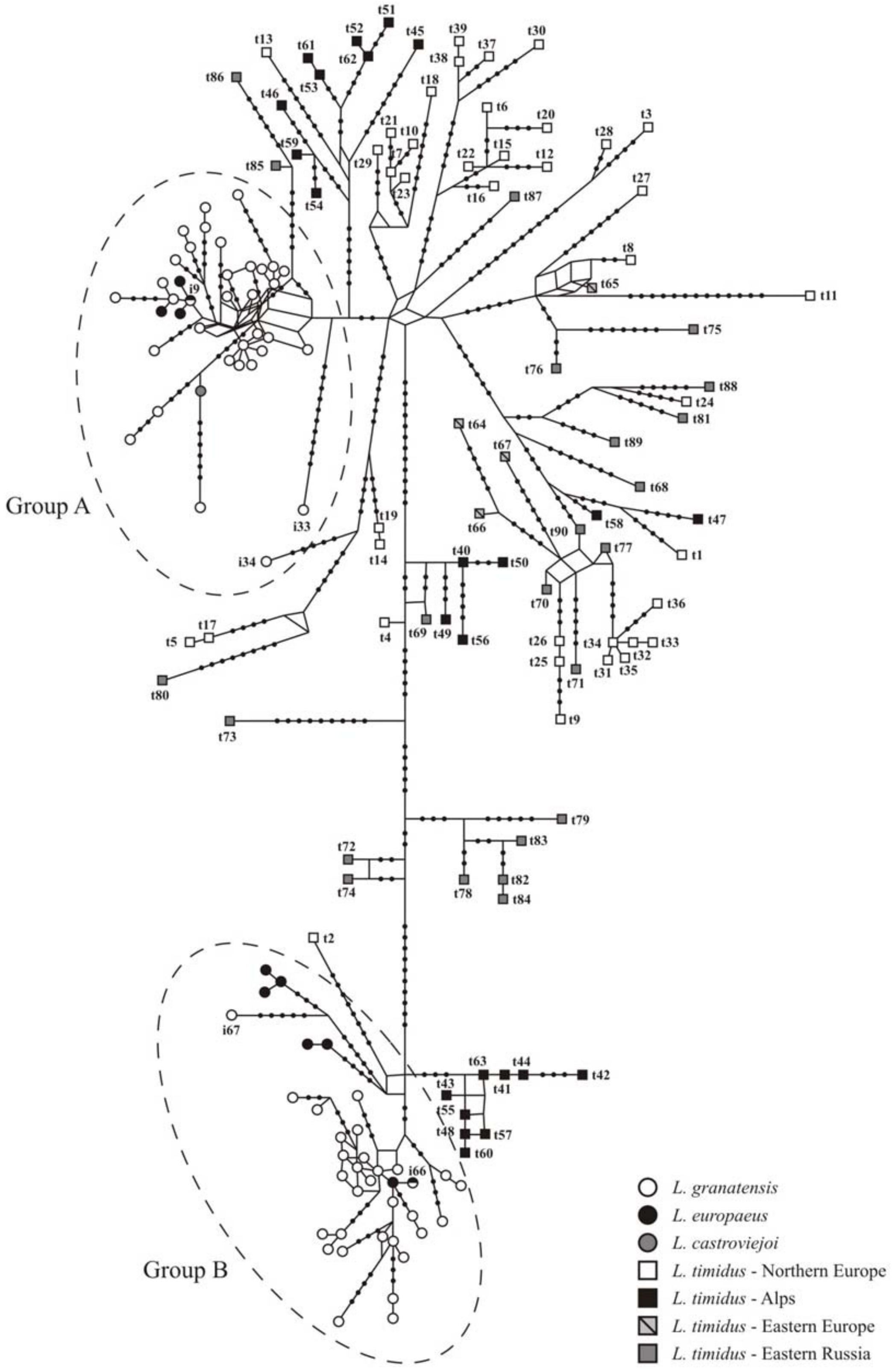
2
1
Lepus timidus
Lepus europaeus
Lepus granatensis
Lepus castroviejoi
Overlap L. timidus / L. europaeus

Fig. 2:


t51
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t3
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t54
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t87
t8
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t67
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Group A
t47
t58
t66
t19
i33
t90
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t1
i34
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t40
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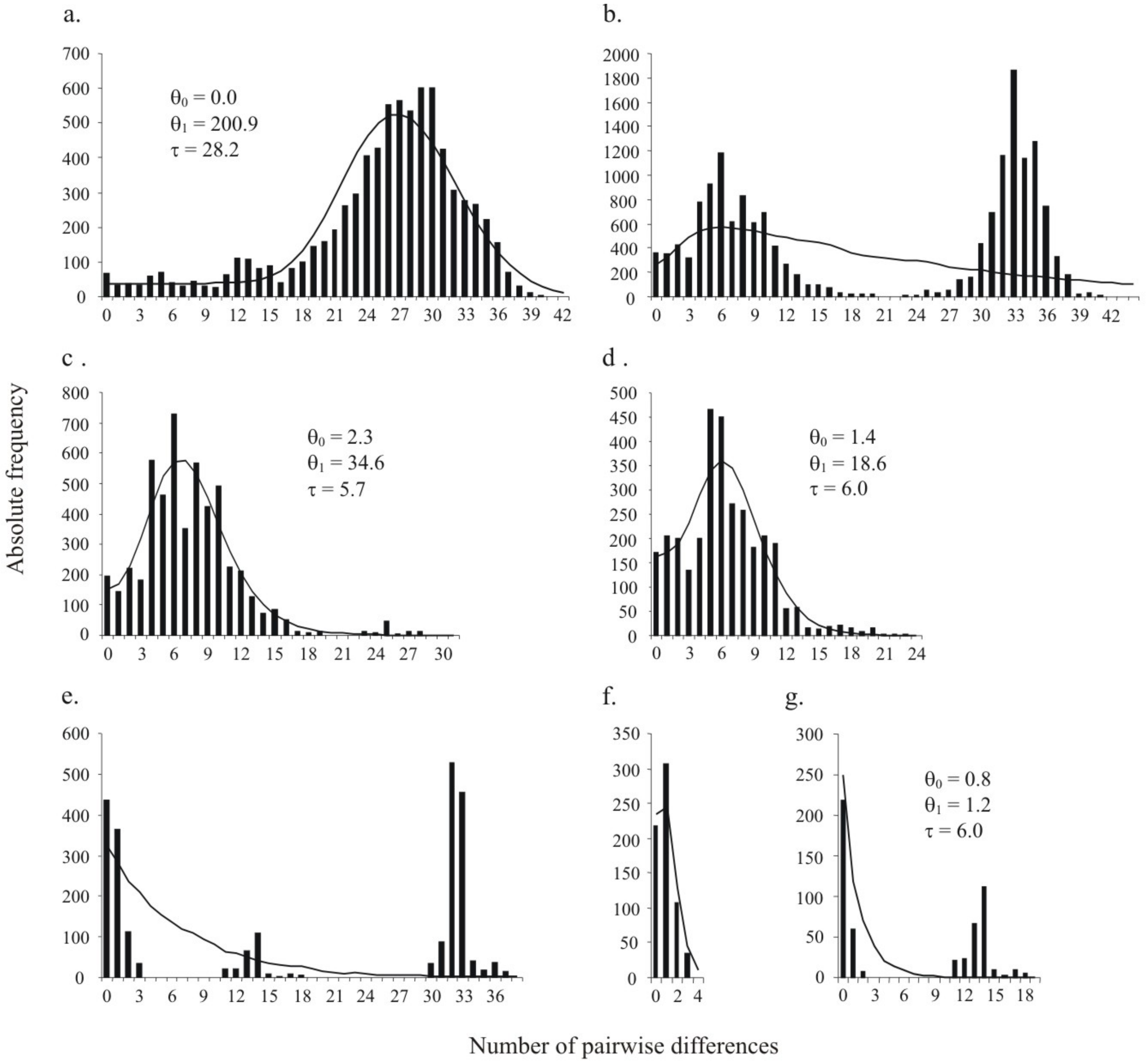
t80
t9
t73
t79
t72
t83
t74
t78
t82
t84
t2
i67
t63
t44
t42
t43
t41
t55
t48
t57
t60
i66
Group B
○ L. granatensis
● L. europaeus
● L. castroviejoi
☐ L. timidus - Northern Europe
■ L. timidus - Alps
▨ L. timidus - Eastern Europe
■ L. timidus - Eastern Russia

Fig. 3:


a.
700
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100
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42
$\theta_0 = 0.0$
$\theta_1 = 200.9$
$\tau = 28.2$
b.
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42
c .
800
700
600
500
400
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30
$\theta_0 = 2.3$
$\theta_1 = 34.6$
$\tau = 5.7$
d .
500
450
400
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18 21 24
$\theta_0 = 1.4$
$\theta_1 = 18.6$
$\tau = 6.0$
e.
600
500
400
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30 33 36
f.
350
300
250
200
150
100
50
0
0 2 4
g.
300
250
200
150
100
50
0
0 3 6 9 12 15 18
$\theta_0 = 0.8$
$\theta_1 = 1.2$
$\tau = 6.0$
Absolute frequency
Number of pairwise differences

Author Information Box

This work is part of a project on the evolutionary relationships in the genus *Lepus* and is also included in the PhD thesis project of J Melo-Ferreira focused on phylogeography and patterns of introgression in hares. PC Alves is a researcher at CIBIO, University of Porto, and his main research area is conservation genetics and evolution of Iberian mammals, particularly Lagomorphs. P Boursot has general interests in molecular evolution, hybridization and speciation and his favourite model is mice. F Suchentrunk has a long-term interest in the evolution of hares. N Ferrand heads the CIBIO, University of Porto, and is interested in a variety of questions in evolutionary and conservation genetics. E Randi is head of conservation biology and genetics at INFS. A Kryukov investigates natural hybridization, molecular phylogeny and phylogeography of birds, mammals and amphibians.

Appendix

Haplotypes with frequencies higher than 1:
Lepus granatensis: i1, 10; i2, 6; i4, 4; i5, 1; i6, 4; i7, 2; i8, 5; i9, 2; i10, 3; i11, 2; i12, 5; i15, 3; i16, 6; i17, 2; i18, 6; i19, 3; i20, 9; i22, 3; i23, 3; i24, 2; i25, 2; i26, 5; i27, 2; i30, 3; i36, 2; i37, 7; i40, 3; i41, 3; i42, 4; i43, 3; i45, 11; i46, 2; i48, 3; i50, 2; i54, 4; i56, 2; i57, 12; i60, 2; i65, 2.
Lepus europaeus: i9, 18; i68, 8; i69, 2; i70, 9; i72, 21; i73, 3; i74, 2; i75, 3; i76, 2;
Lepus timidus: t30, 2; t31, 4; t35, 2; t36, 4; t40, 2; t41, 2; t43, 2; t45, 3; t46, 7; t47, 2; t48, 3; t51, 2; t52, 5; t53, 4; t54, 2 ; t72, 2 ; t82, 2; t83, 2.