

# Invasion from the cold past: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia

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## Abstract

Mitochondrial DNA introgression from *Lepus timidus* into *Lepus granatensis* and *Lepus europaeus* was recently reported in Iberia, although *L. timidus* presumably retreated from this region at the end of the last ice age. Here we assess the extent of this ancient mtDNA introgression by RFLP analysis of 695 specimens representing the three hare species present in Iberia. The introgressed *L. timidus* lineage was found in 23 of the 37 populations sampled. It is almost fixed in *L. europaeus* across its Iberian range in the Pyrenean foothills, and in *L. granatensis*, which occupies the rest of the peninsula, it is predominant in the north and gradually disappears further south. We also found it in *Lepus castroviejoi*, a species endemic to Cantabria. Multiple hybridizations and, potentially, a selective advantage for the *L. timidus* lineage can explain the remarkable taxonomic and geographical range of this mitochondrial introgression.

**Keywords:** cytochrome *b*, Iberian Peninsula, introgression, *Lepus*, mitochondrial DNA, PCR-RFLP

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## Introduction

The cyclic oscillations of the climate during the Pleistocene induced major changes in species distribution, forcing many of them to retract into fragmented distribution ranges during glaciations (Hewitt 1996). These repeated fragmentations produced a large taxonomic diversity in Iberia and in other southern European peninsulas as these places represent the major ice age refugial areas (Hewitt 1996). The genus *Lepus* reflects this pattern as shown in the two endemic hare species in the Iberian Peninsula: the broom hare, *Lepus castroviejoi*, restricted to the Cantabrian Mountains in the northwest, and the Iberian hare, *Lepus granatensis*, present throughout the peninsula except in the northeast, which is inhabited by the brown hare, *Lepus europaeus*. Despite earlier taxonomic controversies, studies based on morphology (Palacios 1989), protein markers (Bonhomme *et al.* 1986; Alves *et al.* 2000) and mitochondrial DNA (Pérez-Suárez

*et al.* 1994; Alves *et al.* 2003) support the species status of the two endemic Iberian hares.

Repeated range variations during the glacial oscillations also set the conditions for periodic secondary contacts between species. The genus *Lepus* is widely represented in Europe with five extant species, and although their distributions occasionally overlap, so far, few cases of natural hybridization have been reported. The best documented case describes the hybridization and unidirectional mitochondrial introgression between native mountain hares, *Lepus timidus*, and introduced brown hares in Sweden (Thulin *et al.* 1997; Thulin & Tegelström 2002). More surprisingly, mitochondrial introgression of *L. timidus* into *L. europaeus* and *L. granatensis* of the Iberian Peninsula has recently been described (Alves *et al.* 2003), although according to palaeontological data (Altuna 1970), *L. timidus* presumably disappeared from the Iberian Peninsula at the end of the last ice age. Here we assess the geographical pattern of this introgression and show that mtDNA lineages of *L. timidus* origin are prevalent in the northern Iberian populations of *L. granatensis*, *L. europaeus* and *L. castroviejoi*. We argue that, for this pattern

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**Table 1** Relative frequencies of the mitochondrial RFLP haplotypes in 37 sample locations in the Iberian Peninsula and association to the mitochondrial lineage

Sp	Pop code	Location	RFLP Haplotype									<i>n</i>
			AA	AB	EA	BB	BE	FB	CC	DD	GD	
gra	Ala1	Álava	0.72			0.28						47
	Alb	Albacete	0.96			0.04						25
	Alc	Alcañiz	0.21			0.79						24
	Ali	Alicante	0.90			0.10						20
	Alj	Aljustrel	1									25
	Ben	Benavente	0.12		0.04	0.84						25
	Brg	Bragança	0.18			0.82						11
	CBr	Castelo Branco	1									24
	Cac	Cáceres	0.45		0.55							20
	Crđ	Córdoba	0.83		0.17							6
	Crn	La Coruña	1									6
	CRé	Ciudad Real	0.92	0.08								25
	Cue	Cuenca	0.95			0.05						20
	Grn	Granada	1									25
	Hue	Huesca				1						7
	Mad	Madrid	0.44			0.32			0.24			25
	Mer	Mértola	1									12
	Mur	Múrcia	1									7
	Nav1	Navarra	0.04			0.96						25
	Pan	Pancas	1									25
	Ptm	Portimão	1									13
	SE	Serra da Estrela	1									6
	San	Santarém	1									16
	Sen	Sendim	0.57		0.24	0.19						21
	Sev	Sevilla	0.88		0.12							8
	Sor	Soria	0.23			0.77						26
	TC	Tierra de Campos	0.36		0.05	0.59						22
	Tol	Toledo	0.73			0.27						26
	Tor	Tordesillas	0.77			0.23						22
	Val	Valência	0.80			0.20						5
Zar	Zaragoza	0.11			0.89						36	
eur	Ala2	Álava				1						24
	Can	Cantabria				0.67			0.33			9
	Jac	Jaca				0.92			0.08			12
	Nav2	Navarra				0.75			0.25			8
	Vlc	Villarcayo				0.68	0.32					28
cas	Cas	Cantabria				0.11				0.78	0.11	9
mtDNA lineage			gra	gra	gra	tim	tim	tim	eur	cas	cas	Total 695

Sp, species; Pop, population, *n*: sample size.

to be the result of random drift alone, multiple hybridizations have to be postulated, which raises the question of a possible influence of natural selection in the spread of this ancient mitochondrial introgression.

## Materials and Methods

Tissue samples of 695 hare specimens (605 *Lepus granatensis*, 81 *Lepus europaeus* and 9 *Lepus castroviejoi*) were collected

in 37 populations across the Iberian Peninsula (Table 1, Fig. 1). Species identification was assessed in the field on the basis of morphologic characters (see Palacios 1989). Total genomic DNA was extracted from frozen liver or ear tissue using standard methods similar to those described in Sambrook *et al.* (1989). Polymerase chain reaction (PCR) primers LGCYF (Alves *et al.* 2003) and LCYTBR, 5'-CCGAGAAGGTCAGGAGAGAA-3', were used to amplify a 669 bp mitochondrial cytochrome *b* (cyt *b*) fragment.

**Table 2** *AluI* (A) and *Hpy* CH4V (H) restriction sites (asterisks) for RFLP haplotypes detected in four different hare mtDNA lineages in the Iberian Peninsula. New GenBank Accession nos are indicated (+)

Base	72	77	96	164	177	282	345	372	435	486	489	mtDNA lineage	GenBank Accession No.
Res Enz	A	H	A	H	A	H	A	A	A	H	A		
AA	*			*	*	*				*		gra	AY176190
AB	*			*	*	*						gra	AY942565†
EA	*			*	*	*	*			*		gra	AY942566†
BB	*			*		*		*	*			tim	AY176238
BE	*	*		*		*		*	*			tim	AY942567†
FB	*			*		*		*	*		*	tim	AY942568†
CC			*							*	*	eur	AY176230
DD	*					*		*				cas	AY176235
GD	*					*		*			*	cas	AY942569†

gra, *Lepus granatensis*; tim, *Lepus timidus*; eur, *Lepus europaeus*; cas, *Lepus castroviejo*.

Based on several published *L. granatensis*, *L. europaeus*, *L. castroviejo* and *L. timidus* *cyt b* sequences (Halanych *et al.* 1999; Pierpaoli *et al.* 1999; Alves *et al.* 2003), we searched for restriction enzymes that would allow species recognition. Although a single enzyme would have sufficed, we selected two, *AluI* and *Hpy*CH4V, to reduce the possibility of misclassification due to homoplasy. The PCR products were digested with these two enzymes separately, and the restriction patterns revealed after electrophoresis in 3% high resolution agarose gels. Fragments giving restriction patterns not predicted by the available sequences were sequenced (628 bp, using the PCR primer LGCYF) following the ABI Prism BigDye Terminator Cycle Sequencing 3.0 (Applied Biosystems) standard protocol.

A minimum-spanning network among the RFLP haplotypes and the uncorrected distances between haplotype sequences were computed using the program ARLEQUIN 2.001 (Schneider *et al.* 2000).

## Results

Table 2 describes the different restriction sites encountered after digesting the amplified 669 bp mitochondrial *cyt b* fragment in the hare samples listed in Table 1, using *AluI* (seven restriction patterns named A to G) and *Hpy*CH4V (five restriction patterns, A to E). Joint information with the two enzymes defines nine haplotypes that can be unambiguously attributed to the species-specific lineages: three to *Lepus granatensis* (AA, AB and EA), three to *Lepus timidus* (BB, BE and FB), one to *Lepus europaeus* (CC) and two to *Lepus castroviejo* (DD and GD) – Fig. 2. Five of these haplotypes (AB, EA, BE, FB and GD) contained restriction site variations not predicted by the available sequences. Although this did not impede their classification based on the other restriction sites (Fig. 2), we sequenced them to confirm their species-specific lineage assignment based on all variable sites in the fragment. All new sequences were deposited in GenBank (Table 2; Accession nos

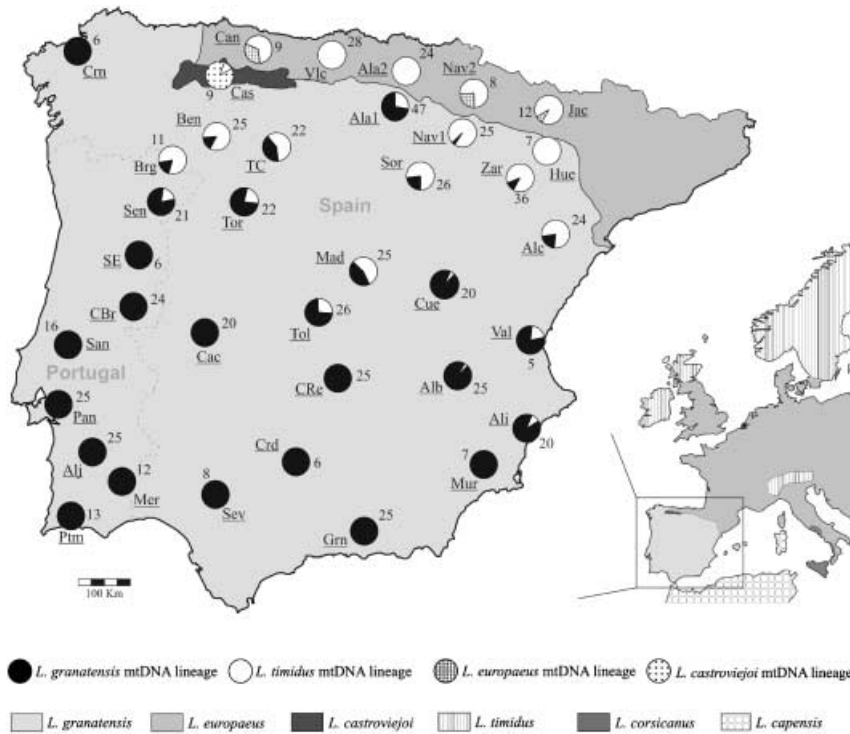
AY942565–AY942569). The ranges of *d* values between the introgressed and the resident species haplotypes, obtained from sequence data, are tim vs. gra *d* = 0.100–0.104, tim vs. eur *d* = 0.097–0.100 and tim vs. cas *d* = 0.026–0.032. These values are similar to those obtained by Alves *et al.* (2003).

The geographical distribution and frequencies of each species-specific mtDNA lineage in the hare populations from Iberia are shown in Fig. 1. The *L. timidus* mtDNA lineage was detected in 32% (193) of the *L. granatensis* specimens, which were all from the northern and central regions. Its frequency increases towards the north where the *L. granatensis* range meets that of *L. europaeus*. Introgression affects 93% (75) of the specimens and all populations of the latter species. We also detected introgression in one of our nine *L. castroviejo* specimens.

## Discussion

Although mitochondrial introgression in contact zones is not uncommon (e.g. Ferris *et al.* 1983; Carr *et al.* 1986; Tegelström 1987; Bernatchez *et al.* 1995; Arnold 1997; Ruedi *et al.* 1997; Goodman *et al.* 1999), the observation of *Lepus timidus* mtDNA in a vast area of the Iberian Peninsula and its presence at high frequencies in the populations of three different hare species is remarkable.

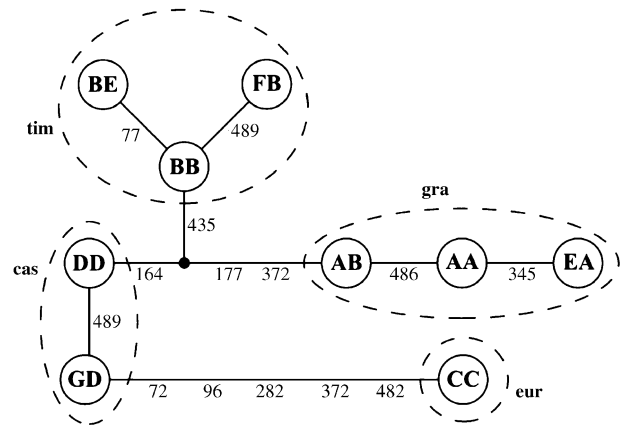
The fact that the mountain hare is now extinct from this region complicates the reconstruction of the history of this introgression. This species is only found in northern latitudes or at high altitudes so it is reasonable to assume that, during warmer periods of the glacial oscillations, the distribution of *L. timidus* in Iberia shrank while the habitats typical of the southern *Lepus granatensis* expanded. There is phylogeographical evidence that this occurred in other European species, such as the wild rabbit (Branco *et al.* 2002). The present limit of *L. timidus* mtDNA could therefore mark the southernmost historical position of a hybrid zone that fluctuated with the climate. The only known palaeontological



**Fig. 1** Species ranges of hares in western Europe (according to Mitchell-Jones *et al.* 1999) and geographical distribution and frequencies of the four mitochondrial lineages present in the three species from the Iberian Peninsula. Sample sizes and population codes depicted in Table 1 are shown next to the pie charts.

records of *L. timidus* are in the extreme north of Iberia, in Guipúzcoa, Basque Country, Spain (Altuna 1970), but their apparent absence in other parts of Iberia could be due to the uncertainty of species identification, which resulted in a long-standing taxonomic confusion in the genus *Lepus*. We suggest that the available *Lepus* fossil records in the Iberian Peninsula should be re-analysed.

A history of repeated geographical shifts of the contact zone between *L. timidus* and *L. granatensis* and widespread hybridization, especially given the amplitude of the mtDNA introgression, could have left traces in the nuclear genome of the introgressed species. The available allozyme and molecular data do not provide evidence of differentiation in the nuclear background between the introgressed and nonintrogressed populations (Alves & Ferrand 1999; Alves 2002; Alves *et al.* 2003). However, the genomic and geographical coverages of these data are limited and further investigation is needed. Male-biased colonization, recurrent backcrossing and demographic pressure during the postglacial expansion of *L. granatensis* could, nevertheless, have contributed to supplant the nuclear genome of *L. timidus*, but not the resident mtDNA. Such a cytonuclear genomic dissociation has been described in several mammal species such as pocket gophers and, more recently, in African elephants (Ruedi *et al.* 1997; Roca *et al.* 2005). In plants, numerous cases of chloroplast capture without evidence of nuclear introgression are known (see Rieseberg & Soltis 1991). In European oaks, for example, differences in dispersal abilities between *Quercus robur* and *Quercus*



**Fig. 2** Minimum-spanning network among assessed RFLP haplotypes. Dashed lines group each species mtDNA lineage (gra: *Lepus granatensis*; tim: *Lepus timidus*; eur: *Lepus europaeus*; cas: *Lepus castroviejoi*). The size of each haplotype is not proportional to its frequency (see Table 1 for relative frequencies). The numbers indicate the polymorphic sites detected in the RFLP analysis.

*petrea* and hybridization asymmetries have been suggested to explain the lack of differentiation in chloroplast DNA between them (Petit *et al.* 2004).

Naturally, we cannot know the type of interactions between *L. timidus* and *L. granatensis*. The only available evidence of hybridization in hares concerns *L. timidus* and *L. europaeus* in Sweden, where unidirectional introgression of the native *L. timidus* mtDNA occurs in the introduced *L. europaeus*

(Thulin *et al.* 1997; Thulin & Tegelström 2002). Asymmetries in species abundance or mating preferences were suggested to explain this pattern of mtDNA introgression (Thulin & Tegelström 2002). In captivity, female *L. timidus* spontaneously breed with *L. europaeus* males, while the reverse mating does not occur, although artificial insemination proved successful (Gustavsson & Sundt 1965). A similar asymmetry could explain the massive introgression of *L. timidus* mtDNA into Iberian *L. europaeus*. However, it is not known whether *L. timidus* ever coexisted in Iberia with *L. europaeus*, which most probably arrived only recently in western Europe, as attested by palaeontological and molecular data (Lopez-Martinez 1980; Pierpaoli *et al.* 1999). In the absence of a contact between these two species, *L. timidus* mtDNA could have entered *L. europaeus* via a *L. granatensis* intermediate. It should be possible to estimate the number of independent introgressions from *L. timidus* into the Iberian species by analysing mtDNA sequence variation of the introgressed haplotypes.

The same questions arise concerning the origin of the introgression into *Lepus castroviejoi*. The history of this species is poorly understood, and our data is too limited to obtain a reliable estimate of the extent to which this species is introgressed. It is however, remarkable that this third Iberian species has also been affected by mtDNA introgression from *L. timidus*.

To explain three similar introgression events into three different species, each with a different history, ecology and behaviour by purely stochastic, demographic or historical factors, requires a rather complex scenario, even if possible breeding asymmetries are taken into account. A selective hypothesis, assuming some advantage for the introgressed *L. timidus* mtDNA in different nuclear backgrounds, deserves consideration. Although mitochondrial DNA has long been considered an innocent bystander of population history for the convenience of phylogeographical reconstruction, evidence is accumulating that it is subject to selection pressures of various sorts (reviewed for instance in Arnold 1997; Rand 2001; Ballard & Whitlock 2004). Selection has repeatedly been invoked to account for massive mtDNA introgression (e.g. Bernatchez *et al.* 1995; Rognon & Guyomard 2003). There is evidence of mitochondrial adaptation in the primate lineage (e.g. Andrews *et al.* 1998) and, more interestingly, some arguments that temperature adaptation might be shaping its regional variation in humans (Mishmar *et al.* 2003; Ruiz-Pesini *et al.* 2004). A contribution of *L. timidus* mtDNA to adaptation to cold could explain its evolutionary success in all the species present in the colder northern part of the Iberian Peninsula. Occasional hybridizations followed by multiple mtDNA selective sweeps could explain the observed situation whatever the histories of the Iberian species. This assumption opposes the suggestion that, in Sweden, brown hares with mountain hare mtDNA express a fitness reduction due to cytonuclear incompatibility (Thulin & Tegelström 2002). Obviously, we do not have empirical

support for the occurrence of selection and either indirect evidence from sequence variation analyses, or more direct evidence revealing specific physiological impacts, is required before this hypothesis of a selective advantage of *L. timidus* mtDNA can be retained. We feel however, that our results should encourage research in that direction.

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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. P.C. 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.

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