

## Are *Lepus corsicanus* and *L. castroviejo* conspecific? Evidence from the analysis of nuclear markers

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### Abstract

Five species of genus *Lepus* occur naturally in Europe: *L. europaeus*, *L. timidus*, *L. granatensis*, *L. corsicanus*, and *L. castroviejo*. Of these, the latter two have restricted ranges, *L. castroviejo* in the Iberian Peninsula and *L. corsicanus* in central and southern Italy.

Morphological data show that *L. castroviejo* and *L. corsicanus* have extensive phenetic similarities, and might be sister taxa, which seems to be supported by a close genetic relationship at the mitochondrial DNA level. This marker also suggests a strong genetic similarity between both *L. castroviejo* and *L. corsicanus* and *L. timidus*.

However, mtDNA introgression seems to be a common phenomenon in hares and may confound any phylogeny based solely in this type of marker. Here, we analyse recently published DNA sequences data from 3 nuclear loci, reassess the genetic differentiation between *L. corsicanus* and *L. castroviejo* and compared it to *L. europaeus*, *L. granatensis*, *L. timidus*, and *L. capensis* (from Africa). This molecular data confirms a very close relationship between *L. corsicanus* and *L. castroviejo*, whereas the other species are phylogenetically clearly separated from each other.

Our results show that *L. corsicanus* and *L. castroviejo* have a strong genetic similarity, supporting the hypothesis that these species are most likely conspecific.

### Introduction

Hares are all included in genus *Lepus* and have a worldwide distribution. The presence of some conservative morphological characteristics among the species of hares often gave rise to taxonomic confusions within this mammal group.

Despite these earlier controversies, the latest taxonomic view accepts five species of genus *Lepus* occurring naturally in Europe: *L. europaeus*, *L. timidus*, *L. granatensis*, *L. corsicanus*, and *L. castroviejo* (fig. 1). Of these, the latter two have allopatric and very restricted ranges, the broom hare (*L. castroviejo*) occurring in the Cantabrian Mountains of the Iberian Peninsula, and the Italian hare (*L. corsicanus*) being present in the Apennines from central and southern Italy, and also in Sicily.

Several molecular studies have been conducted so far to assess the genetic diversity and the phylogenetic relationships of the European hare species (e.g. Pérez-Suárez *et al.* 1994, Thulin *et al.* 1997, Pierpaoli *et al.* 1999, Suchentrunk *et al.* 1999, Alves *et al.* 2000, Estonba *et al.* 2006, Ben Slimen *et al.* 2005). However, only Alves *et al.* (2003) included all five species simultaneously and analyzed both mitochondrial DNA (mtDNA) and a nuclear marker (the transferrin gene).

Apart from the latter study, the most comprehensive analyses of the evolutionary relationships among European hare species focused on RFLPs of the total mtDNA (Pérez-Suárez *et al.* 1994), and on mtDNA control region and cytochrome *b* sequences (Pierpaoli *et al.* 1999). The former, however, did not include *L. corsicanus* and *L. timidus*, and the latter did not include *L. castroviejo*, but suggested that *L. corsicanus* is clearly different from *L. europaeus* as it was traditionally classified (Ellermann and Morisson-Scott 1951, Flux and Angermann 1990).

Indeed, recent data show a clear morphometric differentiation between *L. corsicanus* and *L. europaeus* (Riga *et al.* 2001). Nonetheless, in a previous morphological study on the Italian hare, Palacios (1996) showed that despite some morphological peculiarities, *L. corsicanus* had extensive phenetic similarities to *L. castroviejo*, and both were clearly different from *L. europaeus*, suggesting that *L. corsicanus* and *L. castroviejo* might be sister taxa. On the other hand, analysis based on mtDNA by Alves *et al.* (2003) showed that these two taxa are closely related to *L. timidus*

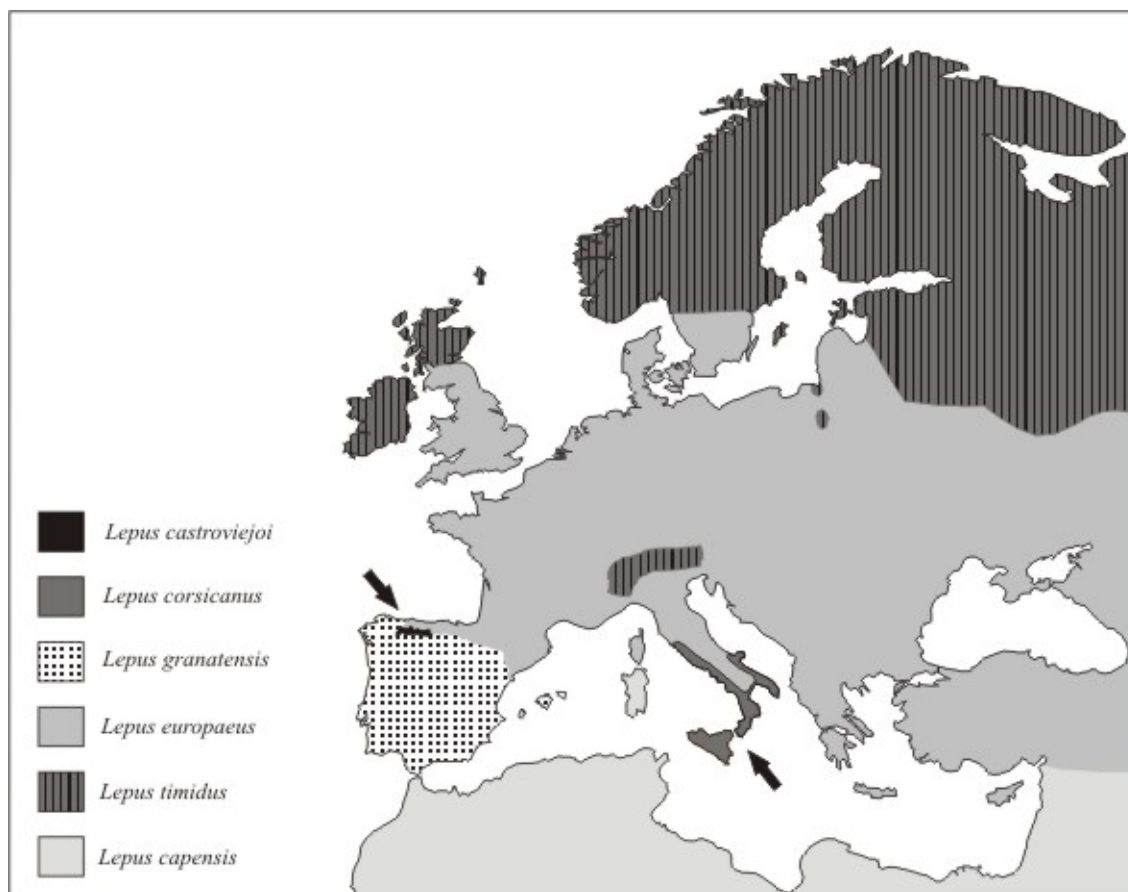
(2.2%-2.7% of divergence), and that the level of differentiation between them is very low when compared with the typical levels among hare species (circa 1.4% *vs.* 9% average between *Lepus* species). Moreover, these authors suggested that this mtDNA similarity to *L. timidus* could be due to ancient mitochondrial introgression similar to the one that occurred into the Iberian species (see also Melo-Ferreira *et al.* 2005; Melo-Ferreira *et al.* 2007).

These mtDNA resemblances led Wu *et al.* (2005) to suggest that both *L. castroviejoi* and *L. corsicanus* should be considered subspecies of *L. timidus*. However, this work did not consider that the mtDNA in hares seems to be the subject of recurrent introgression either due to ongoing or ancient contact and hybridisation (Thulin *et al.* 1997, 2006a, 2006b, Alves *et al.* 2006).

Thus, within genus *Lepus*, the analyses based solely on mtDNA sequences can be misleading and only data from several unlinked markers should produce reliable estimates of the phylogenetic relationships (see Robinson and Matthee 2005, Alves *et al.* 2006, Ben Slimen *et al.* 2007). First data resulting from the combination of mtDNA and nuclear genes shows that *L. corsicanus* and *L. castroviejoi* are genetic similar, and different from *L. timidus* (Alves *et al.*, in press).

In this work we reassess the genetic differentiation between *L. corsicanus* and *L. castroviejoi* and compared it to *L. europaeus*, *L. granatensis*, *L. timidus*, and *L. capensis* (from Africa) by analysing recently published DNA sequences data from 3 nuclear loci.

**Figure 1 - Geographical distribution of the European and northern African hares according to Mitchell-Jones *et al.* (1999) and to Angelici and Luiselli (2001). The arrows point the distribution of *L. castroviejoi* and *L. corsicanus*.**



## Methods

We used recently gathered DNA sequence data from the nuclear gene Transferrin, TF, intron 5 from the Carbonic Anhydrase II gene, CAII, and intron 13 from the Albumin gene, ALB (Alves *et al.* in press).

Four sequence datasets were defined, one for each gene and a fourth for the concatenated characters. The approach outlined by Huelsenbeck and Crandall (1997) was used to test 56 alternative models of evolution, employing PAUP\* 4.0b10 (Swofford 2002) and MODELTEST v3 (Posada and Crandall 1998). The selected models, using the AIC criterion, were the HKY for TF and ALB, K81uf for CAII and TIM for the concatenated dataset. To determine whether the sequences could be concatenated for further analyses we imported them into MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001).

Two distinct runs of 5.000.000 generations with two replicates and were performed, using a conservative burn-in of 1.000.000 in the post runs analysis:

1. using the correspondent model for each gene and unlinked the topologies and branch lengths;
2. using the single model for the concatenated dataset.

The harmonic means of the obtained likelihoods were then compared calculating the Bayes factor (B10; see Nylander *et al.* 2004). The obtained value ( $2\ln B10 = 1.94$ ) indicated that there was no strong evidence against the use of the concatenated dataset. This dataset was imported into PAUP\* 4.0b10 and an unrooted Neighbor-Joining tree was produced using the TIM model of sequence evolution and 1000 bootstrap replicates.

MEGA version 3.1 (Kumar *et al.* 2004) was used to compute the uncorrected p-distances between pairs of species.

**Table 1 - List of specimens analysed in this study, sample locations, abbreviated codes. For GenBank accession numbers of nuclear transferrin (TF), carbonic anhydrase II (CAII) and albumin (ALB) sequences see Alves et al. (in press).**

<i>Species</i>	<i>Collection locality</i>	<i>code</i>	
<i>Lepus castroviejo</i>	Spain (Argovejo)	Lcast1	
	Spain (Maraña)	Lcast2	
	Spain (Cantabrian Mountains)	Lcast4	
	Spain (Cantabrian Mountains)	Lcast5	
	Spain (Cantabrian Mountains)	Lcast6	
	Spain (Cantabrian Mountains)	Lcast7	
	Spain (León province)	Lcast9	
	Spain (León province)	Lcast10	
	Spain (Riano)	Lcast11	
	<i>Lepus corsicanus</i>	Italy (Castello Porziano)	Lcors1
		Italy (Corsica)	Lcors2
<i>Lepus europaeus</i>	Austria (Lassée)	Lass	
	Spain (Navarra)	Nav47	
<i>Lepus granatensis</i>	Portugal (Aljustrel)	Alj107	
	Portugal (Aljustrel)	Alj108	
<i>Lepus timidus</i>	Scotland (Mull)	Esc3	
	Norway	Nor34	
<i>Lepus capensis</i>	Morocco (Rabat)	Raba5	
	Morocco (Tetouan)	Tet2	

## Results

Nineteen individuals representing six hare species were analysed for 1715 bp from three independent nuclear genes (679 bp of CAII, 425 bp of TF and 611 bp of ALB). The Neighbor-Joining tree shows undoubtedly that *L. corsicanus* always shares the genotypes with *L. castroviejoii*, and both are clearly distinct from *L. timidus* (fig. 2). All other species could be clearly separated although their relationships cannot be fully understood given the low bootstrap support of the internal nodes. This result is also illustrated in table 2 where the uncorrected p-distance between pairs of species is 0,000 between *L. castroviejoii* and *L. corsicanus*, while it ranges from 0,004 to 0,013 among the other species.

Figure 2 - Phylogenetic relationships of the European and northern African hares derived from the unrooted Neighbor-Joining analysis of a concatenated nuclear dataset (1715 bp). Bootstrap support is indicated on the branches.

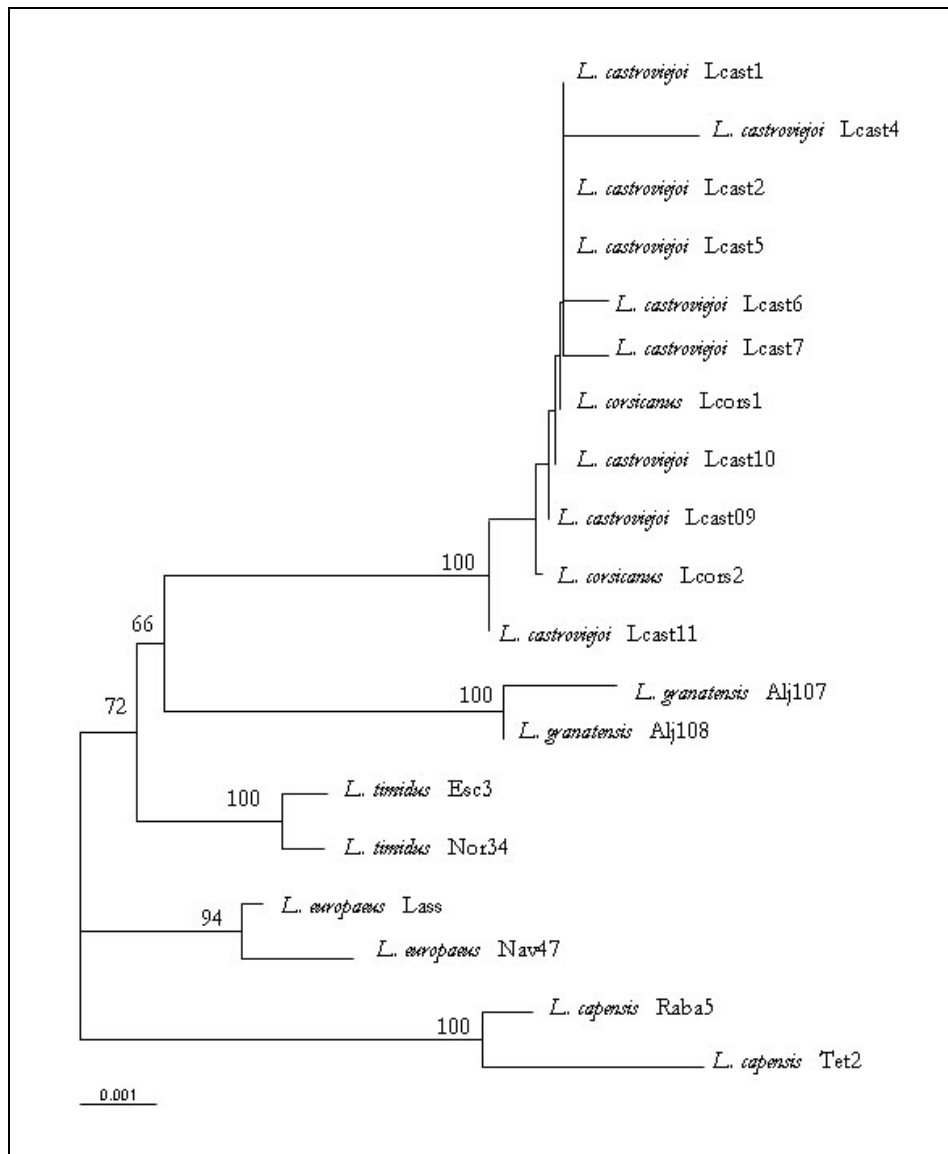


Table 2 - Uncorrected p-distances between the nuclear sequences of pairs of the hare species analysed in this study.

	1	2	3	4	5
1 <i>L. castroviejo</i>					
2 <i>L. corsicanus</i>	0.000				
3 <i>L. timidus</i>	0.006	0.006			
4 <i>L. granatensis</i>	0.010	0.010	0.006		
5 <i>L. europaeus</i>	0.008	0.007	0.004	0.007	
6 <i>L. capensis</i>	0.013	0.013	0.010	0.013	0.010

## Discussion

In the nuclear markers used here, *L. corsicanus* and *L. castroviejo* share their sequences, and none of these sequences occur in any other taxa analysed in this study. Further, the species separation for *L. granatensis*, *L. timidus*, *L. europaeus* and *L. capensis* is confirmed, but not for *L. corsicanus* and *L. castroviejo*.

This absence of genetic differentiation between *L. castroviejo* and *L. corsicanus* is in agreement with the mitochondrial DNA (see Alves *et al.* 2003, Wu *et al.* 2005, Alves *et al.*, in press), and with microsatellite and allozyme data (Suchentrunk *et al.* 2006, 2007, this volume). On the other hand, this genetic similarity is also in accordance with the overall morphological similarity suggested by Palacios (1996), who considered them sister taxa.

Despite the present allopatric distribution of *L. corsicanus* (Italy) and *L. castroviejo* (Spain), it seems that these two species had a recent common ancestor. Their common ancestor could have had a larger distribution range in Europe during the Pleistocene and it is plausible that the posterior climatic changes split it into two allopatric refugia in the Iberian and Italian Peninsulas.

The expansion of *L. corsicanus* and *L. castroviejo* from these putative refugia could have been prevented by competition with other hare species during various climatically different phases. In the Iberian Peninsula, *L. castroviejo* could have faced competition by *L. granatensis* and by *L. timidus* as well, when this species was present in northern Spain in Late Pleistocene (Altuna 1970). Regarding *L. europaeus* as another possible ecological competitor to *L. castroviejo*, no convinced fossil or molecular data exist that might give a clue to whether or not this species was present in late Pleistocene in Iberia, or whether it immigrated from a rapidly expanding postglacial population outside Iberia (e.g., Pierpaoli *et al.* 1999, Kasapidis *et al.* 2005). Similarly, in the Italian Peninsula, the northwards expansion of *L. corsicanus* could have been restricted by competition with *L. timidus* and *L. europaeus*.

Curiously, similar phylogeographic scenarios of allopatric refugia in these two regions have been also suggested for other species, namely the chamois, *Rupicapra pyrenaica* (Massini and Lovari 1988). The two species *R. pyrenaica* and *R. rupicapra* appear to have differentiated prior to the Würm glacial period. However, *R. pyrenaica* is geographically split into two groups, one in the Pyrenees (Spain), and the other in Italy (central-South Apennines), while *R. rupicapra* invaded Europe during the Würm and perhaps prevented postglacial expansion of *R. pyrenaica* from its refugia (Massini and Lovari 1988).

Although the nuclear genes confirm the genetic similarity between *L. corsicanus* and *L. castroviejo*, the genetic proximity between this group and *L. timidus* is not supported. In fact, the combined nuclear data set show that these two groups (*L. timidus* and *L. castroviejo/L. corsicanus*) are well differentiated (tab. 2). This contradicts the suggestion based on mtDNA alone that *L. corsicanus* and *L. castroviejo* should be considered subspecies of *L. timidus* (Wu *et al.* 2005). The genetic similarity observed between these two species and the *L. timidus* could be due to an ancient hybridisation and introgression of mtDNA from *L. timidus* into the ancestral *L. corsicanus/L. castroviejo* population or to shared ancestry and incomplete lineage sorting at the mtDNA. Given the low support at internal nodes in the nuclear phylogeny presented here, the phylogenetic relationships between the species cannot be revealed, and therefore this question remains unsolved. Maybe increasing the length of the sequences and the number of sampled loci could help to clarify this issue.

To conclude, our results based on three independent nuclear loci support the specific status of four species (*L. granatensis*, *L. europaeus*, and *L. timidus* and *L. capensis*), but suggest that *L. corsicanus* and *L. castroviejo* have a strong genetic similarity, and are distinct from *L. timidus*.

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