Phylogenetic Aspects of Nuclear and Mitochondrial Gene-Pool Characteristics of South and North African Cape Hares (*Lepus capensis*) and European Hares (*Lepus europaeus*)

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Introduction

Hares and jackrabbits (genus *Lepus*) are a notoriously difficult group, taxonomically, due mainly to broad phenotypic variation within taxa and wide overlap of traditional morphological characters (e.g., Angermann 1965, 1983; Flux 1983; Flux and Angermann 1990) across groups. However, several recent studies have demonstrated that forms representing superficially similar phenotypes but distinct evolutionary units can be differentiated by thorough analyses of morphological and phenetic characters and with the use of appropriate statistics (e.g., Palacios 1989 for hares from the Iberian Peninsula and Riga et al. 2001 for *Lepus corsicanus,* Italian hare). On the other hand, conspicuous phenotype differences or significant morphological or morphometric distinction might not always indicate differentiation at higher evolutionary level. For instance, the many domestic rabbit (*Oryctolagus cuniculus f. dom.*) breeds with all their different sizes and phenotypes have been created only very recently in evolutionary terms by anthropogenic selection and are still capable to be interbred. Similarly, in the genus *Lepus* it is conceivable that more or less strong selective pressure on relatively few genes, such as coat color genes or genes controlling for body size, could have led to conspicuous phenotypic adaptation to local or regional environments in forms that might otherwise still interbreed when they meet (again) in the wild.

Molecular data suggest fairly old ancestry of the genus *Lepus* (e.g., Halanych and Robinson 1999; Robinson and Matthee 2005). However, the currently traced *Lepus* lineages might represent offshoots of ancient lineages that were typical of ancestral taxa such as *Trischizolagus*, *Serengentilagus*, or *Hypolagus* (albeit there is no way to test this hypothesis, as these genera are all extinct). In effect, fossil evidence suggests that the whole genus *Lepus* has experienced its major adaptive radiation only recently in evolutionary terms

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(approx. within the last 2–2.5 million years), and probably many forms (species) are of much younger age. Therefore, overall genetic differentiation among many taxa might be relatively small. Moreover, in accordance with late Pleistocene climate changes and concomitant environmental perturbations, recent molecular data suggest complicated evolutionary scenarios for at least some species or forms, including phases of secondary contact and introgressive hybridization, as well as incomplete lineage sorting and presence of shared ancestral polymorphism (e.g., Thulin et al. 1997a; Alves et al. 2003; Kasapidis et al. 2005; Melo-Ferreira et al. 2005; Wu et al. 2005; Thulin et al. 2006; Ben Slimen et al. 2007). Such complex patterns of molecular evolution might lead to paraphyly for certain taxa (comp. e.g., Pérez-Suárez et al. 1994; Ben Slimen et al. 2007) and wrong systematic conclusions, particularly if only a single molecular marker system with limited power of resolution is applied and/or only few samples are studied (Alves et al. 2006; Ben Slimen et al. 2007).

As for other parts of the world, evolutionary relationships and systematics of various forms of hares from Africa must be considered provisional (e.g., Angermann 1983; Flux 1983; Flux and Angermann 1990; Robinson and Matthee 2005; Hoffmann and Smith 2005). To address only some of the systematic uncertainties, Ellerman and Morrison-Scott (1951) acknowledged the separate species status of *Lepus atlanticus* in northwest Africa, whereas Petter (1959), based on morphological arguments (particularly body size), hypothesized that cape hares (*Lepus capensis* L., 1758) include various forms from East Africa and all forms from North Africa except for an isolated occurrence of African savanna hares (*Lepus crawshayi*, sensu Petter, syn. to *Lepus victoriae* Thomas, 1823, cf. Flux and Angermann 1990, but see Hoffmann and Smith 2005) in North West Algeria. Petter (1961) even included all forms of the European hare (*Lepus europaeus* Pallas, 1778) into *L. capensis*, and most later authors implicitly acknowledged the presence of *L. capensis* in North Africa (e.g., Setzer 1958 for Egyptian hares, Petter and Saint Girons 1972 and Pérez-Suaréz et al. 1994 for Moroccan hares). Based on phenotypic and morphological comparisons, Angermann (1965) suggested the presence of *L. europaeus* in addition to *L. capensis* in North Africa, but was later on (Angermann 1983) somewhat unsecure about northwest African forms, that she related tentatively to "*granatensis*". *Lepus granatensis* (Rosenhauer, 1856) from the Iberian Peninsula and the Balearic Islands was earlier included either in *L. capensis* or *L. europaeus*, but morphological and molecular data undoubtedly demonstrate that *L. granatensis* must be considered a species distinct from *L. europaeus* and *L. capensis* (e.g., Bonhomme et al. 1986; Palacios 1989; Pérez-Suaréz et al. 1994; Alves and Ferrand 1999; Alves et al. 2000, 2003). Ellerman and Morrison-Scott (1951) acknowledged the presence of *Lepus arabicus* Ehrenberg, 1833 in parts of North Africa (Libya), a form that is currently listed as a subspecies of *L. capensis* (see e.g., Wilson and Reeder 1993) or possibly represents a separate species (Hoffmann and Smith 2005). Petter (1961) also retained *L. arabicus* as separate species, albeit with

its distribution restricted to the Arabian Peninsula. The above-mentioned African Savanna hare (*L. victoriae*) has recently been renamed as *L. microtis* Heuglin, 1865 by Hoffman and Smith (2005). However, herein we follow the conclusive arguments of Petter (1959) and Angermann (1965), and consider this latter name as "*nomen dubium*" (a note on this taxonomic issue will be published elsewhere).

In this work, we present molecular clues to the evolutionary relationships between African cape hares and European hares and test Petter's (1959, 1961) hypotheses that North African hares with simple grooves in the first upper incisors (i.e., all forms except those from the environs of Beni Abbes, Algeria, that are presently considered *L. victoriae*) belong to *L. capensis*, and that this species also includes the European hare (*L. europaeus*). Specifically, we examine published molecular data in respect to nuclear and mitochondrial gene-pool differentiation among cape hares from South and North Africa and European hares.

Overall close gene-pool relationships would correspond to the null hypothesis of conspecificity of all samples, whereas distinct gene-pool divergence would agree with the current systematic view of separate species (i.e., *L. capensis* for South Africa and *L. europaeus* for Europe). In addition, considerable molecular divergence between *L. capensis* from South and North Africa would suggest differentiation on the species level, corresponding to the view of several earlier authors that considered some North African forms as separate species (see also Hoffmann and Smith 2005). To calibrate gene-pool divergence levels among our samples, we included allozyme data of mountain hares (*Lepus timidus* L., 1758) from three regions in Europe and sequences from diverse regions of Eurasia. On the one hand, mountain hares represent a "good *Lepus* species" (despite introgressive hybridization in wild populations, see below) with an evolutionary history clearly different from the samples of the "*L. capensis*/*europaeus* complex" (sensu Angermann 1983) and on the other hand they should also provide levels of within-species differentiation for comparison.

Comparative Analysis of Nuclear and Mitochondrial Gene-Pool Data

Recent molecular studies in the genus *Lepus* suggest a lower level of differentiation in nuclear gene-pools than in mtDNA. This seems to hold by and large for both within and between species comparisons (comp. e.g., Bonhomme et al. 1986; Hartl et al. 1993; Pérez-Suárez et al. 1994; Pierpaoli et al. 1999; Suchentrunk et al. 1999, 2000a; Alves and Ferrand 2000; Mamuris et al. 2001, 2002; Koh et al. 2002; Vapa et al. 2002, 2007; Alves et al. 2003; Suchentrunk et al. 2003; Fickel et al. 2005; Kasapidis et al. 2005; Sert et al. 2005; Waltari and Cook 2005; Wu et al. 2005; Ben Slimen et al. 2005; Estonba et al. 2006; Ben Slimen et al. 2007; Thulin et al. 2006). Therefore, we used both nuclear and mitochondrial gene-pool

evidence. Specifically, we re-analyzed published multilocus allozyme and partial sequence data of the hypervariable domain 1 of the mtDNA control region of South African cape hares, hares from central Tunisia that are currently considered cape hares (e.g., Flux and Angermann 1990), and European hares.

Examination of Nuclear Gene-Pool Variability

To analyze nuclear gene-pool variability and differentiation among taxa we used published data of allelic variation at 29 structural (allozyme) gene loci (Hartl et al. 1993; Suchentrunk et al. 1999; Ben Slimen et al. 2005). All these data have been produced in our laboratory in Vienna under a standardized protocol (e.g., Grillitsch et al. 1992) by using marker samples in all gels for comparison of band patterns. Our set of loci was similar to that screened earlier in diverse hare species (e.g., Bonhomme et al. 1986; Grillitsch et al. 1992; Hartl et al. 1993; Suchentrunk et al. 1998, 1999, 2000a, 2001, 2003; Alves et al. 2001; Cervantes et al. 2002; Vapa et al. 2002; Ben Slimen et al. 2005; Sert et al. 2005); it encompassed the following 13 monomorphic loci (locus acronym, E.C. number, and locus in parentheses): lactate dehydrogenase (LDH, 1.1.1.27, Ldh -1), malate dehydrogenase (MOR, 1.1.1.37, Mor -1), malic enzyme (MOD, 1.1.1.40, Mod-1), catalase (CAT, 1.11.1.6, Cat), 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, -3), fumarate hydratase (FH, 4.2.1.2, Fh), and aconitase (ACO, 4.2.1.3, Aco-2). The 16 polymorphic loci are listed in Table 1. We used diverse software packages (Genetix, vers. 4.02, Belkhir 1999; Arlequin 3.01, Excoffier et al. 2005; Phylip pc package, Felsenstein 1995; PCO program, Anderson 2003) to calculate allele frequencies, linkage disequilibrium between polymorphic loci, diverse genetic distances for pairwise comparisons of differentiation among populations/taxa, to construct dendrograms of genetic relationships, to perform a metric principal coordinate (PCO) analysis of the Cavalli-Sforza-Edwards (CSE) chord distance matrix, and to assess the relative amount of nuclear gene-pool variability attributable to populations or groups of populations. In addition, we run assignment tests for each individual based on a Bayesian approach (Rannala and Mountain 1997, Paetkau et al. 2004 simulation algorithm, as implemented in GeneClass 2.0.g, Piery et al. 2004) to check for proportions of correct population assignment.

Examination of Mitochondrial Sequence Variability

For studying levels and patterns of mtDNA variability and differentiation we used published CR-1 sequences available on GenBank from African cape hares, European hares, and mountain hares. We selected a segment of 415 bp that allowed the alignment of 33 South African cape hare sequences, 60 European

hare sequences, seven sequences of cape hares from north-central Tunisia, and 18 Eurasian mountain hare sequences (for accession numbers, provenances, and references see the appendix). The European hare sequences represented two phylogenetic groups of lineages, one occurring in several eastern Mediterranean islands and in some hares from Bulgaria (A-clade), and another one occurring in hares from most parts of Europe (B-clade) (see Kasapidis et al. 2005). The sequences were aligned with Clustal X (1.83) (Thompson et al. 1997), and a neighbor joining (NJ) dendrogram based on Tamura and Nei (1993) distances (TN93) was constructed with MEGA 2.1 (Kumar et al. 2001). The same program was used to evaluate the robustness of the NJ tree topology by bootstrapping (1,000 repetitions) and to confirm the NJ topology by running a maximum parsimony analysis (MP) without indels, with the close-neighbor-interchange option with search level 1 and with initial tree by random addition (10 repetitions) and 1,000 bootstrap repetitions. As a further alternative for portraying phylogenetic relationships among sequences, we subjected the TN93 distance matrix to a metrical PCO analysis (Anderson 2003). The resultant individual sequence coordinates were tested for variation among taxa by generalized least square regression (GLS), with a restricted maximum likelihood approach for variance homogeneity, separately for each dimension by using the S-Plus 6.2 program. For all series of tests, sequential Bonferroni corrections were applied to account for multiple tests (Rice 1989).

Patterns of Nuclear Gene-Pool Differentiation

Sixteen allozyme loci exhibited polymorphism (see Table 1 for allele frequencies and locus details). There was no linkage disequilibrium between any pair of polymorphic loci when tested separately in each taxon/population and when accounting for multiple tests. None of the samples revealed diagnostic alleles at any locus, and most common alleles were the common ones in all taxa or populations. However, mountain hares showed almost diagnostic alleles at the Sdh and the Acp-1 loci; occurrence of few alternate alleles in two European and Mountain hare populations was most likely due to introgressive hybridization (Suchentrunk et al. 2005). This allele pattern produced quite distinct gene-pool divergence between mountain hares and all samples of the "*L. capensis/europaeus* complex". However, nuclear gene-pool differentiation between Tunisian and European hares was only marginally higher than that found among populations of central European hares. South African cape hares showed a differentiation level that was clearly lower than that found between European and mountain hares. A NJ dendrogram based on CSE chord distances is given in Fig. 1.

The relatively close genetic relationships of South African *L. capensis*, presumed *L. capensis* from north-central Tunisia, and the central European *L. europaeus* populations were confirmed by the 2D PCO model, which explained all variation of the CSE chord distance matrix (see Fig. 2 for the

Table 1 Allele frequencies at polymorphic allozyme loci (*details in footnote*). See Hartl et al. (1993) for allozyme data of *L. europaeus*, Suchentrunk et al. (1999) for allozyme data of *L. timidus*, and Ben Slimen et al. (2005) for allozyme data of *L. capensis*. *N* = sample size

Locus	Allele	*L. capensis* (South Africa) *N* = 9	*L. capensis* (north-central Tunisia) *N* = 45	*L. europaeus* (range over five populations from central Europe) *N* = 200	*L. timidus* (range over three regions in Europe) *N* = 200
Sdh	100	1.0	1.0	1.0	0.0–0.041
	300	0.0	0.0	0.0	0.959–1.0
Ldh-2	100	0.944	0.935	1.0	1.0
	83	0.0	0.048	0.0	0.0
	105	0.056	0.016	0.0	0.0
Mor-2	100	1.0	1.0	0.964	1.0
	79	0.0	0.0	0.036	0.0
Idh-1	100	0.944	1.0	1.0	1.0
	121	0.056	0.0	0.0	0.0
Idh-2	100	0.611	0.978	0.933–1.0	0.321–0.954
	130	0.389	0.022	0.0–0.067	0.0–0.046
	83	0.0	0.0	0.0	0.0–0.679
Pgd	100	0.833	0.978	0.786–1.0	0.964–1.0
	170	0.0	0.0	0.0–0.052	0.0–0.36
	129	0.0	0.0	0.0–0.018	0.0
	117	0.0	0.0	0.0–0.036	0.0
	64	0.0	0.011	0.0–0.143	0.0
	79	0.111	0.0	0.0	0.0
	92	0.056	0.0	0.0	0.0
	69	0.0	0.011	0.0	0.0
Hk-2	100	1.0	1.0	0.969–1.0	0.973–1.0
	67	0.0	0.0	0.0–0.031	0.0–0.027
Es-1	−108	0.0	0.0	0.0–0.021	0.1–0.143
	−100	1.0	0.907	0.569–0.833	0.843–0.9
	−75	0.0	0.093	0.167–0.414	0.0
	−42	0.0	0.0	0.0–0.017	0.0
	−132	0.0	0.0	0.0	0.0–0.14
Pep-1	100	1.0	0.978	1.0	1.0
	83	0.0	0.022	0.0	0.0
Pep-2	100	0.0	0.856	0.768–0.875	0.0–0.053
	104	0.444	0.1	0.125–0.232	0.906–1.0
	114	0.556	0.022	0.0	0.0–0.058
	94	0.0	0.022	0.0	0.0
Acp-1	100	1.0	0.932	1.0	0.0–0.036
	115	0.0	0.0	0.0	0.964–1.0
	81	0.0	0.068	0.0	0.0
Mpi	100	0.444	0.956	0.914–1.0	0.929–0.964
	126	0.556	0.044	0.0–0.086	0.014–0.071
	77	0.0	0.0	0.0	0.0 –0.023

(*Continued*)

Table 1—Continued

Locus	Allele	*L. capensis* (South Africa) $N = 9$	*L. capensis* (north-central Tunisia) $N = 45$	*L. europaeus* (range over five populations from central Europe) $N = 200$	*L. timidus* (range over three regions in Europe) $N = 200$
Aco-1	100	1.0	1.0	1.0	0.979–1.0
	60	0.0	0.0	0.0	0.0–0.021
Mod-2	100	1.0	1.0	1.0	0.929–1.0
	110	0.0	0.0	0.0	0.0–0.071
Glud	100	1.0	0.944	1.0	1.0
	96	0.0	0.056	0.0	0.0
Es-D	100	0.978	1.0	0.75–0.875	0.810–0.929
	141	0.0	0.0	0.125–0.328	0.071–0.148
	72	0.0	0.0	0.0	0.0–0.042
	60	0.022	0.0	0.0	0.0

Isozyme/-system, abbreviation, E.C. number, and respective structural gene loci: sorbitol dehydrogenase (SDH, 1.1.1.14, Sdh), lactate dehydrogenase (LDH, 1.1.1.27, Ldh-2), malate dehydrogenase (MOR, 1.1.1.37, Mor-2), malic enzyme (MOD, 1.1.1.40, Mod-2), isocitrate dehydrogenase (IDH, 1.1.1.42, Idh-1,-2), 6-phosphogluconate dehydrogenase (PGD, 1.1.1.44, Pgd), glutamate dehydrogenase (GLUD, 1.4.1.3, Glud), hexokinase (HK, 2.7.1.1, Hk-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), mannose phosphate isomerase (MPI, 5.3.1.8, Mpi)

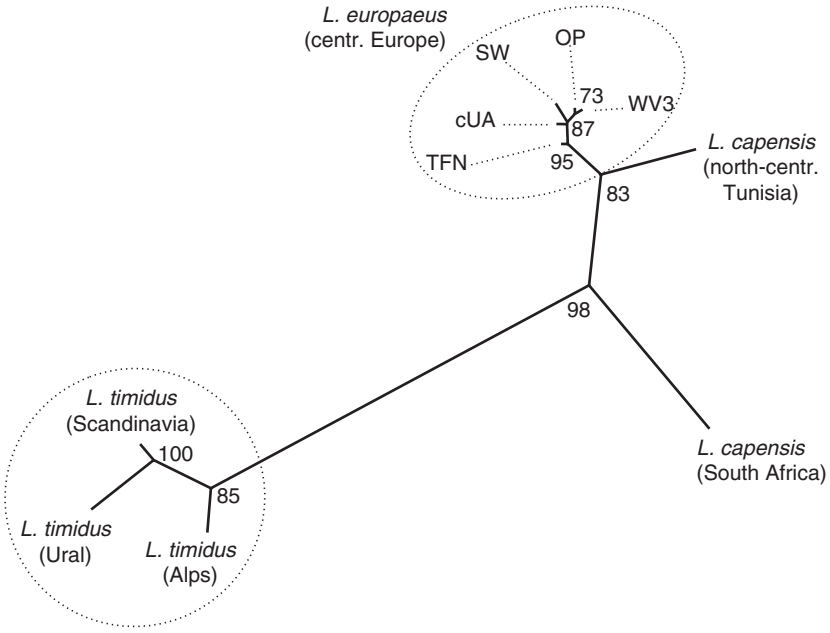

Fig. 1 Neighbor-joining dendrogram of *Lepus* taxa/populations studied presently, based on Cavalli-Sforza/Edwards distances calculated from 29 allozmye loci. Bootstrap support values (100 repetitions) above 50% are given at respective internal nodes. For European hare populations, see Hartl et al. (1993; cUA combines OWN, OWS, OIV, OKT); for mountain hare populations, see Suchentrunk et al. (1999); and for *L. capensis* populations see Ben Slimen et al. (2005)

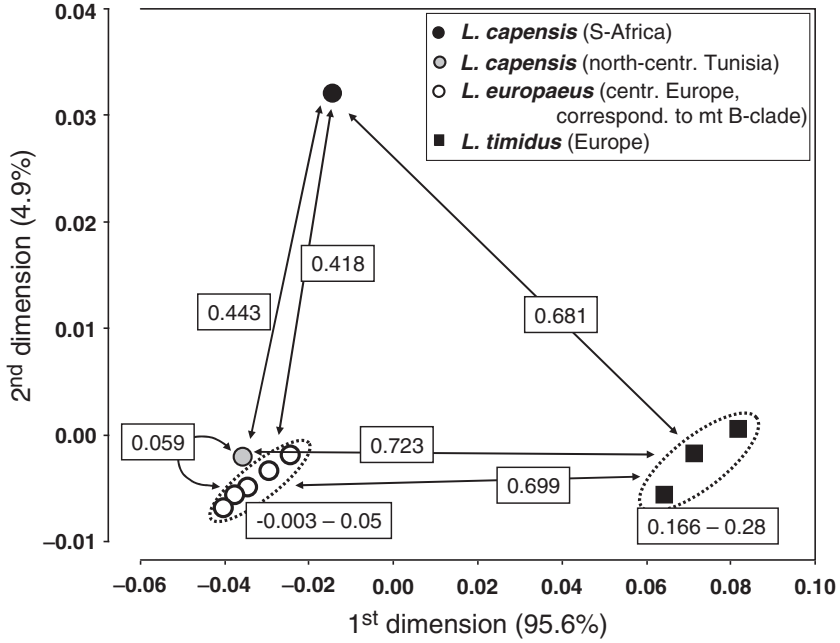

Fig. 2 Scatterplot of coordinates of hare taxa/populations as obtained from the 2D PCO analysis of the Cavalli-Sforza/Edwards chord distance matrix based on 29 allozyme loci. *Double-headed arrows* and associated values indicate mean pairwise Θ values (relative genetic differentiation) among taxa. Respective ranges of Θ values for among-population comparisons within central European hares and mountain hares are also given

scatterplot of population coordinate scores). Relative genetic differentiation (Θ values, see Fig. 2) was generally significant for pairwise comparisons between all taxa/populations, except for comparisons between populations of *L. europaeus* and between mountain hares from the Alps and Scandinavia, when based on Bonferroni corrections. An analysis of molecular variance (AMOVA) revealed that 12.57% of the total allozymic variance were due to variation between the *L. europaeus* and *L. capensis* (from both South and North Africa) samples (FCT = 0.126, n.s.), whereas 7.43% were due to differentiation among populations within those two groups (FSC = 0.085, $p < 0.00001$), and 80.0% were due to variation among individuals within populations (FST = 0.2, $p < 0.00001$). For calibration, a second AMOVA model, based on the comparison between populations of *L. europaeus* and *L. timidus*, revealed that 55.99% of the variation were due to separation into the two species (FCT = 0.559, $p = 0.0147$), 3.5% were due to differentiation among populations within each species (FSC = 0.0795, $p < 0.00001$), and 59.5% were due to variation among individuals within populations (FST = 0.595, $p < 0.00001$). The assignment tests were in essence concordant with the revealed pattern of gene-pool partitioning: all mountain hares were assigned correctly to one of

the mountain hare populations, albeit only a small fraction was assigned correctly to the population; also, all South African cape hares were assigned correctly to the South African population, but only 68.9% of the Tunisian hares were assigned correctly, 8.9% were assigned to the South African cape hare population, and 22.0% were collected to one of the European hare populations. Reversely, only 49.0% of all European hares were assigned correctly to the species, with a very low proportion of assignment to the correct population, and the remaining 51.0% of European hares were assigned either to the Tunisian population (43.5%) or to the South African population (5.5%).

Differentiation of mtDNA CR-1 Sequences

A total of 117 sequences downloaded from GenBank (see Appendix) could be aligned for a 415-bp-long fragment of the mtDNA CR-1 and used for phylogenetic analysis. Due to the somewhat shorter alignment relative to several of those sequences published earlier, some of the downloaded original haplotypes were now identical. In the NJ dendrogram (Fig. 3), haplotypes of European hares from both the A- and B-clade clustered in two well-supported and closely related groups and the Tunisian haplotypes were also relatively close to the European hare haplotypes. In contrast, South African haplotypes were distinctly separate from both European hares and Tunisian hares. Surprisingly, Mountain hare haplotypes were closer to European hares and Tunisian hares than were the South African Cape hares. This tree topology was in essence confirmed by our MP analysis (175 variable sites, 147 parsimony informative sites, 28 singletons; iCI = 0.300156, iRI = 0.861709, for general explanation see e.g., Nei and Kumar 2000); for MP bootstrap values see also Fig. 3.

An eight-dimensional PCO analysis explained the total variation (101.1%) of the TN93 distance matrix, and the GLS analyses revealed significant differentiation of individual PCO scores of the sequences for the first five dimensions explaining 94.02%. The plots of the individual PCO values of the first four dimensions (Fig. 4) gave a pattern of distance relationships very similar to that in the NJ and MP trees.

Contrasting Patterns of Differentiation between Mitochondrial Lineages and Nuclear Gene-Pools

All analyses of partial CR-1 sequences demonstrate distinct mtDNA divergence among South African Cape hares (*L. capensis*), Tunisian Cape hares, and European hares (*L. europaeus*). However, this clear differentiation pattern is not fully paralleled by nuclear gene-pools. While our multilocus allozyme approach separates the South African Cape hares somewhat from the European hare populations, both in terms of absolute and relative genetic dif-

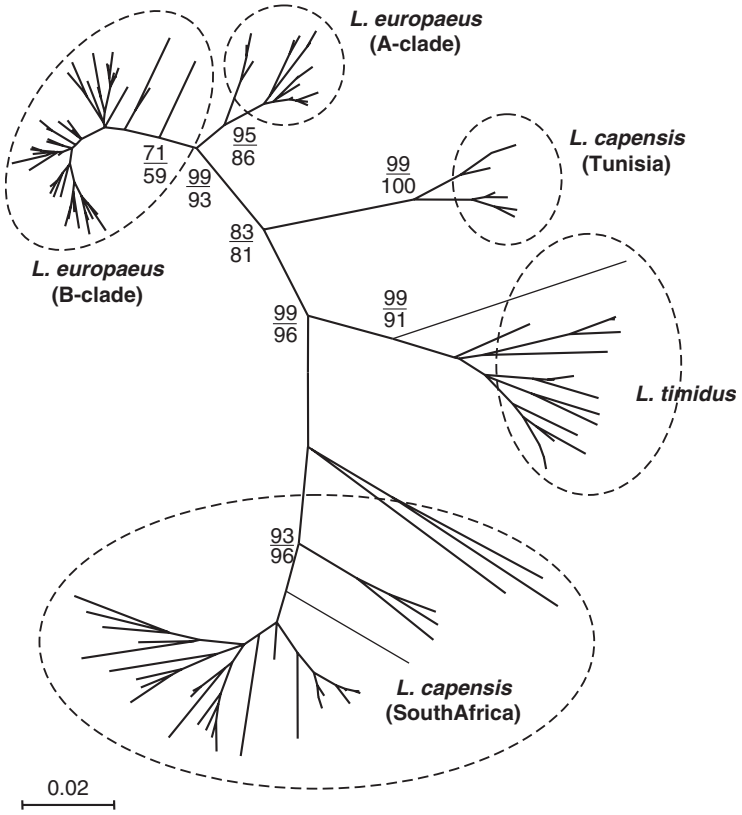

Fig. 3 NJ dendrogram of partial (410-bp) mtCR-1 sequences of South and North African *L. capensis*, European *L. europaeus,* and Eurasian L. *timidus* based on Tamura/ Nei (1993) distances. Bootstrap support values >50% are given for respective internal nodes for both the NJ (*upper values*) and the MP (*lower values*) majority rule consensus trees that had in essence the same topology as the presented NJ tree

ferentiation, hares from north-central Tunisia are fairly closely related to the European hares. North African hares are considered as belonging to *L. capensis* (e.g., Petter 1959; Flux and Angermann 1990; Wilson and Reeder 1993; but see Hoffmann and Smith 2005). The divergence level between South African Cape and European hares is approximately half of that between *L. europaeus* and *L. timidus* populations. The somewhat elevated divergence level between South African Cape hares and European hares is exclusively due to the pronounced differences in allele frequencies at some loci, but not due to alternately fixed alleles. In spite of the relatively high number of alleles at polymorphic loci that occur exclusively either in the South African Cape hares, the Tunisian hares, or the European hares ("private alleles"), the general nuclear gene-pool architecture is by and large the same for all these samples.

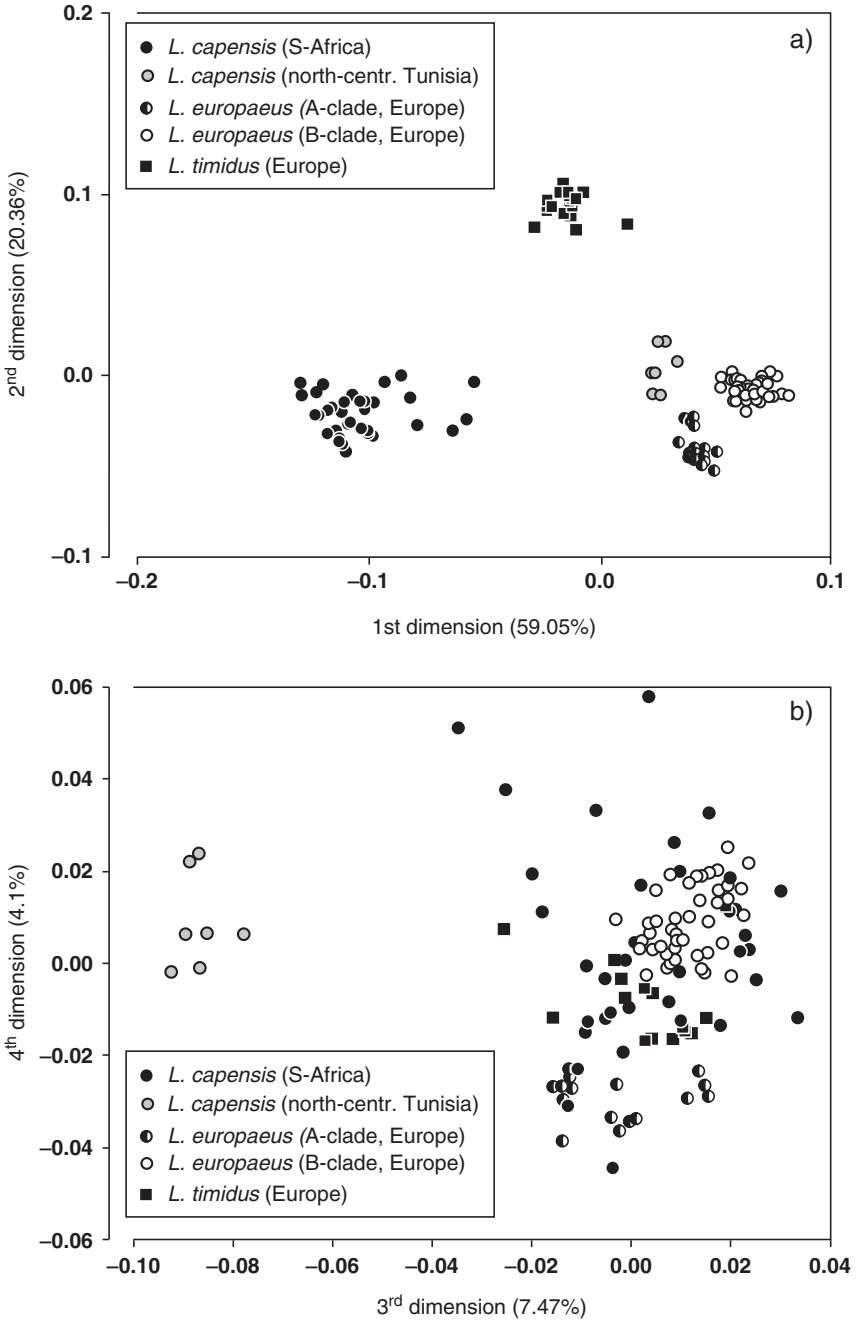

Fig. 4 Scatterplots of individual PCO scores of partial mtCR-1 sequence data as derived for the first two coordinate dimensions (**a**) and the third and fourth dimensions (**b**) of the eight-dimensional model

Alleles that are alternately fixed or almost alternately fixed occur only at two loci (Sdh, Acp-1) between *L. timidus* and *L. capensis* samples on the one hand and between *L. timidus and L. europaeus* on the other (Table 1). The few European hare-type alleles in Mountain hares at these two loci are considered as resulting from current or historic introgressive hybridization between European and Mountain hares from the Swiss Alps (Suchentrunk et al. 2005). Both the Sdh and the Acp-1 loci contribute to the high genetic differentiation between Mountain hares and all other samples.

Our set of allozyme loci identifies the Mountain hares as a clearly separate evolutionary unit, in accordance with their separate species status. Similar sets of loci revealed separate evolutionary lineages for "good hare species", such as Iberian hares (*L. granatensis*), Japanese hares (*Lepus brachyurus*), and Italian hares (*L. corsicanus*) (Alves and Ferrand 1999; Suchentrunk et al. 1999; Suchentrunk et al. unpubl. data). Thus, we consider our nuclear gene-pool results, based on 29 unlinked allozyme loci, appropriate for inferring evolutionary relationships among our samples; particularly because they rest on a wide range of rapidly, moderately, or slowly evolving structural gene loci (see also e.g., Richardson et al. 1986 for the general use of allozyme data for phylogenetic and speciation studies). On the contrary, mtDNA can be viewed rather as one linkage system, reflecting the evolution of the organelle rather than that of organisms. Therefore, mtDNA data might cause erroneous conclusions on species differentiation, if applied without concomitant data of multiple nuclear markers (e.g., Ballard et al. 2002; Ballard and Whitlock 2004). Inferences on phylogenetic relationships of *Lepus* species that have exclusively been drawn from mtDNA data (e.g., Pierpaoli et al. 1999; Waltari and Cook 2005; Wu et al. 2005), in spite of published evidence for various introgression scenarios for mtDNA in diverse hare species (see Thulin et al. 1997a; Alves et al. 2003; Melo-Ferreira et al. 2005), must therefore be considered preliminary at best.

Our finding that differentiation among South and North African Cape hares and European hares is clearly lower for the nuclear gene pool than for the mtDNA is in accordance with recent studies of European and Anatolian European hares. While nuclear gene-pool differentiation as assessed by allozyme and RAPD analysis indicated relatively little divergence between Anatolian and European populations on the one hand, and between Greek and Central European populations on the other, mtDNA RFLP and sequence data indicated a clearly higher level of differentiation (cf. Mamuris et al. 2001; 2002; Suchentrunk et al. 2003; Kasapidis et al. 2005; Sert al. 2005). The discordance between nuclear and mtDNA is probably due to higher gene flow in males and more pronounced philopatry in females. For Central European hares, such a sex-specific difference in gene flow was evident even on small geographic scale between neighboring populations (Fickel et al. 2005); and it was also concluded from molecular data for South African Cape hares and Scrub hares (*Lepus saxatilis*) by Kryger (2002).

Regarding mtDNA data, there are many more *Lepus* control region sequences available on the GenBank than the ones presently used in our

phylogenetic analysis. However, we aimed to compare patterns of nuclear and mitochondrial gene-pool differentiation among three phenotypically similar taxa (*L. capensis* from South and North Africa, and *L. europaeus*), rather than to develop a model of phylogenetic relationships within the genus *Lepus*. The present results clearly show distinct divergence of all analyzed mtDNA sequences into three monophyletic clades (i.e., South African *L. capensis*; North African *L. capensis;* and *L. europaeus* with two subclades) without any hint of paraphyletic lineages as regards the presently studied taxa. This might indicate that the mtDNA data in this study are free of possible cases of introgression or nuclear representations of mtDNA ("numts"). However, in view of the relatively high sequence divergence in the South African Cape hare clade and one divergent haplotype in the Mountain hare clade, we do not fully exclude the occurrence of numts.

Reticulate Evolution in the Genus *Lepus* and Consequences for Phylogeny Reconstruction

As pointed out by Alves et al. (2006) and Ben Slimen et al. (2007), a phylogenetic analysis within the genus *Lepus* must include nuclear evidence because of the possibility of unrecognized presence of introgressed mtDNA. Phases of reticulate evolution cannot be excluded for some species, and this could lead to erroneous conclusions, if phylogenetic inferences are based exclusively on mtDNA. Thulin et al. (1997a), Alves et al. (2003), and Melo-Ferreira et al. (2005) have demonstrated introgressive hybridization of *L. timidus* type mtDNA into *L. europaeus* and *L. granatensis*, and occurrence of foreign mtDNA in other *Lepus* species is very plausible (see e.g., Pierpaoli et al. 1999 for *Lepus starcki* (Ethiopian Highland hare) and *Lepus habessinicus* (Abyssinian hare); Alves et al. 2003; Ben Slimen et al. 2007). Preliminary data (Suchentrunk et al. 2005) reveal substantial bidirectional introgressive hybridization in both nuclear and mtDNA of wild living European hares and Mountain hares from Switzerland, but limited morphological consequences in higher generation hybrids. Introgression might be even more likely in cases of secondary contact of less differentiated (conspecific) gene-pools of hares, as found between the European and inferred Anatolian/Middle Eastern mtDNA lineages of European hares (Kasapidis et al. 2005), and such evolutionary scenarios might inflate mtDNA variability within species. The presently compared South African *L. capensis* sequences show a relatively high phylogenetic heterogeneity. Their range of pairwise distances (up to 15.8%) is somewhat greater than that of the Tunisian hares and that found between the two clades of European hares from the eastern Mediterranean (A-clade: from inferred Anatolian Pleistocene range, B-clade: southeastern European Late Pleistocene range). The latter two clades from Southeast Europe and the Aegean Islands show an average nucleotide divergence of 6.6% (Kasapidis et al. 2005).

Most likely, the increased sequence heterogeneity of the South African *L. capensis* sequences results from a distinct phylogeographic partitioning in Africa (Kryger 2002). However, even for one local sample from the northern Cape Province Ben Slimen and Suchentrunk (in press) revealed a substantial divergence level (up to almost 8%) of mitochondrial lineages. Relatively high levels of differentiation (up to 7.3% sequence divergence) were also found for partial control region sequences of other hare species, such as Palaearctic Mountain hares (*L. timidus*) by Thulin et al. (1997b), and this was interpreted as resulting from the existence of ancestral lineages in a (little structured) continuous population that existed during the last glaciation in Europe. Similarly, persistence of shared ancestral polymorphism could not be excluded for Palaearctic lineages of Mountain hares (Ben Slimen et al. 2007). On the other hand, inflated mtDNA variability and hence a tendency towards taxonomic inflation might result from continuous large effective population sizes of tropical species in contrast to species from more northern latitudes that might have experienced bottlenecks during the climatic changes in the (Late) Pleistocene (Harris and Froufe 2005). Whether nuclear gene-pool differentiation of South African Cape hares shows a similarly high level of heterogeneity or fits the above hypothesized general mode of higher nuclear gene flow relative to gene flow in mtDNA of hares, or whether those South African hares currently considered cape hares should on the contrary be split into separate species, remains to be studied by nuclear gene-flow analyses and other biological characters. Our preliminary allozyme, microsatellite, and mitochondrial sequence data of some cape hares, however, suggest conspecificity despite quite substantial sequence divergence in one (conspecific) South African population (Ben Slimen and Suchentrunk, in press).

Revival of Petter's (1961) Hypothesis of Conspecificity of *L. europaeus* and *L. capensis*

The presently found close nuclear gene-pool relationships between South African and Tunisian cape hares and central European hares fit the hypothesis of conspecificity of *L. capensis* and *L. europaeus*, put forward by Petter (1961) on morphological grounds. Similarly, Angermann (1965), also based on morphological comparisons, considered the occurrence of European hares in parts of North Africa. Later on she was still not clear about some forms of the *L. capensis* / *L. europaeus* complex, and wrote: "*L. capensis* s. l. may consist of parapatric forms in various stage of divergence—subspecies, semispecies or allospecies" (Angermann 1983). However, in a provisional summary of the genus *Lepus*, Flux and Angermann (1990) considered European hares absent from Africa. Flux (1983) also pointed towards the taxonomic uncertainty of forms of *L. capensis* and *L. europaeus*, and European hares were indeed accepted as subspecies of *L. capensis* for a while by checklists or various authors during the second half of the 20th century (see e.g., checklist in Myers

and McInnes 1981). Petter (1959, 1961) also included all hares from North Africa (except one isolated population of *L. victoriae* in the region of Beni Abbes, NW Algeria) into *L. capensis*, and our data of Tunisian hares (see also Ben Slimen et al. 2005) support his hypothesis. Moreover, mtDNA PCR-RFLP data (Ben Slimen et al. 2006) suggest close genetic relationships among South African Cape hares, north-central Moroccan and north-central Tunisian hares. In addition, partial CR-1 sequence data (Ben Slimen et al. 2007) and microsatellite data for 11 loci (Ben Slimen et al. unpubl. data) indicate close phylogenetic association between Tunisian and desert hares from northwestern Egypt and relatively high gene flow among those populations. Certainly, we cannot exclude the possibility that the little overall divergence indicated by the presently studied allozyme loci might to some degree be due to concordant selection effects at those loci, and that the differentiation pattern revealed by eleven microsatellite loci (Ben Slimen et al. unpubl. data) is due to possible length homoplasy for some of those loci. Also, even single genes that have currently not been studied might indicate a different species and could thus lead us to a false conclusion. But the most parsimonious interpretation of our results on nuclear gene-pool relationships would be that South African and Tunisian Cape hares and probably also European hares are conspecific, thus matching Petter's (1959, 1961) hypothesis.

Gene-Pool Relationships and Species Concepts

In contrast to the allozyme results, the present results on mtDNA differentiation are not congruent with Petter's (1961) hypothesis of conspecificity of *L. capensis* and *L. europaeus*. However, we suggest that the presently revealed distinct evolutionary divergence of the highly variable mtDNA control region fragment between South African Cape hares, North African hares, and European hares might result from regional anagenesis within a network of geographically separated populations that are still cohesive in evolutionary terms, fitting an "interbreeding species concept" (Lee 2003). Screening many contiguous populations of hares that are currently considered *L. capensis* for mtDNA variation might reveal more or less continuous mtDNA gene-pool among those hares that are currently included in *L. capensis*, *L. europaeus*, and perhaps also *L. tolai*, albeit there is apparently a (relatively recent?) distributional gap between South and East African populations (e.g., Flux and Angermann 1990). A high level of mtDNA variability but relatively low differentiation in nuclear DNA is rather the rule for hares from north-central Tunisia and for European hares from diverse parts of Europe and Anatolia (Mamuris et al. 2001, 2002; Suchentrunk et al. 2003; Kasapidis et al. 2005, Sert et al. 2005; Ben Slimen et al. 2005, 2006, 2007; see also Fickel et al. 2005). Alternatively, mitochondrial lineages of "*L. capensis* sensu latu" (forms included in the cape hare by Flux and Angermann 1990) might represent regional offshoots from an ancestral gene pool of a basal *Lepus* species that gave raise to the evolution of all modern *Lepus* species. Such an interpretation

is backed up by a more comprehensive comparison of mitochondrial DNA haplotypes by Ben Slimen et al. (2007).

In conclusion, our comparison of nuclear and mitochondrial gene-pool differentiation suggests that *L. europaeus* might go (once again) into *L. capensis* L., 1758. Variation of external phenotypes (e.g., coat color types, external measurements) of these hares might be paralleled only at a low level of gene-pool differentiation. This was shown for Anatolian and Tunisian hares (Sert et al. 2005; Ben Slimen et al. 2005, 2007; see also Suchentrunk et al. 2000b), and preliminary mtDNA data of over 100 hares from different parts of Tunisia revealed only a little effect of coat color types (four types considered) on partitioning of sequence variance. Phenotypic characters such as body size, coat color, ear length, etc. might be under more or less strong selection by environmental characteristics. A good example is given by domestic rabbits that have evolved an enormous amount of phenotypic forms including coat color and size and shape characteristics within a very short period of evolutionary time (albeit under very strong selection by breeders). European hares that were introduced from Britain to New Zealand less than 150 generations ago have already adapted in their body size to the new environments following Bergman's rule (Flux 1990) and European hares from our breeding station in Vienna exhibit already significant reduction in skull (body) size after less than 20 years (unpubl. data). In mammals, many (quantitative, epigenetic) characters are likely controlled by a relatively small number of structural gene loci. If such phenotypic characters of cape and European hares are not linked to representative gene-pool compartments or to genes that are important in the context of reproduction, overemphasizing them in phylogenetic analyses might lead to a portrayal of habitat characteristics to which the hares were exposed in their local evolution, rather than a picture of overall evolutionary relationships.

To test Petter's (1961) hypothesis of conspecificity of Cape and European hares, gene-flow analyses are necessary for many nuclear markers (preferentially allozymes, nuclear gene sequences, microsatellites) of many neighboring populations in Africa, the Middle East and Europe, hence, a population genetic approach would be necessary. Inferences coming from mtDNA phylogeography certainly will help to understand historic population demography and relationships in these hares (see e.g., Kryger 2002; Kasapidis et al. 2005). Given that even *Lepus* species with very divergent mtDNA, such as Iberian, European and Mountain hares, may exhibit a considerable level of introgressive hybridization in natural populations (Thulin et al. 1997a; Alves et al. 2003; Melo-Ferreira et al. 2005; Suchentrunk et al. 2005; Thulin et al. 2006; Ben Slimen et al. in press), we might expect a gradual change of gene-pool characteristics in chains of intergrading populations between South African *L. capensis* and *L. europaeus*, similar to "ring species" scenarios (see e.g. Irwin et al. 2005). The current distributional gap between southern African and more northern populations (e.g., Flux and Angermann 1990) renders the populations allopatric and excludes natural gene flow between the southern African Cape hares and presumed northern Cape hare populations

per se. However, this distributional gap might be very recent in evolutionary terms, and gene-pools of South African Cape hare populations and those from north of the distributional gap might thus be similar, particularly if large effective population sizes were present before the gap has formed, and if no serious bottlenecks have occurred afterwards. Such a scenario would in principal not contradict an "interbreeding species concept" or a "cohesive species concept" (see e.g., Lee 2003). European hares from continental Europe and the British Isles (Suchentrunk et al. 1998, 2001) as well as the disjunct populations of Mountain hares from Scandinavia, Scotland, Ireland, and the Alps (see Thulin et al. 1997a; Suchentrunk et al. 1999) serve as examples of allopatric though conspecific populations, respectively. Our present view is in contradiction to the position of Hoffmann and Smith (2005) who tend to split "*L. capensis* sensu latu" into several species, based on the argument there would be indications of restricted gene flow in *L. capensis*. However, at least all nuclear gene-pool data (the present ones and those of Kryger 2002 on microsatellites of South African *L. capensis*) indicate a rather low level of relative genetic differentiation among populations (i.e., low pairwise F_{st} and R_{st} values). Despite distinct mitochondrial gene-pool separation, Kryger (2002) reported gene flow among populations at a level that translated into clearly more than one individual theoretically migrating per generation between populations. This would be theoretically sufficient to counteract genetic differentiation due to random genetic drift in the populations. In our opinion, the "*L. capensis/europaeus* complex" (as coined by R. Angermann 1983) represents an exciting taxon for studies on speciation and evolution in mammals. Its systematic resolution needs a combined approach of phylogenetics, phylogeography, and population genetics, based on various nuclear and mitochondrial markers, and including other biological characteristics, such as phenotypic and morphometric data.

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Appendix

List of sequences downloaded from GenBank and used for our alignment (sequences with nucleotide ambiguities were not considered)

Species	Accession number in GenBank	References
L. capensis (South Africa)	AF491353 to AF491385	Kryger et al. (2002), direct submission
L. europaeus (Bulgaria, Greece and various Aegean Islands, Cyprus, northern Israel)	AY466782 to AY466853; except AY466827, AY466798, AY466799, AY466800, AY466813, AY466823, AY466824, AY466833, AY466836, AY466838, AY466850, AY466851	Kasapidis et al. (2005)
L. timidus (Sweden, Norway, Scotland, Russia, China)	AY422309 to AY422325; except AY422318 AJ287976 and AJ287977	Waltari et al. (2004) Wu et al. (2005), direct submission
L. capensis (north-central Tunisia)	DQ207740 to DQ207746	Ben Slimen et al. (2007)