Molecular genetic aspects of *Lepus corsicanus*

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Abstract

We report some molecular (multi-locus allozyme, microsatellite, and mitochondrial HV-1 sequence) data collected recently from some *Lepus corsicanus* specimens, to (1) help evaluating the phylogenetic position of this species, to (2) evaluate possible phylogeographic approaches, and to (3) evaluate the chances of detecting possible introgression of introduced brown hares (*L. europaeus*) into *L. corsicanus* where the two species occur sympatrically.

Our results are in agreement with the recently suggested hypothesis that *L. corsicanus* is phylogenetically closely related, probably conspecific, with *L. castroviejoi* from the Cantabrian Mountains in the Iberian Peninsula. They further suggest that mtHV-1 sequences are useful for phylogenetic inferences in this species, e.g., for studying introductions or translocations, and that our applied microsatellite marker system is useful for detecting introgession events possibly occurring between *L. corsicanus* and *L. europaeus*.

Introduction

The Appenine hare (*Lepus corsicanus* De Winton, 1898) has been redescribed only recently by Palacios (1996) as a separate species, after it has conventionally been considered a subspecies of the brown hare (*L. europaeus* Pallas, 1778) during a certain period of time in the 20th century.

Whereas this recent species redescription was based on phenotypic and morphological characters (see also Riga *et al.* 2003 for a comprehensive summary), some few molecular data have recently been gathered that confirmed the separate species status (Pierpaoli *et al.* 1999, Alves *et al.* 2003). However, inadequate interpretation of mitochondrial (mt) sequences caused Wu *et al.* (2005) to consider *L. corsicanus* merely a subspecies of moutain hares (*L. timidus*).

Recently, Suchentrunk *et al.* (2006) examined the mt HV-1 sequence characteristics of the remains of a hare collected as roadkill in Corsica. It could not be assigned morphologically to either *L. europaeus* or *L. corsicanus*, due to the fact that only some few tissue parts were available. They concluded from the mtDNA characteristics that the examined tissue sample was from *L. corsicanus*.

They phylogenetically analysed the obtained sequence together with *L. corsicanus* sequences downloaded from GenBank, and concluded that their Corsican sample had close phylogenetic relationships to Sicilian lineages. This was, however, in disagreement with the conclusion from (unpublished) sequence data from M. Pierpaoli reported in Riga *et al.* (2003), which indicated close phylogenetic relationship between some Corsican *L. corsicanus* and mainland Italian *L. corsicanus*.

A further preliminary molecular examination of *L. corsicanus* and *L. europaeus* in southern Italy (Fulgione *et al.* 2006) suggested occasional introgression of the latter species in *L. corsicanus*. However, all these few molecular studies warrant further examination.

Here we report on some further molecular data that we have gathered in the recent past, which might help understanding phylogenetic and biological characteristics of the Appenine hare. Specifically, we use (1) multi-locus allozyme and microsatellite approaches to help evaluate the phylogenetic position of *L. corsicanus*, we use (2) some additonal samples to comment on the use of mt sequence data for phylogeographic inferences, and we use

(3) microsatellite data of some few *L. corsicanus* samples to get a first idea about their usefulnes for inferences about possible cases of introgression from *L. europaeus* and/or differentiation between the two species.

Methods

Allozyme electrophoresis and statistical analysis

Frozen (-20°C) liver and kidney samples of one *L. corsicanus*, shot at Castel Porzano (north of Rome) was used for horizontal starch gel electrophoresis to resolve allelic variation at isozyme loci. In tissue preparation, electrophoresis, and protein specific staining we followed Hartl and Höger (1986) and Grillitsch *et al.* (1992).

For direct side-by-side comparisons of migrating allozymes the sample was run in the same gels together with samples of two broom hares (*L. castroviejoi*), of brown hare, *L. europaeus* from five local populations in northern and eastern Austria (OIV, n=30, OWN, n=31, WV3, n=28, TFN, n=30, SW, n=29; see Hartl *et al.* 1993), as well as mountain hare, *L. timidus*, from eastern Switzerland (Canton Glarus – CH, n=15), Scandinavia (SC, n=46), and the Ural (UR, n=46) (see Suchentrunk *et al.* 1999). This allowed for allele designations consistent with Hartl *et al.* (1993) and Suchentrunk *et al.* (1999).

The following 18 isozymes/-systems encoded by 28 putative structural gene loci were assayed for allozymic variation by horizontal starch gel electrophoresis (isozyme/-system, abbreviation, E.C.number, and corresponding structural gene loci in parentheses): sorbitol dehydrogenase (SDH, 1.1.1.14, Sdh), lactate dehydrogenase (LDH, 1.1.1.27, Ldh -1,-2), malate dehydrogenase (MOR, 1.1.1.37, Mor -1,-2), malic enzyme (MOD, 1.1.1.40, Mod-1,-2), isocitrate dehydrogenase (IDH, 1.1.1.42, Idh-1,-2), 6-phosphogluconate dehydrogenase, (PGD, 1.1.1.44, Pgd), glucose-6-phosphate dehydrogenase (GPD, 1.1.1.49, Gpd), glutamate dehydrogenase (GLUD, 1.4.1.3,Glud), superoxide dismutase (SOD, 1.15.1.1, Sod-1,-2), purine nucleoside phosphorylase (NP, 2.4.2.1,Np), aspartate aminotransferase (AAT, 2.6.1.1, Aat-1,-2), hexokinase (HK, 2.7.1.1, Hk-1,-2), esterases (ES, 3.1.1.1, Es-1; 4.2.1.1, Es-D), acid phosphatase (ACP, 3.1.3.2, Acp-1), peptidases (PEP, 3.4.11, Pep-1,-2), aconitase (ACO, 4.2.1.3, Aco-1,-2), mannose phosphate isomerase (MPI, 5.3.1.8, Mpi), glucose phosphate isomerase (GPI, 5.3.1.9, Gpi-1). Genotypic interpretation of band patterns were carried out in accordance with quaternary enzyme structures (e.g., Rothe 1994).

All loci scored presently were known to be in genotypic linkage equilibrium in the respective studied brown and mountain hare populations (Hartl *et al.* 1993, Suchentrunk *et al.* 1999). Thus, each locus conveyed independent gene pool information.

The GENETIX 4.05.2 software (see Belkhir *et al.* 2004) was used to calculate allele frequencies and pairwise Nei's (1978) genetic distances corrected for small sample sizes, as well as Cavalli-Sforza Edwards chord (CSE) distances between *L. corsicanus*, *L. castroviejoi*, and all currently used local populations of *L. timidus* and *L. europaeus*.

Based on the CSE distance matrix, we run a Principal Coordinate Analysis (PCA), using the PCO program Anderson (2003).

We also constructed a neighbor joining (NJ) dendrogram based on Nei´s (1978) distances using the PHYLIP vers. 3.57c software package (Felsenstein 1995). Bootstrap support values (1000 repetitions) for internal nodes were calculated with the same program.

Mitochondrial HV-1 sequences

The following four *L. corsicanus* samples were used for mitochondrial (mt) DNA sequence analysis (GenBank accession numbers EU200450-EU200453)

1. LcoAl: muscle tissue sample collected as roadkill near Aléria, Corsica, in 2004 (see Suchentrunk et al. 2006),
2. LcoCP: one liver sample from a hare shot at Castel Porzano, mainland Italy,
3. LcoSI: one ear tissue sample of a hare collected near Siracusa, Sicily in 2003,
4. LcoRA: one ear tissue sample of a hare collected at Randazzo, near Catania, Sicily in 2003,

5. LcoMO: a further sample that was supposed to belong to *L. corsicanus*, turned out to be in fact from L. europaeus (see below): ear tissue sample of a (small/young?) hare collected at Monterufoli near Pisa in 2006, and introgession was suspected; no further information upon its external phenotype was available.

The samples LcoCP, LcoSI, and LcoRA were considered belonging to *L. corsicanus*, based on their external phenotype (see Palacios 1996). For LcoAl no phenotypic species diagnosis was possible, as it represented only remnants of a heavily smashed roadkill; but both mtDNA and nuclear gene sequences were typical for *L. corsicanus* (see Suchentrunk *et al.* 2006, Alves *et al.* in press).

Total genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep kit. We used the forward primer Le.H-Dloop 5´-AAGAACCAGATGCCAGTTATAG-3´ and the reverse primer Le.L-Dloop 5´-AATTCTCTTTAAACTATTCTCTGC-3´ to amplifiy a portion of the control region (hypervariable part-1, HV-1) by polymerase chain reaction (PCR), following by and large the protocol of Kassapidis *et al.* (2005).

The purified PCR products were sequenced (MACROGEN Inc., Seoul) and the sequences as well as those downloaded from GenBank (see below) were aligned with ClustalX (1.83) (Thompson *et al.* 1997).

The hare from Monterufoli, mainland Italy, revealed a HV-1 sequence identical with the Leu2 sequence reported by Pierpaoli *et al.* (1999); hence it was identified as a brown hare sequence.

In a first step a phylogenetic analysis of the remaining four new Apennine hare HV-1 sequences was conducted with the inclusion of all available HV-1 sequences of *L. corsicanus* (Piepaoli *et al.* 1999) and *L. europaeus* (Pierpaoli *et al.* 1999; Fickel *et al.* 2005; Kassapidis *et al.* 2005). The list of downloaded sequences is not shown, but can be requested from the authors.

Since many of the downloaded sequences were shorter than our new four sequences, our first alignment was based on 244 bp (including indels) long HV-1 segments. With this alignment length several of the initially different haplotypes from GenBank became identical. One haplotype of a South African *L. capensis* (AF491353) was used as outgroup. Prior to all further analysis Modeltest 3.06 was used to find the optimal model of DNA substitution for our data set (Posada and Crandall 1998), which was the HKY85 model with TRatio= 7.1409, Shape = 0.2708.

We used PAUP4.0b10 (Swofford 2003) to construct a NJ tree as well as a maximum parsimony (MP) analysis with TBR branch swapping and 10 random taxon addition replicates under a heuristic search, saving no more than 100 equally parsimonious trees per replicate.

Support for the internodes in the trees was assessed by bootstrap percentages after 1000 resampling steps (Felsenstein 1985) also using PAUP. As a third approach, we run a maximum likelihood phylogenetic (ML) analysis, with heuristic ML searches using TBR branch swapping again in PAUP4.0b10. ML nodal support was estimated by using the non-parametric bootstrap (Felsenstein 1985) and was restricted to 100 pseudo-replicates because of limited computing time.

As an additional approach, we constructed median-joining (MJ) networks (Bandelt et al. 1999) using the software Network 4.2.0.1 (available at http://www.fluxus-technology.com/sharenet.htm) including all corsicanus haplotypes.

Our first network was based on the 21 variable positions (with one indel position which was considered as a fifth character) of the 244 bp alignment encompassing all L. corsicanus sequences downloaded from GenBank as well as those of our new samples.

Our second network was based on the 462 bp alignment of the four new haplotypes with 15 variable sites (including one indel position). This latter network was ment to reveal additional information, to confirm or refute our phylogeographic conclusion regarding the Corsican sample LcoAl, as it was based on longer sequences. All positions were equally weighted and MJ networks were constructed setting the parameter ε equal to 0.

Microsatellite-based analysis of nuclear gene pool difference between *L. corsicanus* and *L. europaeus*

To assess nuclear gene pool characteristics and differences between Appenine and brown hares and to evaluate the chances of detecting evidence of possible nuclear introgression of brown hares into Appenine hares and vice versa, we selected the following eleven microsatellite loci with different levels of polymorphism: Sol08, Sol28

(Rico *et al.* 1994), Sol33 (Surridge *et al.* 1997), Lsa 1, Lsa 2, Lsa 3, Lsa 6 and Lsa 8 (Kryger *et al.* 2002 ; see also Ben Slimen *et al.* in press), and Sat2, Sat 8, Sat12 (Mougel *et al.* 1996).

The *L. corsicanus* samples listed above (LcoAl, LcoCP, LcoSI, LcoRA) and the presumed *L. corsicanus* sample LcoMO were genotyped for those loci together with one *L. castroviejoi* sample and samples of 15 *L. europaeus* from northern and eastern Austria on a LI-COR 4200 sequencer following the protocol described in Schappelwein (2007) and Ben Slimen *et al.* (in press). In some few individuals one or the other locus did not yield satisfying genotypes after several repeats.

The brown hare samples were taken from Austria and not from Italy to minimize te chance of samples in the comparison that might a priori be introgessed by *L. corsicanus*, and that migth thereby slur the characteristics of either species.

The GENETIX 4.05.2 software was used to calculate allele frequencies and to run a factorial corrspondence analysis based on the genotypes at the eleven loci.

The STRUCTURE software vers. 2, 13.7.2004 (Pritchard *et al.* 2000) was used to probabilistically infer (population) structuring of the genotype data set, thereby assigning each individual to a population or jointly to more than one population, if their genotypes indicated that they were admixed, under the assumptions of within population Hardy-Weinberg equilibrium and linkage equilibrium. The latter has been tested earlier for brown hares (see Schappelwein 2007, Ben Slimen *et al.* in press). Specifically, we run 15 admixture models for each K=1-10 (1-10 hypothetical populations), with length of Burnin period of 100.000 and 100.000 MCMC repetitions after Burnin.

Results

Allozyme data

Twelve (42.9%) of the 28 isozyme loci revealed allelic variation, with an average of 2.67 alleles per polymorphic locus (see table 1 for allele frequencies at polymorphic loci).

The allelic composition of the *L. corsicanus* and *L. castroviejoi* samples were identical except for two loci; firstly, whereas *L. corsicanus* had only one *Es-1* allele, *L. castroviejoi* had a second allele at this locus, and secondly, at the *Acp-1* locus *L. corsicanus* had two alleles, whereas there was only one observed in *L. castroviejoi.*

This resulted in relatively low genetic distance values between the two species, compared to the values between either species and *L. timidus* as well as *L. europaeus*, and compared to the values between the latter two species (see figure 1 for means of pairwise CSE chord distances between species and between populations within *L. timidus* and *L. europaeus*, respectively).

The PCA of the CSE distance matrix yielded a resolution with two dimensions, explaining already over 100% of the matrix structure (fig. 1). It grouped *L. corsicanus* and *L. castroviejoi* in close vicinity within the two-dimensional space, exhibiting only a slightly higher divergence (particularly along the second dimension) than for the population samples of *L. timidus*.

This close relationship between *L. corsicanus* and *L. castroviejoi* was also revealed by the NJ dendrograms (not shown) based on Nei´s (1978) and CSE distances, which was supported by high bootstrap values.

Mitochondrial HV-1 sequences

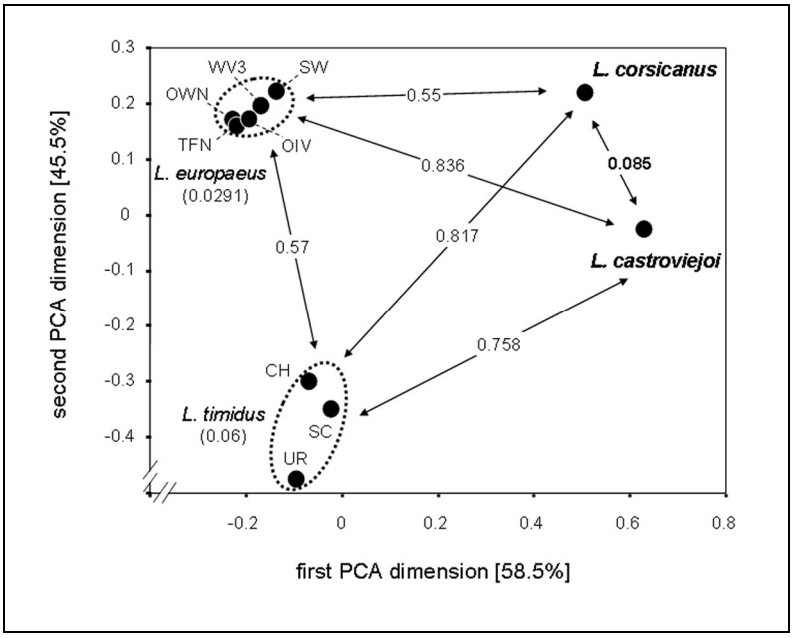
Total sequence information of our HV-1 fragments was 462 bp, including one indel at position 251 for "LcoCP".

No sequence showed an unexpected position in our phenotypic and phylogenetic analyses; hence we considered our data not affected by possible nuclear representations of mtDNA. Both the NJ, MP, and ML dendrograms concordantly revealed two major clusters, one encompassing all *L. europaeus* haplotypes and the other all *L. corsicanus* haplotypes (see fig. 2 A for a NJ tree and bootstrap support values for NJ, MP, and ML analyses).

Whereas the LcoCP (Castel Porziano) sequence was identical with the Lco2 haplotype from Latium (see tab.1 in Pierpaoli *et al.* 1999), our *L. corsicanus* sequences (i.e., LcoSI, LcoRA, LcoAI) clustered with sequences from Sicily

and southern mainland Italy (Lco4, Lco5; see tab.1 in Pierpaoli *et al.* 1999). However, this differentiation pattern within *L. corsicanus* was only modestly supported by bootstrap values (fig. 2 B).

Figure 1 - Two-dimensional plot of principal coordinate analysis (PCA) of populations/taxa based on 28 allozyme loci. For acronyms see "Methods". Numbers attached to arrows indicate pairwise CSE distance values, numbers in parentheses attached to "*L. europaeus*" and "*L. timidus*" indicate means of pairwise CSE distance values for each species.



The MJ network based on the 244bp alignment (fig. 3) revealed separation among Sicilian, central Italian, and southern Italian (Campanian/Calabrian) haplotypes, with the Corsican haplotype (LcoAl) identical with Lco13 from Sicily. The MJ network for the haplotypes LcoAl, LcoCP, LcoRA, LcoSI based on 462 bp revealed close phylogenetic relationship of the Corsican LcoAl and the Sicilian LcoRA and LcoSI sequences (fig. 4). However, the LcoMO haplotype clustered within the *L. europaeus* sequences; in fact it was identical with the Leu2 haplotype reported by Pierpaoli *et al.* (1999).

Microsatellites

We revealed a total of 71 alleles, i.e., an average of 6.5 alleles per locus, and a range from 2 (Sol-33, Sat-8) to 15 (Sat-2). Of all eleven loci only 2 (Sol-8, Lsa-6) revealed differentially diagnostic alleles for the *L. corsicanus* and *L. europaeus* comparison; i.e., our two (very limited) species samples did not have any allele in common at those two loci.

For the comparison between the *L. corsicanus* and *L. castroviejoi* samples, three loci (Lsa-2, Lsa-3, Sat-2) showed differentially diagnostic alleles. The factorial correspondence analysis (fig. 5) revealed three-dimensional separation of *L. corsicanus* and *L. europaeus* individuals at a relatively low level of variation (a total of 33.4% of variation for the first three axes). Whereas it showed clearly different positons for the *L. corsicanus* and *L. europaeus* individuals, respectively, the position of the *L. castroviejoi* sample was slightly closer to the Sicilian *L. corsicanus* samples than was the mainland Italian LcoCP specimen. The mainland Italian LcoMO specimen was positioned somewhere in between the *L. corsicanus* and the *L. europaeus* specimens (fig. 5). The Bayesian structural population analysis as implemented in the STRUCTURE software suggested tentatively highest likelihood for a significant structuring of the 22 individual samples into three populations (K=3, ln P (D) mean over 15 runs = -610.5, range from -625.5 to –596.0; for K<3 and K=4-15, ln P (D) mean over 15 runs per K: < -668.8, range from –1827.4 to – 603.7).

Table 1 - Allele frequencies at polymorphic loci in *Lepus corsicanus* (Lco), *L. castroviejoi* (Lca), and respective ranges for the local populations of *L. timidus* (Lti) and *L. europaeus* (Leu). * - the Sdh A allele is differentially diagnostic for *L. europaeus*, its presence in some mountain hares from the Swiss Alps is due to introgressive hybridization (see Suchentrunk *et al.* 2005).

locus	*allele*	*Lco*	*Lca*	*Lti (range)*	*Leu (range)*
Sdh	A	0.0	0.0	0.0 – 0.67*	1.0
	B	1.0	1.0	0.933 – 1.0	0.0
Mor-2	A	1.0	1.0	1.0	0.967 – 1.0
	B	0.0	0.0	0.0	0.0 – 0.033
Idh-2	B	1.0	1.0	0.321 – 0.867	0.933 – 1.0
	A	0.0	0.0	0.0	0.0 – 0.067
	C	0.0	0.0	0.133 – 0.679	0.0
6-Pgd	A	0.0	0.0	0.964 – 1.0	0.786 – 1.0
	B	0.0	0.0	0.0 – 0.036	0.0 – 0.052
	C	0.0	0.0	0.0 – 0.016	0.0 – 0.018
	D	1.0	1.0	0.0	0.0 – 0.036
	E	0.0	0.0	0.0	0.0 – 0.143
Hk-2	A	1.0	1.0	0.957 – 1.0	0.95 – 1.0
	B	0.0	0.0	0.0 – 0.043	0.0 – 0.05
Es-1	A	0.0	0.0	0.1 – 0.143	0.0 – 0.05
	B	0.0	0.0	0.857 – 0.9	0.569 – 0.833
	C	1.0	0.75	0.0	0.167 – 0.414
	D	0.0	0.25	0.0	0.0 – 0.017
Es-D	A	0.0	0.0	0.818 – 0.933	0.672 – 0.887
	B	1.0	1.0	0.067 – 0.182	0.113 – 0.328
Pep-2	A	1.0	1.0	0.0 – 0.1	0.75 – 0.862
	B	0.0	0.0	0.9 – 1.0	0.138 – 0.25
	C	0.0	0.0	0.0 – 0.057	0.0
Acp-1	A	0.5	0.0	0.0	1.0
	B	0.5	1.0	1.0	0.0
Mpi	A	1.0	1.0	0.9 – 0.929	0.914 – 1.0
	B	0.0	0.0	0.0 – 0.077	0.0 – 0.086
	C	0.0	0.0	0.0 – 0.1	0.0
Acon	A	1.0	1.0	0.978 – 1.0	1.0
	B	0.0	0.0	0.0 – 0.022	0.0
Me-2	A	1.0	1.0	0.929 – 1.0	1.0
	B	0.0	0.0	0.0 – 0.071	0.0

Figure 2 - Phylogenetic relationships of mtDNA sequences. A: NJ tree of HV-1 haplotypes based on 244 bp and HKY85 (alpha=0.2708) distances. Bootstrap values are indicated for NJ/MP/ML if >50%. B: Magnification of L. corsicanus cluster in the NJ, with geographic sample details for haplotypes. For acronyms see "Methods" and Pierpaoli *et al.* 1999.

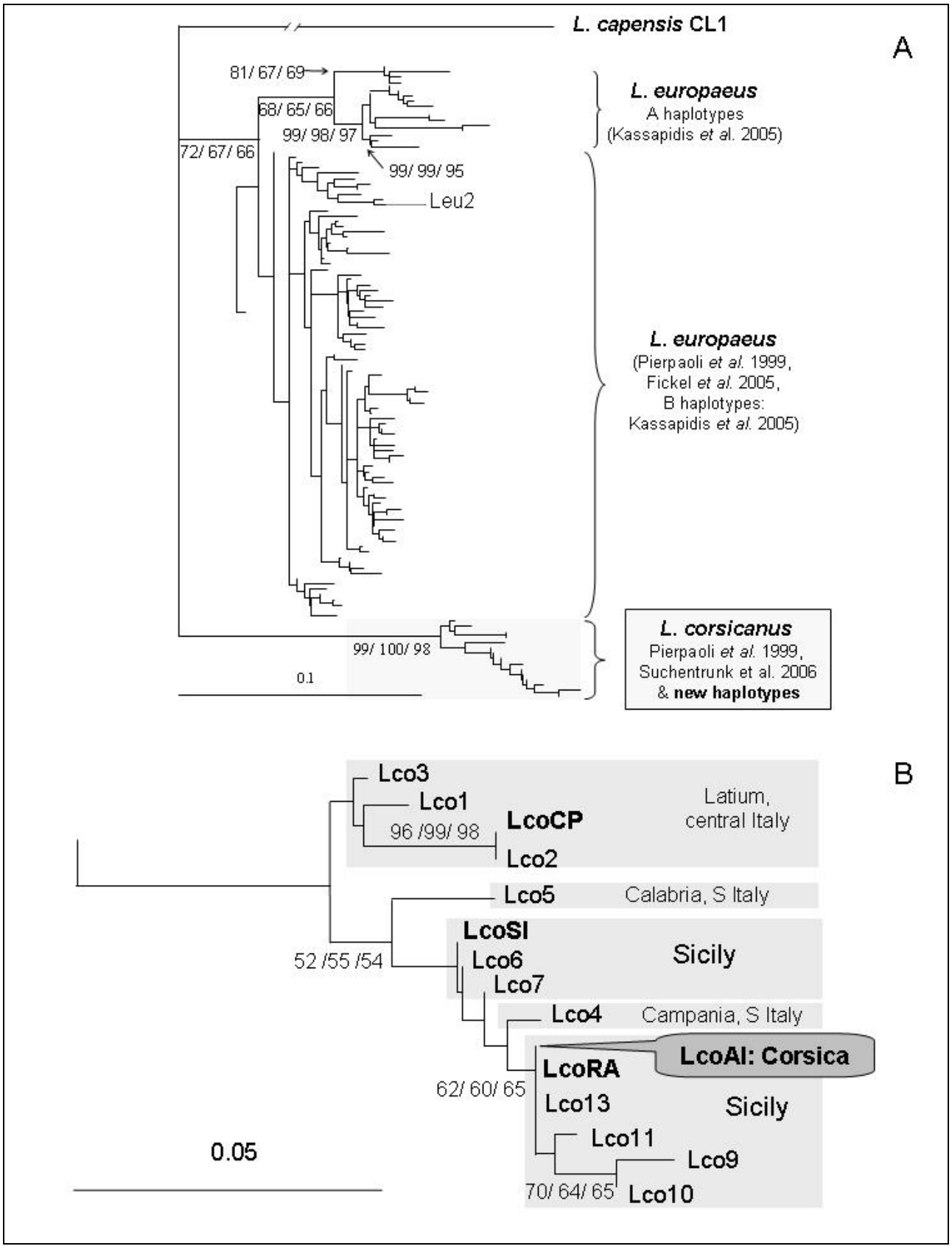


Figure 3 - Median-joining network of mtHV-1 haplotypes based on 244 bp and 21 variable sites (including one indel position coded as fifth character). For haplotype acronyms see "Methods" and Pierpaoli *et al.* 1999. Each bar on a connecting line between haplotypes (full cirlces) indicates a single site substitution. Small open circles indicate inferred haplotypes.

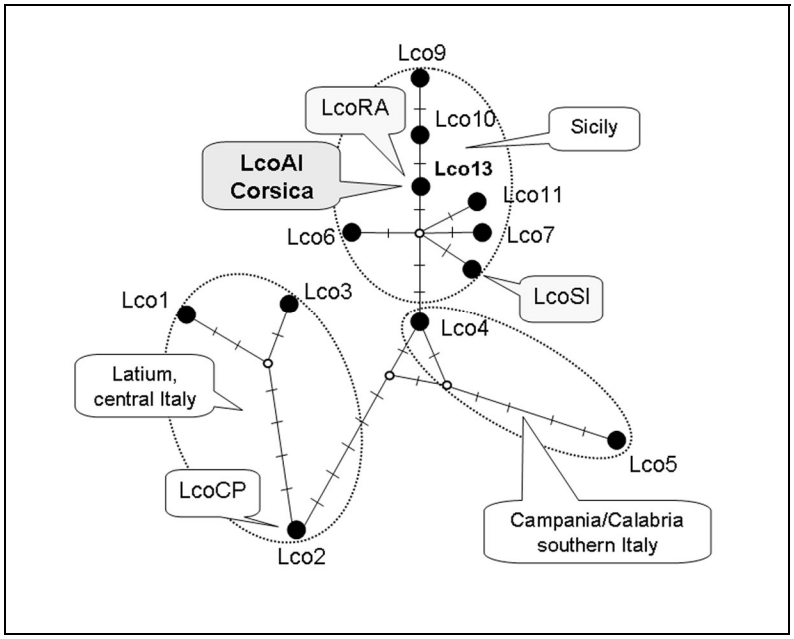
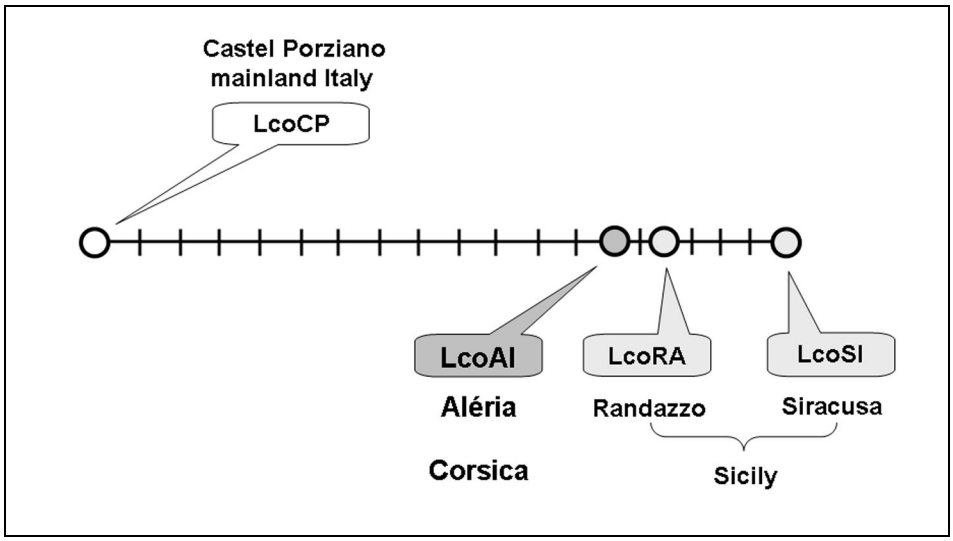


Figure 4 - Median-joining network of mtHV-1 haplotypes based on 462 bp and 15 variable sites.



The summary plot of estimates of individual membership coefficients (Q) in each inferred population is depicted in figure 6. High individual Q values for a single population assignation were observed for each of the three inferred populations (one for *L. corsicanus*, two for *L. europaeus*), concordant with the interpretation of structuring into three populations.

The samples LcoAl, LcoRA, LcoCP, and LcoSI were assigned into one inferred population, which contained the *L. castroviejoi* sample too. However, the supposed *L. corsicanus* sample LcoMO was assigned to one of the *L. europaeus* populations.

This latter result was concordant with its mtHV-1 sequence characteristics.

Figure 5 - Three-dimensional plot of factorial correspondence genotype analysis for eleven microsatellite loci. Each cube indicates an individual´s position in the three-dimensional space. For acronyms see "Methods".

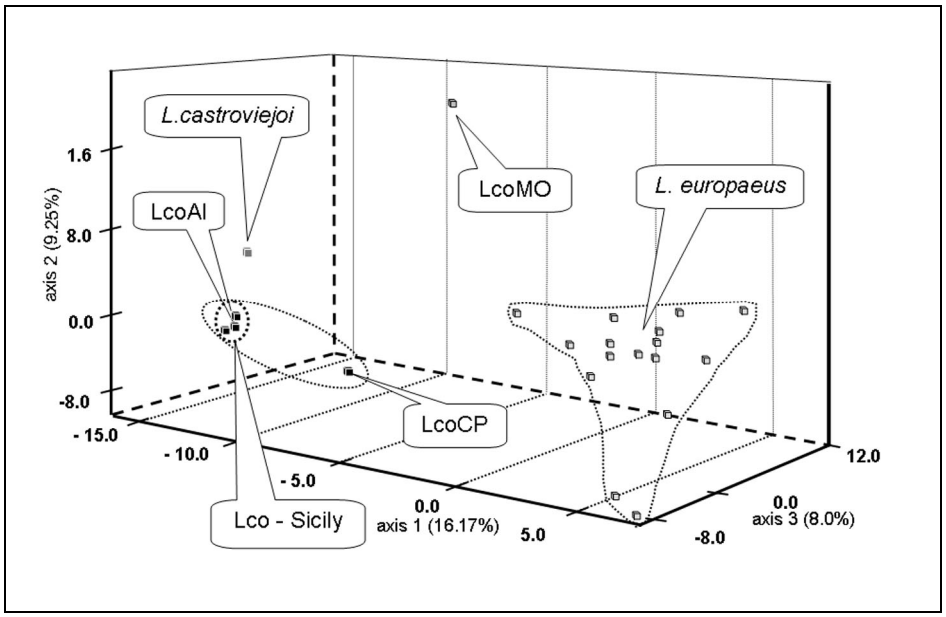
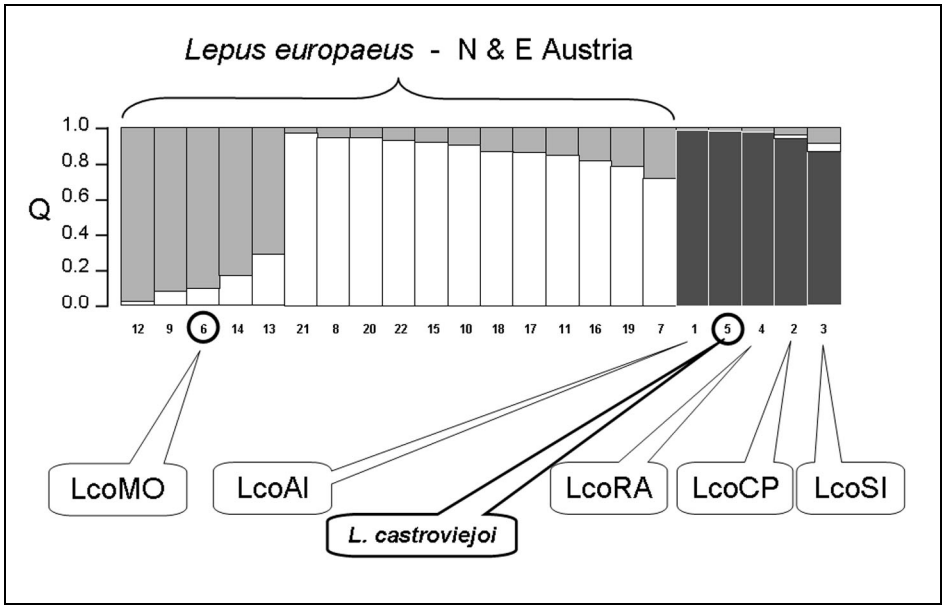


Figure 6 - Structure Analysis, based on individual genotypes at eleven microsatellite loci (see "Methods" and "Results"). Summary plot of estimates of individual membership coefficients (Q) in each inferred cluster (K=3). Each column represents an individual. Each column is partitioned into K=3 differently shaded segments, that represent that individual´s estimated membership fraction in each of the 3 inferred genetic clusters. Numbers represent current individual numbers in the data file. For acronyms of *L. corsicanus* samples see "Methods".



Discussion

In spite of the limited samples sizes, our data allow some molecular issues to be raised.

Firstly, the allozyme results indicate distinct phylogenetic separation of the nuclear gene pool of *L. corsicanus* from both *L. europoaeus* and *L. timidus*, but close relationship with *L. castroviejoi* from the Cantabrian Mountains in the Iberian Peninsula. This finding contradicts the interpretation of Wu *et al.* (2005), who, based on mtDNA results,

interpreted both taxa as subspecies of *L. timidus*. Our findings rather are concordant with those of Alves *et al.* (2003), who described close sequence similarity for the two taxa both in the mtDNA and for one nuclear gene (transferrin). The latter authors hypothesized that their ranges currently restricted to the Iberian Peninsula on the one hand and the Appenine Peninsula as well as Sicily on the other could be the results of southern (late) Pleistocene refuges of a once more widely distributed common ancestor.

The close morphological and phylogenetic relationships between the two species has been pointed out also already by Palacios (1996). However, recently Alves *et al.* (in press), based on sequence data for three nuclear gene loci, reasoned that *L. corsicanus* and *L. castroviejoi* were conspecific (see also Alves *et al.*, this volume).

Our allozyme data are not in contradiction to this interpretation. Even though based on only three individuals, the relatively high number of (unlinked polymorphic) loci allows such inferences.

Quite a number of theoretical and empirical studies have demonstrated that increasing the number of unlinked loci has a larger and more important effect on the sampling variance than has the sample size on the species level (e.g., Nei 1978, Gorman and Renzi 1979, Shriver *et al.* 1993). Therefore, our set of loci with varying degree of polymorphism might counterbalance the small sample sizes for the two taxa in respect to genetic distance estimates.

When basing a divergence time estimate for our *L. corsicanus* and *L. castroviejoi* samples from the *L. timidus* samples on Nei´s (1978) distances values corrected for small sample sizes and the average rate of codon substitution (D/2 x alpha, with alpha = 10^{-7}, Nei 1975), we arrive at a range from 635.000 to 840.000 ybp. This matches perfectly with the estimate obtained by Pierpaoli *et al.* (1999) for their mt cytochrome b sequences between *L. corsicanus* and *L. timidus* (800.000 ybp). The divergence time estimate between our *L. corsicanus*/*L. castroviejoi* and *L. europaeus* samples ranges between 540.000 and 815.000ybp, which, however, clearly contrasts with the cyt b sequence-based estimate of Pierpaoli *et al.* (1999), which was 3,000.000ybp between the *L. timidus*/*L.corsicanus* and the *L. europaeus*/*L.starcki*/ *L.habessinicus* clades.

The divergence time estimate between our *L. timidus* and *L. europaeus* samples, disregarding the sample form Switzerland, because of bilateral nuclear introgression between the two species in this part of the Alps (Suchentrunk *et al.* 2005), ranges between 500.000 and 675.000ybp. This latter estimate range roughly complies with that given in Grillitsch *et al.* (1992) and with the palaeontological record for the *timidus* and *europaeus*-lineages (see also Suchentrunk *et al.* 1999). For *L. corsicanus* and *L. castroviejoi* our allozyme data did not reveal a distance value significantly above zero; thus, we refrained from calculating a divergence time estimate.

As regards the mtHV-1 sequences, Suchentrunk *et al.* (2006) have used them for inferences on the origin of *L. corsicanus* introduced to Corsica. Three major source regions would be possible for such introductions: central or southern mainland Italy, or Sicily, as these are the only regions where *L. corsicanus* is indigenous.

In principle, Pierpaoli *et al.* (1999) considered the mt control sequences as useful for such studies in *L. corsicanus*, as they showed quite some phylogeographic structuring. For the study reported in Suchentrunk *et al.* (2006), one Appenine hare was available from Corsica (i.e., LcoAl) and the neighbor joining analysis suggested that it had a sequence characteristic similar to those from Sicily reported by Pierpaoli *et al.* (1999) rather than to those from mainland (central) Italy. This interpretation was, however, somewhat in contrast with unpublished mtDNA data from M. Pierpaoli, reported by Riga *et al.* (2003, p. 128). According to the latter authors these unpublished data indicated phylogenetic relationships between some Corsican *L. corsicanus* samples and those ones from mainland Italy, but not from Sicily. In essence the same result was obtained for the same LcoAl sample in the recent NJ and MP analyses by Alves *et al.* (in press).

As *L. corsicanus* has possibly been first introduced to Corsica during the occupasion period of the Republics of Genua and Pisa (northern and central Italy) after the 15th century (see Riga *et al.* 2003), their interpretation would be quite plausible. Nevertheless, all our mtHV-1 sequence results of the LcoAl sample strongly suggest that (at least) this hare was closely phylogenetically related to *L. corsicanus* from Sicily: our sequence comparison and phylogenetic analysis of the LcoAl sample and all other *L. corsicanus* mtHV-1 sequences available from GenBank were based on the best fitting substitution model as evaluated by MODELTEST and all three tree-building approaches used (NJ, MP, ML) yielded essentially the same cluster topology regarding the relationship between the Corsican sample, the mainland Italian and the Sicilian samples.

Nevertheless, at low intraspecific evolutionary levels tree-building approaches might still be somewhat misleading, as suggested by the comparison of the cluster results and the median-joining networks that we also

constructed. In fact, our first network based on the shorter (244bp) alignments involving all available GenBank sequences revealed some slight discrepancies with all three trees (NJ, MP, ML) regarding some haplotype positions.

For instance, according to the network in fig. 3 the Sicilian LcoSI haplotype shows the same evolutionary divergence to the Sicilian haplotypes Lco 6, Lco7, and Lco11 as to the Corsican LcoAl (i.e., only one substitution difference). But the trees suggested a somewhat greater divergence between LcoSI and Lco11 than between LcoSI and Lco6 and Lco7. Both networks, however, confirmed our interpretation that our Corsican *L. corsicanus* sample was phylogenetically closer to the Sicilian haplotypes than to all available mainland Italian ones.

Whether Appenine hares have been introduced to Corsica from both mainland Italy and Sicily remains to be investigated by bigger sample sizes. For such phylogeographic analyses our findings recommend using network analyses rather than tree-based approaches.

The relatively rich allelic diversity revealed at our eleven employed microsatellite loci suggests that they are a useful and representative marker system for diverse inferences on the nuclear gene pool characteristics of both *L. corsicanus* and *L. europaeus*, such as the detection of possible introgressions in either species.

Actually, Fulgione *et al.* (2006) have reported on microsatellite results suggesting some introgression of *L. europaeus* into *L. corsicanus* from the Cilento and Vallo di Diano National Park (South Italy), where indigenous Appenine hares are living sympatrically with introduced *L. europaeus*.

Similarly to the latter authors, we found a quite reasonable distinction of nuclear gene pools of the two species when applying multivariate and Bayesian statistics, altough diagnostic alleles were rather rare, if at all present. Our samples suggested differentially diagnostic alleles at two loci, but as this was based on rather low sample sizes, we might expect them to disappear when more individuals are screened for allelic variation. Nevertheless, as stated above, we are convinced that upon sophisticated statistical treatment identification of possibly introgressed individuals can be identified with reasonable probability. Our Bayesian population structure analysis confirmed with high probability the assignment of the Corsican LcoAl-individual to *L. corsicanus*, in accordance with our mtDNA result as well as with sequences of three nuclear gene loci (Alves *et al.* in press). It also confirmed the present allozyme results as well as the sequence data of Alves *et al.* (in press) indicating that *L. corsicanus* and *L. castroviejoi* are closely related and probably conspecific. In addition, it revealed that one supposed *L. corsicanus* individual (LcoMO), of which we did have only vage information on its external appearance, was in fact a (pure) *L. europaeus*; it was assigned perfectly to one of the two inferred Austrian brown hare populations.

In conclusion, we consider the presently implemented molecular marker systems as useful for the examination of diverse phylogeographic and differentiation analyses of the Apennine and the brown hare.

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