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Molecular evidence of conspecificity of South African hares conventionally considered *Lepus capensis* L., 1758

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Abstract

Conventionally, *Lepus capensis* is considered to range across large parts of Africa, the Middle East, Central and Far East Asia. However, a recent morphological study restricts cape hares tentatively to a small range in the Western Cape Region of South Africa and groups all other *L. capensis*-type hares from South Africa into a new species: *L. centralis*. Here, we studied molecular relationships among *L. capensis*-type hares from South Africa. Phenotypically and morphologically the individuals matched either the newly described *L. capensis* or *L. centralis*. We examined 66 hares for allelic variation at 13 microsatellite loci and for sequence variation of the hypervariable domain 1 of the mitochondrial control region. All tree-generating analyses of the currently obtained sequences and all South African cape hare sequences downloaded from GenBank revealed monophyly when compared to sequences of various other *Lepus* species. A network analysis indicated close evolutionary relationships between hares of the "*L. capensis*-phenotype" and the "*L. centralis*-phenotype" (according to Palacios et al. 2008) from the southwest of the Western Cape, relative to their pronounced evolutionary divergence from all other more central, northern, and north-eastern *L. capensis*-type hares. F-statistics, a Bayesian admixture STRUCTURE model, as well as a principal coordinate analysis of microsatellite data indicated close genetic relationships among all South African *L. capensis*-type hares studied presently. A coalescence model-based migration analysis for microsatellite alleles indicated gene flow between most of the considered subspecies of cape hare, including *L. capensis capensis* and *L. capensis centralis*, theoretically sufficient to balance stochastic drift effects. Concordantly, AMOVA models revealed only little effects of partitioning microsatellite variation into the two suggested morpho-species "*L. capensis*" and "*L. centralis*". Under an "Interbreeding Species Concept" (e.g. a strict or relaxed Biological Species Concept), the current molecular data demonstrate conspecificity of the two proposed morpho-species "*L. capensis*" and "*L. centralis*". Based on the present molecular data the differentiation of subspecies of cape hares from southern Africa is discussed.

Keywords: *Lepus capensis*; Cape hare; mtDNA; Microsatellites; Phylogeny

Introduction

Cape hares (*Lepus capensis* L., 1758) in the broad sense (*Lepus capensis* sensu lato) have commonly been

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considered as members of a polytypic species with a wide distribution across Africa, the Middle East, and parts of Central and Far East Asia (Angermann 1965, 1983; Flux 1983; Flux and Angermann 1990; Hoffmann 1993). Only recently Hoffmann and Smith (2005) were inclined to restrict cape hares to South Africa, and suggested other species names for *L. capensis*-type hares (i.e., hares traditionally considered belonging to *L. capensis*) from East, West, and North Africa, Arabia, and the Middle East. They argued that there was no indication of gene flow between the South African range and more northern ranges in Africa, due to a distribution gap (see e.g., Flux and Angermann 1990; Collins 2005), and that the intervening areas were inhabited by other *Lepus* species, in particular *L. microtis* (i.e., *L. victoriae*: in our opinion "*L. microtis*" must be considered a "*nomen dubium*", as convincingly argued by Petter 1959; see also Angermann 1965; Angermann and Feiler 1988; Suchentrunk et al. 2007).

By citing molecular results of Kryger (2002), Robinson and Matthee (2005) suggested that in southern Africa cape hares (*L. capensis* sensu stricto) were limited to the south-central region, whereas another closely related taxon occurred north of that region. However, the two significant evolutionary units of southern African cape hares (a south-central and a northern population) identified by Kryger (2002) were substantiated only by distinct mitochondrial divergence, whereas the nuclear markers suggested high gene flow across all localities studied. Ben Slimen et al. (2008b) considered *L. capensis*-type hares from North and South Africa conspecific, based on a microsatellite data set. As to our knowledge, no convincing molecular data exist to demonstrate differentiation of southern African *L. capensis*-type hares into two or more species.

However, based on a rigorous morphological examination and chorological data Palacios et al. (2008) split the *L. capensis*-type hares from diverse regions in southern Africa into two provisional species. They considered only *L. capensis*-type hares with a particular set of phenotypes (coat pattern, skull, and dental characters), by and large from the range of *L. c. capensis* (see Roberts 1951), as *L. capensis* and all other studied hares with simple grooves in their principal incisors from other parts of southern Africa as a new species, i.e. *Lepus centralis*. According to their data, those two species have a small overlap zone, where they can be distinguished by a set of morphological characters. Thus, the range of *L. capensis* would likely be confined to a relatively small area in the Western Cape Region.

Here, we studied molecular characteristics of *L. capensis*-type hares from different regions in South Africa including specimens of *L. capensis* and *L. centralis* sensu Palacios et al. (2008), to prove whether or not they were sufficiently genetically separated to warrant their different species status. More specifically, using migration data derived from the distribution of molecular markers, we examined whether the straightforward application of a "Phenotypic (morphological) Species Concept" to South African cape hares by Palacios et al. (2008) was concordant with an "Interbreeding Species Concept"; in particular with the "Biological Species Concept" (BSC; e.g., Mayr 1963, 1995; see also Coyne and Orr 2004).

Material and methods

Specimens

We studied molecular characteristics of 66 *L. capensis*-type hares from different locations in the R.S.A. Among those, 13 specimens (including four fetuses of two females) were collected in June and August 2007 by vehicle-based spotlight shooting at night (a special permit was issued to U. Kryger by "Cape Nature" at Jonkershoek) at the "Good Hope Shooting Range" near Atlantis, owned by the SANDF (SA National Defence Force; 33° 37′ 15″ S/ 18° 28′ 16″ E), the Mudriver Farm (owned by Geoff Duckitt; 33° 29′ 30″S/ 18° 19′ 50″E), and the Yzerfontein Farm (owned by Willem De Villiers; 33° 19′ 46″S/ 18° 10′ 15″E). Those 13 hares were from within the range of *L. c. capensis* as described by Roberts (1951) and the range of the morpho-species "*L. capensis*" as delineated by Palacios et al. (2008). They featured external phenotypes (pelage) as well as non-metric skull and tooth characteristics concordant with those described by Palacios et al. (2008) for the morpho-species "*L. capensis*". The remaining 53 individuals were from different locations across the R.S.A., collected in the course of an earlier project. They have been assigned to different subspecies of *L. capensis* by Kryger (2002) following Roberts (1951). Several of the latter specimens (particularly those from Victoria West determined as *L. capensis centralis* according to Roberts 1951) were available to check non-metric skull and dental characters, which corresponded to that of "*L. centralis*" sensu Palacios et al. (2008). Their sampling locations were outside the range of "*L. capensis*" sensu Palacios et al. (2008) but from within the range of "*L. centralis*" sensu Palacios et al. 2008 (and from within the range of *L. capensis* as conventionally understood, e.g., Hoffmann and Smith 2005; Collins 2005). The respective molecular data of nine of those latter specimens (all from the environs of Victoria West; 31° 24′ 16″S/23° 06′ 56″E) have already been published by Ben Slimen et al. (2008a, b), and have been integrated in the current analyses. For sampling locations, sample sizes, and assignment to subspecies of

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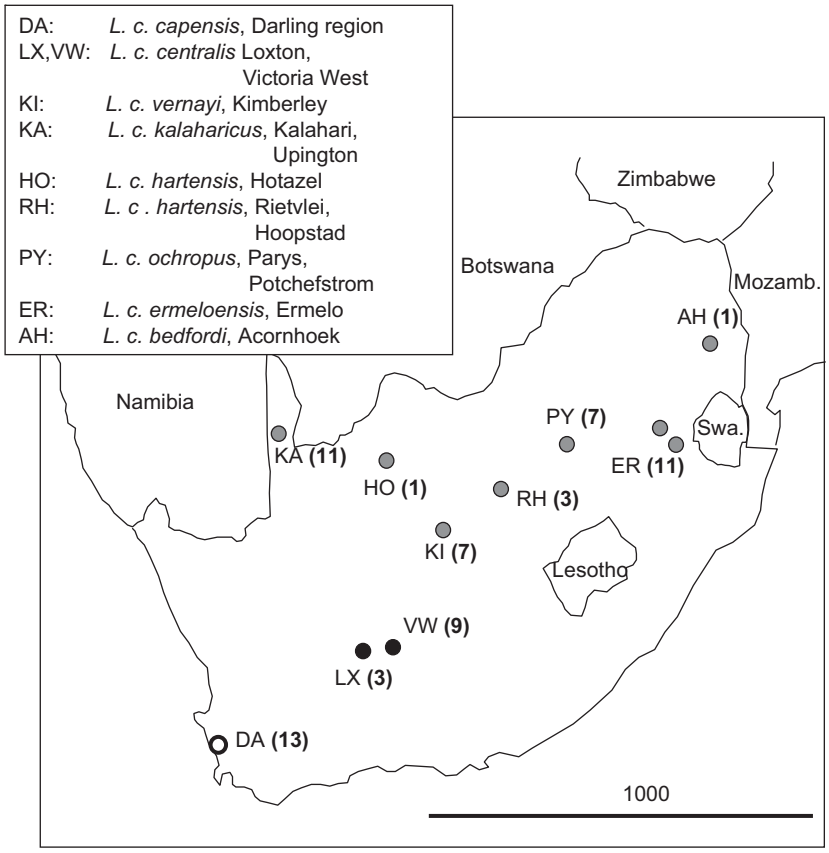

Fig. 1. Geographical sample distribution of *L. capensis*. Individual numbers are in parentheses. White circle: *L. capensis* according to Palacios et al. (2008), black and grey circles: *L. centralis* acc. to Palacios et al. (2008), black circles: localities explicitly within the range of *L. centralis* as described by Palacios et al. (2008). For acronyms see inset. Subspecies designation according to Roberts (1951).

L. capensis according to Roberts (1951; see also Kryger 2002) of specimens see Fig. 1.

Mitochondrial HV1 sequences and phylogenetic analyses

DNA extraction, PCR, and sequencing (Macrogen®) of the hypervariable domain I of the control region (mtHVI) was performed for 53 specimens following Ben Slimen et al. (2007). Sequencing of the remaining 13 individuals was not necessary because they were either embryos ($n = 4$) or their sequences were already available (nine hares from Victoria West; see Ben Slimen et al. 2008a). A 464-bp long HV1 fragment (corresponding to positions 15423 and 15951 of the complete rabbit (*Oryctolagus cuniculus*) mtDNA; Gissi et al. 1998) was sequenced as described in Ben Slimen et al. (2007).

The obtained 53 mtHV1 sequences (see Table 1 for haplotype acronyms, GenBank accession numbers) were aligned using CLUSTALX (1.83) (Thompson et al. 1997), and alignments were also checked by eye. The following indices of DNA polymorphism for the different *L. capensis* subspecies (according to Roberts 1951; see also Kryger 2002) were estimated by using DNASP version 4.1 (Rozas et al. 2003): haplotype diversity (h), nucleotide diversity (π), and mean number of pairwise differences (k). The amount of variation due to partitioning into the presently considered subspecies was calculated by an AMOVA model using ARLEQUIN 1.1 (Schneider et al. 2000). A second AMOVA model was used to test for significant partitioning of sequence variability due to the two morpho-species "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008).

In an initial phylogenetic analysis we included all reliable sequences available from GenBank that could be aligned to our sequences with a reasonable number of base pairs (for a list of GenBank accession numbers see Ben Slimen et al. 2007; Scandura et al. 2007; *L. yakardensis*: AY859501 to AY859525; AY821659 to AY821663; AY823309; AY820781 to AY820802). Prior to a Neighbor Joining (NJ) analysis we used Modeltest 3.06 to find the best-fit model of DNA substitution (Posada and Crandall 1998), which was the HKY85 model with T-Ratio = 7.4852, Shape = 0.8187, Pinvar = 0.3221. The NJ tree was constructed with

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Table 1. South African *Lepus capensis* – mtHV1 haplotypes. Haplotype names, GenBank accession numbers, frequencies (sequence numbers), acronym (see Fig. 1) of sampling locality/region, and subspecies designation according to Roberts (1951) are given. SA haplotypes have already been published by Ben Slimen et al. (2008a). [f]denotes fetal sequences.

Haplotype	GenBank accession nr.	Frequency (sequence nr.), locality (for acronyms see Fig. 1), subspecies
SA1	EF543150	3, VW, *L.capensis centralis*
SA2	EF543151	3, VW, *L.capensis centralis*
SA3	EF543152	1, VW, *L.capensis centralis*
SA4	EF543153	1, VW, *L.capensis centralis*
SA5	EF543154	1, VW, *L.capensis centralis*
DL11		2, (DL11, 13), DA, *L. c. capensis*
DL12		1, DA, *L. capensis capensis*
DL107		5 (DL 107,108,101,111,112), ER, *L. capensis ermeloensis*
DL150		2 (DL150, 151), PY, *L. capensis ochropus*
DL139		2 (DL139, 147), PY, *L. capensis ochropus*
DL137		2 (DL137, 143), KI, *L. capensis verneyi*
DL117		4 (DL117, 118, 119, 121), HO, *L. capensis hartensis*
DL125		3 (DL125, 128, 129), KA, *L. capensis kalaharicus*
DL103		2 (DL103, 109), ER, *L. capensis ermeloensis*
DL34		5 (DL34, 36, 37, 39[f], 40[f]), DA, *L. capensis capensis*
DL35		4 (DL35, 38, 41[f], 42[f]), DA, *L. capensis capensis*
DL136		1, KI, *L. capensis verneyi*
DL145		1, KI, *L. capensis verneyi*
DL149		1, KI, *L. capensis verneyi*
DL146		1, PY, *L. capensis ochropus*
DL135		1, KI, *L. capensis verneyi*
DL142		1, PY, *L. capensis ochropus*
DL148		1, HO, *L. capensis hartensis*
DL116		1, KA, *L. capensis kalaharicus*
DL120		1, KA, *L. capensis kalaharicus*
DL138		1, PY, *L. capensis ochropus*
DL144		1, PY, *L. capensis ochropus*
DL124		1, KA, *L. capensis kalaharicus*
DL134		1, PY, *L. capensis ochropus*
DL122		1, KA, *L. capensis kalaharicus*
DL141		1, KA, *L. capensis kalaharicus*
DL127		1, KA, *L. capensis kalaharicus*
DL140		1, KA, *L. capensis kalaharicus*
DL130		1, KA, *L. capensis kalaharicus*
DL126		1, KA, *L. capensis kalaharicus*
DL104		1, ER, *L. capensis ermoelensis*
DL110		1, AH, *L. capensis bedfordi*
DL33		1, DA, *L. capensis capensis*
DL131		1, LX, *L. capensis centralis*
DL132		1, LX, *L. capensis centralis*
DL133		1, LX, *L. capensis centralis*

PAUP4.0b10 (Swofford 2003), which we also used to run 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 phylogenetic trees was assessed by bootstrap percentages after 1000 resampling steps (Felsenstein 1985) with PAUP. The NJ tree was rooted using the HV1 sequence of *O. cuniculus* (GenBank Accession N° NC001913). Both analyses were based on an alignment of 337 bp (indels were excluded), with 37.56% conserved sites, 56.33% variable sites, 47.28% parsimony informative sites, and 9.05% singletons.

In a second approach we performed a NJ, a MP, and a maximum likelihood (ML) analysis separately for the 53 sequences of *L. capensis*-type hares currently revealed from South Africa, as they were all grouped into one clade in the overall phylogenetic analyses. The NJ analysis was based on a total length of 464 bp including indels (455 bp excluding indels), and on HKY85 (TRatio = 7.8087, Shape = 0.8712, Pinvar = 0.5395) distances, as that was the best fitting substitution model. A scrub hare (*L. saxatilis*) sequence (GenBank acc. number FJ 829854), derived from a road kill on the R317, some 9 km south of Stormsvlei, Western Cape, R.S.A., collected on 14 August 07, was used to root the NJ tree. The MP analysis was performed 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 phylogenetic trees was assessed by bootstrap percentages after 1000 resampling steps (Felsenstein 1985) with PAUP. The ML analysis with TBR branch swapping was also run in PAUP; nodal support was estimated by using the non-parametric bootstrap (Felsenstein 1985) and was restricted to 100 pseudo-replicates to limit computing time.

In addition, we constructed a median-joining (MJ) network (Bandelt et al. 1999) including all currently produced *L. capensis* haplotypes based on their variable positions (134 positions, indels as a fifth character) with equally weighted positions and $\varepsilon = 0$ by using Network 4.2.0.1 (available at http://www.fluxus-technology.com/sharenet.htm). To explore levels and patterns of evolutionary divergence among taxa (*L. capensis* subspecies), we calculated numbers of mutation steps (NMS) as revealed from the MJ network between all pairs of haplotypes within each subspecies and between geographically next subspecies/sampling regions, respectively (see Fig. 1). We tested for significant variation of NMS across subspecies and between pairs of geographically neighbouring populations by two separate Kruskal Wallis (KW) tests. For those tests we used randomized rather than original NMS values, to achieve statistical independence, which is a necessary precondition. Sample sizes (i.e., numbers of randomized NMS values)

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equalled the respective original numbers of haplotype comparisons, to avoid an inappropriate inflation of the data sets. Prior to the tests we performed Spearman rank correlations between hare numbers and respective means and standard deviations of NMS values per subspecies to check for independence of NMS values from sample sizes in within- and between-subspecies comparisons, which was the case.

In parallel to the observed relatively low π values of *L. c. capensis*, which might reflect a founder event (see e.g. Valdiosera et al. 2007), we used a KW test to test for significant variation of randomized pairwise subspecies-specific HKY85 values across the six subspecies. Sample sizes for the KW test corresponded to the numbers of all possible comparisons between specimens (not haplotypes) per subspecies; subspecies-specific π values were correlated (linear Pearson correlation model) with numbers of hares per subspecies.

Finally, we examined whether the mtHV1 sequences exhibited a significant spatial structure by allocating each sequence to a cell of a grid that we applied to the map of the sample locations. We used the resulting (spatial) grid coordinates to calculate Moran's statistics (e.g., Epperson 2003) and associated 95% confidence intervals for ten spatial distance classes based on all individual sequences (i.e., using identical haplotypes repeatedly, if present in more than one individual) by running 1000 permutations using the SGS program vers.1.0c (Degen 2000; see also e.g., Sokal and Oden 1978; Sokal 1998).

Microsatellite analyses and population genetic statistics

We used the following thirteen microsatellite loci with different levels of polymorphism: Sol08, Sol28, Sol30 (Rico et al. 1994), Sol33 (Surridge et al. 1997), Sat 2, Sat 8, and Sat 12 (Mougel et al. 1997), Lsa 1, Lsa 2, Lsa 3, Lsa 6 and Lsa 8 (Kryger et al., 2002) and INRACCDDV0001 (N4) (Chantry-Darmon et al. 2005). Polymerase chain reaction (PCR) amplification was performed for the Sol and the Lsa primers as described in Ben Slimen et al. (2008b). For the Sat primers amplification was performed in 25 µl reaction volumes with the following components: 1 µl of genomic DNA, 0.2 µl of each primer, 2.5 µl of 2 mM dNTP mix, 0.5 µl $MgCl_2$, 0.1 µl Taq DNA Polymerase and 2.5 µl 10 × reaction buffer. After a preliminary denaturation step at 95 °C for 4 min, PCR amplification was performed for 30 cycles of 30 s denaturing at 95 °C, 30 s of annealing at locus-specific temperatures (55 °C for Sat 2 and Sat 12, and 60 °C for Sat 8) and 30 s of extension at 72 °C, with a final 10 min extension step at 72 °C. The PCR products were electrophoresed on a LI-COR 4200 automated sequencer along with a fluorescently labelled size standard (50–350 bp sizing standard; LI-COR® Biotechnology Division). Allele lengths were determined using Gene ImageIR ver. 3.52.software (LI-COR, Inc., © 1990–1998).

We tested the microsatellite loci for deviation from Hardy-Weinberg equilibrium (HWE) using the Markov chain method implemented in GENEPOP version 3.4 (Raymond and Rousset 1995) and the default parameter settings of 1000 demomorizations, 100 batches and 1000 iterations per batch. Significance levels were adjusted using strict Bonferroni correction for multiple comparisons (Rice 1989). The probability of the existence of null alleles was checked with the program Microchecker 2.2.3 (van Oosterhout et al. 2005). Allele frequencies, mean number of alleles (A), observed (H_o) and expected (H_e) heterozygosity were calculated for each locus and for each subspecies (sampling locality) with GENETIX (Belkhir 2004). We used this program also to test for genotypic linkage disequilibrium and to calculate overall and population-specific Weir and Cockerham (1984) estimators of F_{IS} and respective significance levels for deviation from zero by permutation tests (10.000 permutations). We further used it to calculate F_{ST} and associated 95% confidence intervals (CI) between "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008), as well as between *L. c. capensis* (i.e., hares with a phenotype corresponding to "*L. capensis*" sensu Palacios et al. 2008), *L. c. centralis* (i.e., individuals from Victoria West and Loxton), and all other studied *L. capensis* subspecies combined. To test for significant allocation of allelic variability among subspecies (according to Roberts 1951) and among the two morpho-species "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008) we run two AMOVA models by using ARLEQUIN 1.1 (Schneider et al. 2000).

To evaluate whether or not homoplasy might have effected our calculations of microsatellite differentiation among taxa, we plotted subspecies-specific and locus-specific he values against respective locus-specific A values. Based on the pattern of locus-specific saturation of he, we created two sets of loci, one encompassing loci with low and one with high allelic variability (see Ben Slimen et al. 2008b). We then calculated F_{ST} and Cavalli-Sforza & Edwards (1967) chord (CSE) distances between "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008) separately for both sets of loci using GENETIX, expecting higher values for the set of loci with low allelic variability if homoplasy plays a role.

We also quantified the degree of differentiation between and across subspecies using Weir and Cockerham's (1984) estimator (θ) of Wright's F_{ST}, as calculated by the Microsatellite Analyser MSA programme (Dieringer and Schlötterer 2002). Significance levels were adjusted by strict Bonferroni corrections for multiple comparisons after 10.000 permutations. Although a generally valid microsatellite mutation

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model is still not available (e.g., Li et al. 2002), the stepwise mutation model (Ohta and Kimura 1973) provides a good fit to the distribution of allele sizes in microsatellites with short repeat units (Shriver et al. 1993; Valdes et al. 1993). Thus, to obtain an evolutionary signal in addition to pure stochastic/historic allelic population data in connection with gene flow patterns, we also estimated population differentiation by calculating pairwise *rho*, an unbiased estimator of R_{ST} (Slatkin 1995), that takes into account differences in population size and variance of loci, by using the R_{ST}-CALC vers. 2.2 program (Goodman 1997). All *rho* calculations were performed with 10.000 permutations and 1000 bootstrap steps to determine significant deviations from zero.

To test for the amount of gene flow we calculated coalescence theory derived maximum likelihood estimates for the current migration rates (Beerli and Felsenstein 2001) among the different subspecies based on our microsatellite data and using a Markov chain Monte Carlo (MCMC) approach as implemented in MIGRATE Version 1.1 (Beerli 1997–2001). In addition, we examined individually based spatial genetic structuring using a Bayesian statistical approach, i.e. a likelihood analysis to assess the most likely number of population groupings compatible with the observed genotypic distribution using STRUCTURE (Pritchard et al. 2000; Falush et al. 2003). The likelihood when assuming different numbers of populations (k) was calculated under the following specifications: ''admixture model'', ''correlated allele frequencies among populations'', burn-in = 10.000, MCMC = 10.000, 1–11 k and 10 iterations for each k. Mean, maximum, minimum, and standard deviation of ln [Pr(X|k)] were calculated for each k. Moreover, we calculated ΔK for each k, based on the second order rate of change of the likelihood function with respect to k, which is a useful ad hoc statistic for finding the most likely value of k (Evanno et al. 2005). As we did for the F-statistics, we also run the STRUCTURE analysis for both sets of loci (low and high variability). Prior to the STRUCTURE analysis we checked for possible spatial structuring of the microsatellite data that might blur the STRUCTURE result. Pronounced spatial clustering of genotypes/alleles would indicate absence of statistically independent cases; for instance deviation from random mating not caused by genetic discontinuities might lead to overestimation of genetic clustering. Thus, we checked for significant spatial autocorrelation of allele frequencies through calculating pairwise Gregrorius (1978) distances among populations separately for each of five spatial distance classes and testing for significantly higher or lower values than expected by chance (1000 permutations, 95% c.i.). We used Gregrorius (1978) genetic distances due to their superior performance compared to Nei (1972) distances, which where the second option available in the SGS programme. The populations used in this procedure were concordant with the six subspecies given in Table 1 (see also Fig. 1; *L. c. hartensis*, and *L. c. bedfordi* were disregarded due to their too small sample sizes). Spatial coordinates for those populations were obtained from the same grid as used for the spatial autocorrelation analysis of HV-1 sequences. In addition to the spatial autocorrelation analysis we checked specifically for geographic structuring following an ''isolation by distance model'' by a Mantel test (1000 permutations) of ln-transformed geographical distances (straight lines) between sampling localities of subspecies and associated $F_{ST}/(1\text{-}F_{ST})$ values using the GENETIX programme. We further used the Bayesian and the distance-based (Cavalli-Sforza and Edwards (1967) chord (CSE) distances) approaches of population assignment (Cornuet et al. 1999; Bayesian method of Rannala and Mountain (1997) and computing probabilities following the resampling algorithm of Paetkau et al. (2004)) implemented in the GENECLASS 2 program (Piry et al. 2004), to estimate the likelihood of an individual's multilocus genotype to be assigned to the subspecies from which it was sampled.

In line with the suspected founder event for *L. c. capensis* in the Darling region as inferred from reduced mtDNA diversity, we tested our microsatellite data set for such a signal as well using the Bottleneck program Version 1.2.02 (16.II.99) (Cornuet and Luikart 1996). The tests in that program package refer to the observation that under a significant drop of effective population size and a constant (equilibrium) population size allelic diversity (number of alleles) is reduced faster than heterozygosity. Strictly speaking, this is true under the Infinitive Allele Model (IAM), whereas the Stepwise Mutation Model (SMM) is not necessarily associated with a heterozyogsity excess. We ran one-tailed Wilcoxon tests, which has reasonable power for our data sets, for the IAM and the Two-phased model (TPM) with default settings, as most microsatellite data sets fit better to the latter (Di Rienzo et al. 1994). Because of reduced sample sizes for some loci in some populations, we ran the tests only for our *L. c. capensis*, *L. c. centralis*, *L. c. vernayi*, and *L. c. ochropus* samples, and set the level of significance at $p<0.018$ following the B-Y method of accounting for multiple testing (Narum 2006).

We calculated pairwise CSE distances between single individuals and subjected them to a principal coordinate analysis (PCO) using the PCO program of Anderson (2003). Subsequently, we used S-PLUS 2000 Profess. Rel. 2 (© 1988-1999 MathSoft Inc.) to test individual PCO scores for significant variation between the two subspecies *L. c. capensis* and *L. c. centralis* sensu Roberts (1951) and between the two ''morpho-species'' *L. capensis* and *L. centralis* (i.e., *L. capensis* vs. all other individuals) sensu Palacios et al. (2008) with a

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Table 2. Mean, standard deviation (upper row), range (lower row) of pairwise HKY85 distances between subspecies of *L. capensis*. Respective values within each subspecies are given in the diagonal. Numbers of haplotypes for each subspecies are given in parentheses.

	centralis (8)	*capensis* (5)	*ochropus* (5)	*kalaharicus* (7)	*vernayi* (5)	*ermeloensis* (3)
centralis	0.0581 ± 0.0204					
	0.0157–0.0907					
capensis	0.0789 ± 0.0150	0.0140 ± 0.0077				
	0.0591–0.1130	0.0022–0.0251				
ochropus	0.1624 ± 0.0135	0.1612 ± 0.0065	0.0457 ± 0.0214			
	0.1334–0.1860	0.1519–0.1751	0.0022–0.0664			
kalaharicus	0.1659 ± 0.0151	0.1622 ± 0.0123	0.0447 ± 0.0165	0.0423 ± 0.0129		
	0.1258–0.1956	0.1383–0.1850	0.0112–0.0697	0.0135–0.0604		
vernayi	0.1515 ± 0.0142	0.1519 ± 0.0080	0.0392 ± 0.0219	0.0518 ± 0.0075	0.0282 ± 0.0252	
	0.1255–0.1793	0.1390–0.1675	0.0022–0.0638	0.0384–0.0666	0.0022–0.0608	
ermeloensis	0.1540 ± 0.0127	0.1470 ± 0.0054	0.0678 ± 0.0246	0.0698 ± 0.0164	0.0695 ± 0.0354	0.0668 ± 0.0404
	0.1183–0.1779	0.1385–0.1549	0.0181–0.1015	0.0437–0.1021	0.0066–0.0988	0.0205–0.0949

Generalized Least Squares regression model (GLS) with a restricted maximum likelihood approach (REML) for variance homogeneity. We run GLS models separately for each of the 23 PCO dimensions, which together fully explained the variance of the CSE matrix, and we based significance decisions on sequential Bonferroni corrections with a nominal α of 0.5. Eventually, we run a Mantel test (1000 permutations, GENETIX) of pairwise CSE values between subspecies and pairwise mean HKY85 HV1 distances to test for concordance of differentiation in nuclear and mitochondrial gene pools.

Table 3. Measures of genetic diversity in the studied subspecies: h – haplotype diversity, π – nucleotide diversity, k – mean number of pairwise differences.

subspecies (populations)	h	π	k
capensis	0.782	0.01329	6.128
centralis	0.909	0.04973	22.924
ermeloensis	0.607	0.03873	17.893
kalaharicus	0.917	0.03896	18.000
ochropus	0.952	0.03381	15.619
vernayi	0.952	0.02309	10.667
overall	0.979	0.07864	36.175

Results

Mitochondrial sequences

The nucleotide composition of all currently sequenced samples showed the typical low G content (between 10% and 11.5%, with an average of 10.5%), whereas it was higher for the other nucleotides (averaging to 29.2%, 28.9%, and 31.4% for T, C, and A, respectively). This suggested that no nuclear representations of mtDNA (numts) have been included in our analyses. Within subspecies, h ranged from 0.607 to 0.952, π between 0.013 and 0.050, and k between 6.128 and 22.92 (Table 3). The AMOVA revealed that 15.14% of the sequence variability was due to variation among the different subspecies ($p < 0.0001$) and 84.86% was due to variation within subspecies. However, we found no significant (3.8%, n.s.; AMOVA, F-statistics from haplotype frequencies) variation when testing for partitioning due to the two morpho-species *L. capensis* and *L. centralis* senu Palacios et al. (2008).

Both the NJ and the MP analyses of the 337 bp alignments of 392 sequences including the downloaded *Lepus* sequences grouped all sequences of the currently considered *L. capensis*-type hares (i.e., *L. capensis* sensu Roberts 1951; Kryger 2002; Collins 2005) and the *L. capensis*-type sequences of South Africa submitted by Kryger (2002) to GenBank into one clade with bootstrap support of 80% and 75% for the NJ and the MP analyses, respectively (trees not shown).

All presently obtained 53 South African sequences (i.e., excluding the four embryos) represented 36 different haplotypes (461–463 bp), with 26 (72.2%) occurring in single hares, respectively. We used those sequences for detailed phylogenetic analyses together with the five already published haplotypes of *L. c. centralis* from Victoria West (Ben Slimen et al. 2008a). Pairwise HKY85 distances between haplotypes ranged from 0.22% to 19.56%. Table 2 details means, standard deviations, and ranges of HKY85 distances within and between the studied subspecies, and Table 3 lists the three indices of sequence diversity (h, π, k) for each subspecies. The subspecies-specific π values did not depend on sample size (i.e., numbers of hares per subspecies) ($r = 0.584$, $p = 0.224$, linear Pearson correlation

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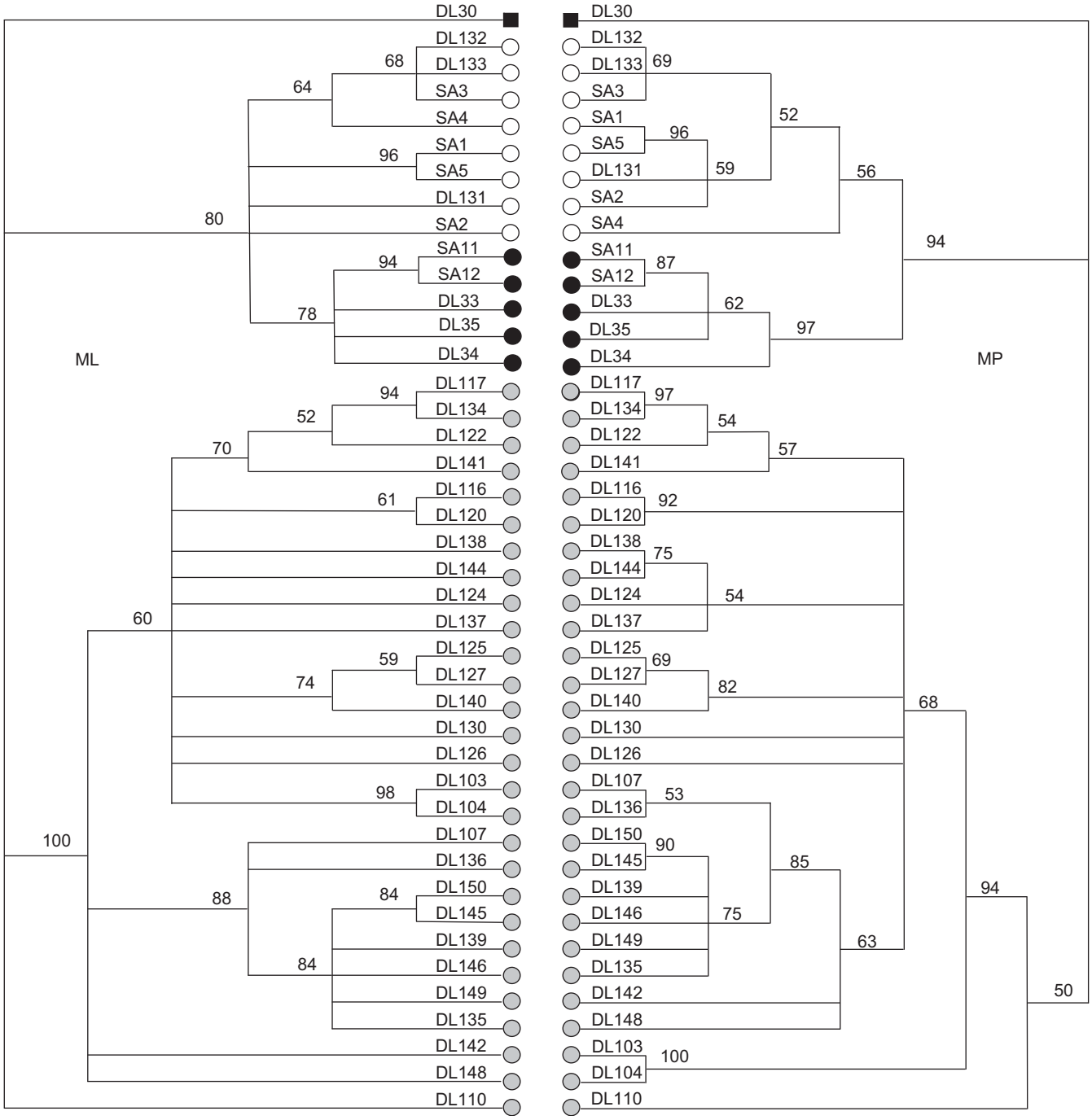

Fig. 2. Maximum likelihood (ML) and maximum parsimony (MP) consensus trees of HV1 haplotypes of *L. capensis* South African cape hares produced presently, with haplotype number and acronym of taxon/sampling region attached. Bootstrap support values are given at internal nodes. The black square indicates a scrub hare (*L. saxatilis*).

model). But the pairwise HKY85 distances varied significantly ($chi^2 = 91.4$, d.f. = 5, $p < 0.0001$ Kruskal Wallis test of randomized values) across the subspecies, with the lowest value in *L. c. capensis* (Table 2).

The ML and MP analyses concordantly revealed two major clades. All haplotypes of *L. c. capensis* from the Darling region and all *L. c. centralis* haplotypes from Victoria West and Loxton clustered together into one clade, and within this clade particularly all *L. c. capensis* haplotypes were grouped into one separate subclade (see Fig. 2 for the respective consensus trees). All other haplotypes of diverse subspecies except *L. c. capensis* and *L. c. centralis* were grouped into the second major clade with several subclades. The NJ analyses showed in essence the same differentiation pattern (dendrogram not shown).

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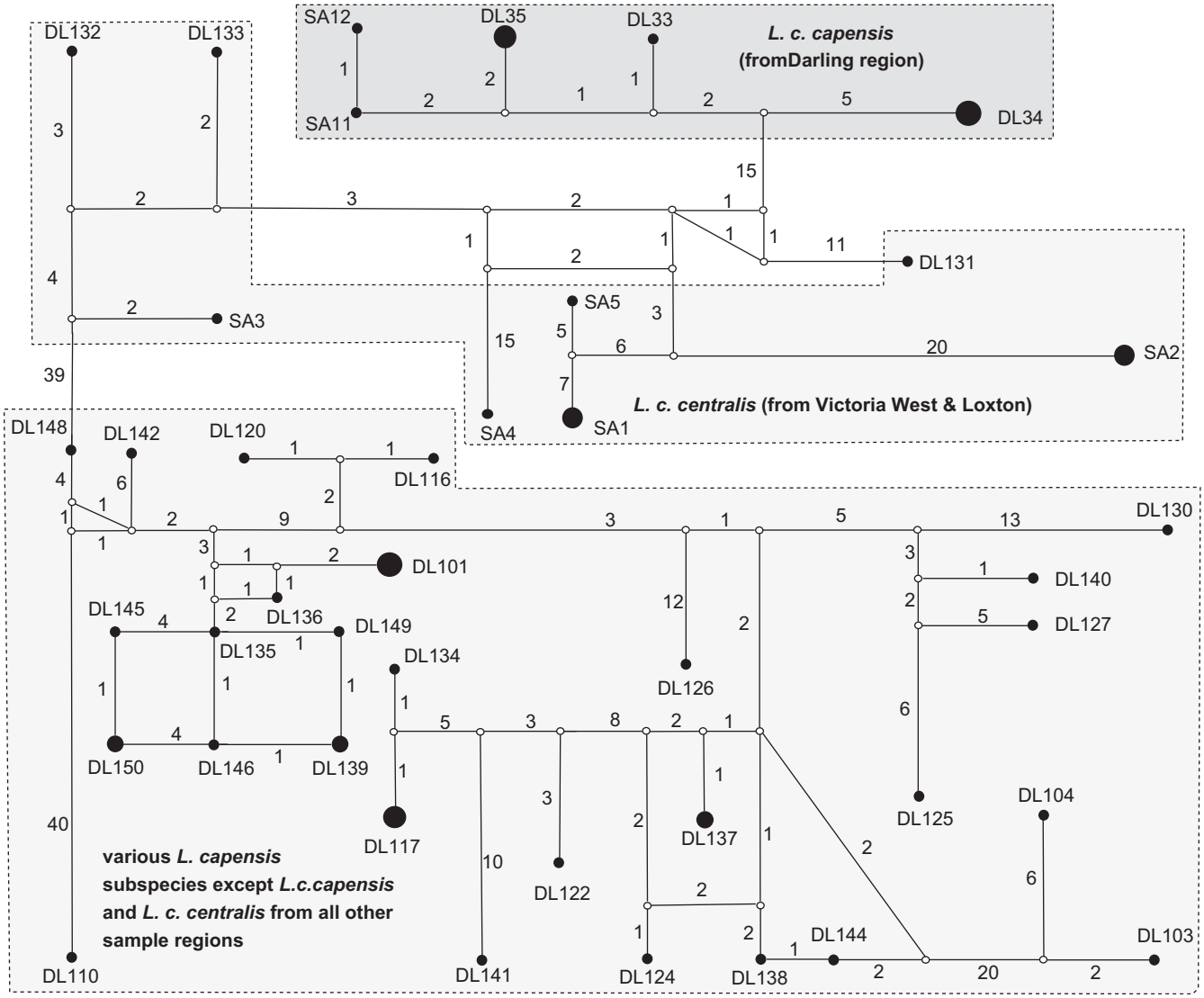

Fig. 3. MJ network of HV1 haplotypes of *L. capensis* specimens studied presently from South Africa. Black cirles indicate haplotypes found; circle sizes reflect numbers of hares with respective haplotype. White circles represent inferred haplotypes and numbers on lines linking two haplotypes indicate numbers of mutation steps between haplotypes.

The MJ network (Fig. 3) grouped all haplotypes of *L. c. capensis* somewhat separate from those of *L. c. centralis* (i.e., hares from Victoria West and Loxton). But the evolutionary divergence between several pairs of the latter hares was greater than for several comparisons between *L. c. capensis* and *L. c. centralis*: e.g., 30 mutation steps between the *L. c. centralis* haplotype DL131 from Loxton and the *L. c. capensis* haplotype DL33 compared to 40 mutation steps between *L. c. centralis* SA2 and SA 4 from Victoria West. Both haplotypes of *L. c. capensis* and *L. c. centralis* were clearly separated evolutionarily from all other cape hare subspecies (from diverse localities and subspecies) studied presently, with the *L. c. centralis* haplotypes connecting between those of *L. c. capensis* and all others. However, we revealed relatively high evolutionary divergence even between haplotypes of single hares within diverse other subspecies/localities: e.g., 28 mutation steps between haplotypes DL150 and DL144 from Parys, 31 mutation steps between DL126 and DL130 from the Kalahari, 27 mutation steps between DL137 and DL145 from Kimberley, or 48 mutation steps between DL104 and DL101 and others from Ermelo (comp. Fig. 3 and Table 1). On the contrary, in several instances only few mutation steps were present between haplotypes of hares from different localities (subspecies) that otherwise showed high within regional/subspecies divergence: e.g., one mutation step only between DL150 from Parys and DL145 from Kimberley or six mutation steps between DL137 from Kimberley and DL124 from the Kalahari (Fig. 3 and Table 1). Both the randomized NMS values and their variances (Fig. 4) varied

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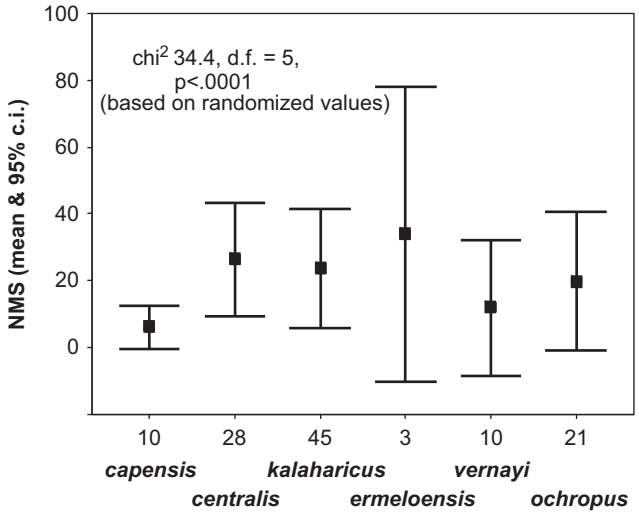

Fig. 4. Variation of numbers of mutation steps (NMS) between haplotypes across subspecies of South African cape hares. NMS as from the MJ network. Numbers associated with subspecies are numbers of pairwise comparisons. Kruskal-Wallis test. Grey zone: range of NMS for within-subspecies comparisons.

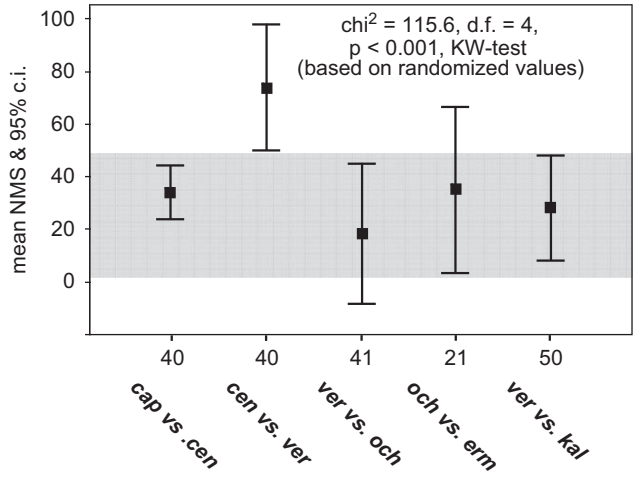

Fig. 5. Variation of numbers of mutation steps (NMS) between haplotypes for pairs of geographically closest subspecies of South African cape hares. NMS as from the MJ network. Numbers associated with pairs of subspecies are numbers of pairwise comparisons. KW-test – Kruskal Wallis test.

significantly across the six subspecies, as did randomized NMS values for pairwise comparisons between geographically next subspecies/sampling regions (Fig. 5). In the between-subspecies comparison, NMS values were highest between *L. c. centralis* and *L. c. vernayi*, whereas NMS values were well within the range of within-subspecies comparisons for the comparison of *L. c. capensis* and *L. c. centralis* (Fig. 5). The spatial autocorrelation analysis revealed significant negative Moran's statistics for five of the ten spatial distance

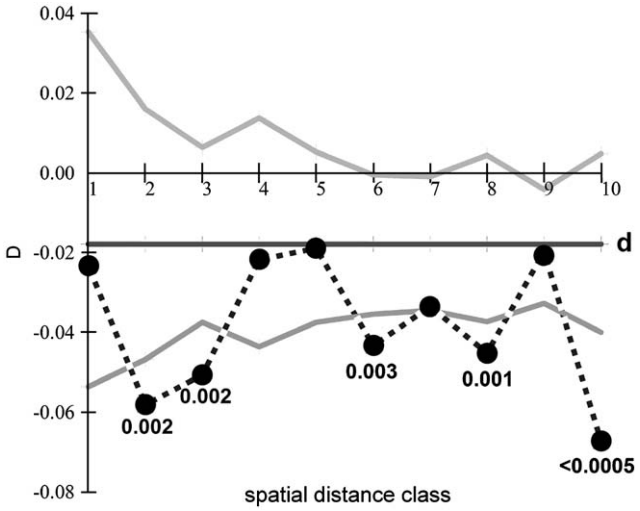

Fig. 6. Spatial autocorrelation of HV1 sequences based on Moran's statistic (D) across 10 geographical distance classes. Black dots indicate D values for distance classes and associated p-values if lower than 0.05. The two grey lines indicate 95% confidence limit and the straight grey line (d) indicates the reference/mean line.

classes (see Fig. 6 for the correlogram, significance values, and 95% c.i. of not significant indices), which indicated negative spatial structuring of the HV1 sequences, both for short and long geographic distances.

Microsatellite data

We determined allelic variation at microsatellite loci for the following six subspecies: *L. c. capensis*, *L. c. centralis*, *L. c. ermeloensis*, *L. c. ochropus*, *L. c. kalaharicus*, *L. c. vernayi.* Together with the few remaining individuals of *L. c. bedfordi* and *L. c. hartensis* we revealed a total of 137 alleles, with an average of 10.54 alleles per locus. The numbers of alleles per locus ranged from 3 (Sat8) to 18 (Sol08), and the total number of alleles per subspecies ranged between 55 (*L. c. capensis*, *L. c. ermeloensis*) and 79 (*L. c. centralis*). Expected (H_e) and observed (H_o) locus-specific heterozygosities, numbers of alleles per locus, allele size ranges (in pb), numbers of private alleles, F_{IS} values, and associated significances, as well as significant deviation of genotypes from HWE are detailed in Table 4. Among all alleles, 30 (21.9%) were private, i.e. found in one subspecies only, whereas the others were shared between two or more subspecies. None of the thirteen loci showed a significant presence of null alleles, there was no hint of allelic drop-out, and there was no linkage disequilibrium for any pair of polymorphic loci, when tested separately for each subspecies. The individual from Acornhoek (i.e., *L. c. bedfordi*) and the four hares from Hotazel and Rietvlei (i.e., *L. c. hartensis*) were not

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Table 4. Allelic variation at 13 microsatellite loci in six cape hare (*L. capensis*) subspecies. Number of alleles per locus (A), allelic size range in bp (R), expected heterozygosity (H_e), observed heterozygosity (H_o) are given. Values that show significant (Bonferroni correction, $\alpha < 0.05$) departure from zero for the inbreeding coefficient F_{IS} are indicated with *. Numbers of private alleles for each subspecies are given in parentheses.

population		Sol08	Sol28	Sol30	Sol33	Lsa 1	Lsa 2	Lsa 3	Lsa 6	Lsa 8	Sat2	Sat8	Sat12	N4	all loci	F_{IS}
L. c. centralis (4)	A	7	9	8	6	4	8	7	3	7	7	2	6	5	79	0.10277
	R	128-160	153-179	165-191	205-219	162-168	235-253	198-210	168-172	180-196	215-241	92-96	110-130	212-222		
	He	0.7449	0.8471	0.8457	0.6500	0.7350	0.8025	0.7350	0.5150	0.7397	0.7407	0.0950	0.6000	0.7350	0.6758	
	Ho	0.8571	0.7273	0.8889	0.7000	0.8000	0.5556	0.5000	0.5000	0.5455	0.8889	0.1000	0.7000	0.6000	0.6433	
L. c. capensis (6)	A	6	9	5	3	3	6	4	3	4	4	1	2	5	55	0.06249
	R	114-134	153-185	163-175	205-213	164-168	243-253	198-208	168-172	184-194	213-221	92	114-118	204-222		
	He	0.6983	0.8077	0.7308	0.6612	0.6657	0.7882	0.4645	0.4615	0.6716	0.4349	0	0.2604	0.7278	0.5671	
	Ho	0.5455	0.8462	0.7692	0.7273	0.6923	0.3333	0.3846	0.4615	0.9231	0.3077	0	0.3077	0.9231	0.5555	
L. c. ermeloensis (3)	A	3	6	7	4	3	4	4	2	5	7	2	5	3	55	0.11111
	R	126-144	155-181	159-183	205-215	162-168	235-251	206-220	168-170	180-196	217-249	92-96	114-134	202-206		
	He	0.5	0.8000	0.8056	0.5800	0.5694	0.7500	0.6944	0.1528	0.7222	0.8200	0.1528	0.7917	0.5694	0.6083	
	Ho	0.664	0.4000	0.6667	0.8000	0.6667	10.000	0.3333	0.1667	0.8333	0.8000	0.1667	10.000	0.5000	0.6154	
L. c. kalaharicus (8)	A	6	7	11	2	5	3	4	3	5	7	1	5	2	61	0.15465
	R	104-144	139-179	157-183	209-211	162-170	241-251	204-214	162-170	186-196	217-245	92	114-130	202-204		
	He	0.82	0.8194	0.8906	0.5000	0.6797	0.6111	0.6389	0.5000	0.7266	0.7639	0	0.7734	0.3750	0.6230	
	Ho	1	0.8333	0.8750	10.000	0.6250	0.6667	0.3333	0.3333	0.7500	0.6667	0	0.6250	0.2500	0.6122	
L. c. vernayi (4)	A	4	8	9	5	4	6	4	4	7	7	2	6	3	69	0.22061*
	R	102-130	159-177	159-191	207-215	162-168	241-251	206-212	162-172	182-196	211-237	90-92	106-132	202-206		
	He	0.6563	0.8571	0.8673	0.7639	0.7245	0.7857	0.6939	0.4592	0.8265	0.7551	0.2449	0.8061	0.4388	0.6830	
	Ho	0.2500	10.000	10.000	0.8333	0.7143	0.5714	0.0000	0.4286	0.7143	0.7143	0.2857	0.5714	0.5714	0.5888	
L. c. ochropus (5)	A	6	8	11	7	4	5	5	4	6	5	2	4	3	70	0.12705
	R	104-136	155-183	159-185	205-217	162-168	241-249	206-220	162-174	184-194	213-237	90-92	114-126	202-206		
	He	0.8056	0.8367	0.8980	0.8333	0.7449	0.7639	0.7361	0.3673	0.7755	0.7600	0.2449	0.6020	0.5000	0.6822	
	Ho	0.6667	0.8571	10.000	10.000	0.7143	0.8333	0.3333	0.4286	0.4286	0.8000	0.2857	0.7143	0.4286	0.6531	
all samples	A	18	17	16	9	5	9	11	5	9	18	3	8	9	137	0.21908*
	R	102-160	139-185	157-191	205-221	162-170	235-253	198-220	162-174	180-196	211-257	90-96	106-132	202-222		
	He	0.8803	0.9088	0.9129	0.8306	0.7409	0.8437	0.8151	0.6046	0.7955	0.8670	0.1045	0.7405	0.7909	0.7566	
	Ho	0.6500	0.7963	0.8571	0.8182	0.6964	0.5814	0.3208	0.3818	0.6897	0.6800	0.1091	0.5965	0.5965	0.5980	

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Table 5. Pairwise θ values (upper diagonal, upper row) and pairwise Rho values (lower diagonal, upper row) for the six *L. capensis* subspecies as obtained from the microsatellite data. Asterisks denote values significantly ($p<0.05$ after Bonferroni corrections) higher than zero. Values in lower rows indicate numbers of migrating individuals as calculated by the MIGRATE analysis for either direction (above diagonal: source population of migration as indicated in the first line; below diagonal: receiving population as indicated by the first column).

	L. c. centralis (1)	*L. c. capensis* (2)	*L. c. ermeloensis* (3)	*L. c. kalaharicus* (4)	*L. c. vernayi* (5)	*L. c. ochropus* (6)
(1)	-	0.09748*	0.11897*	0.14403*	0.09788*	0.12643*
		1.019	0.4686	0.3374	1.0681	1.6533
(2)	0.2254*	-	0.21035*	0.19775*	0.14740*	0.15513*
	1.3425		1.7227	2.0727	2.2536	1.0749
(3)	0.3032*	0.3961*	-	0.14959*	0.11247*	0.09152*
	0.5137	1.2450		0.3819	1.7221	2.5626
(4)	0.3279*	0.3758*	-0.0549	-	0.01606	0.01607
	1.0176	2.1076	0.6634		0.3146	0.8788
(5)	0.3673*	0.3062*	0.1571	0.0574	-	-0.02047
	2.4794	1.8154	1.7787	0.4188		1.5977
(6)	0.3327*	0.2766*	0.1498	0.0852	-0.0236	-
	1.0583	0.6810	0.4572	0.8384	1.0961	

included in this subspecies comparison due to the too small sample sizes.

Pairwise F_{ST} values between subspecies ranged from −0.02047 to 0.21035 (Table 5), with only three (20%) of the 15 pairwise F_{ST} values not differing significantly from zero. Pairwise Rho values ranged from −0.0236 to 0.3961 (Table 5), with six (40%) values not differing significantly from zero. For the comparison of "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008) F_{ST} amounted to 0.0779 (95% CI: 0.0467–0.1082), and for the comparison of *L. c. capensis* (i.e. "*L. capensis*" sensu Palacios et al. (2008)), *L. c. centralis* (from Victoria West and Loxton), and all remaining *L. capensis* subspecies F_{ST} amounted to 0.0984 (95% CI: 0.0548–0.1467). The AMOVA model testing the subspecies effect on partitioning nuclear gene pool diversity yielded 11.14% ($p<0.00001$), whereas within-subspecies diversity was 88.86%. But there was no significant effect due to partitioning genetic variability between "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008) (6.76%, $p=0.186$, AMOVA). The maximum likelihood estimates for the current migration rates between the considered subspecies ranged between 0.3374 (from *L. c. kalaharicus* to *L. c. centralis*) and 2.2536 (from *L. c. vernayi* to *L. c. capensis*), with 63.3% of all migration values above 1.0. All values but one involving *L. c. capensis* as recipient population were above 1.0 (Table 5).

The scatter plot (not shown) of the subspecies-specific He vs. A values for each locus revealed that He remained fairly constant for ten loci (Sol8, 28, 30, 33, Lsa2, 3, 8, Sat 2, 12, 30, INRACCDDV0001) with A equal to or greater than eight. When calculated separately for this set of "highly variable loci" and the set of "less variable loci" (i.e., Lsa1, Lsa6, Sat8), we obtained F_{ST} and CSE distances between "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008) as follows: overall $F_{ST}=0.1075$ (locus-specific values: -0.002 – 0.2138) and CSE = 0.132 for the "highly variable set of loci", and $F_{ST}=0.0454$ (locus-specific values: 0.013 – 0.0861) and CSE = 0.112 for the "less variable set of loci". These results indicate absence of a serious homoplasy effect, because under homoplasy we would have expected actually the opposite; i.e., higher F_{ST} and CSE values for the "less variable set of loci".

Table 6. Summary of Bayesian STRUCTURE results of microsatellite genotypes. Ln(Pr) is the natural logarithm of the probability that K is the correct number of populations according to the model used (see Material and methods). SD is the standard deviation calculated from ten independent runs per K. ΔK values reflect the second order rate change of the likelihood function with respect to K (see Evanno et al. 2005); generally, ΔK cannot be calculated for K = 1, and it could also not be calculated for K = 11 for our data set, as that was the maximal K used in our approach. Values in bold indicate that K = 3 is the most likely result.

K	Mean Ln (Pr)	SD	ΔK
1	−2376.54	7.076	-
2	−3020.93	1483.618	1.047
3	**−2264.93**	**37.006**	**22.674**
4	−2336.74	76.89	2.14
5	−2303.43	58.41	2.682
6	−2327.29	124.531	1.488
7	−2374.9	85.409	2.663
8	−2403.43	153.908	1.852
9	−2527.27	325.289	1.361
10	−2459.12	154.234	2.408
11	−2542.97	219.175	-

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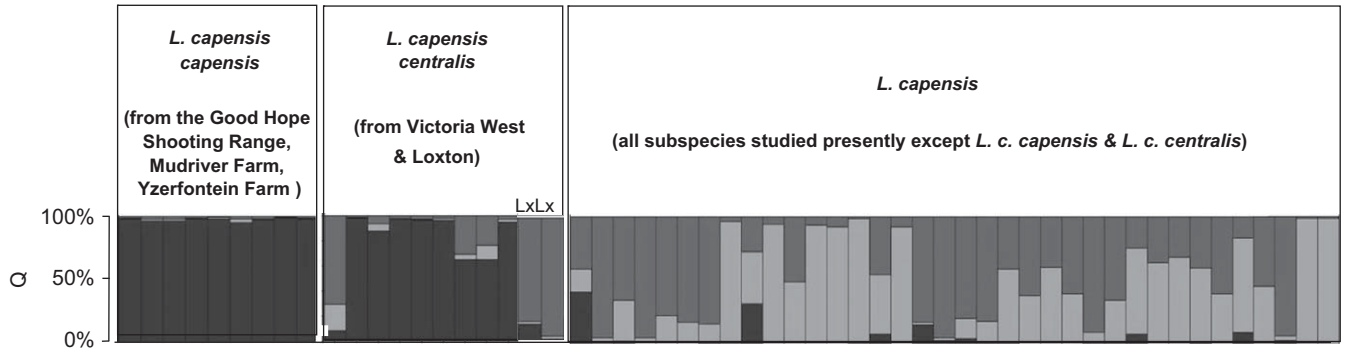

Fig. 7. STRUCTURE analysis of microsatellite data of South African cape hares. Proportions (Q) of the K = 3 populations as inferred from the applied admixture model (see Table 6) for each individual. Lx = Loxton. Embryos not included in the analysis.

The genetic STRUCTURE analysis yielded k = 3 most likely populations underlying the revealed overall genotypic variability. Table 6 lists the ΔK values for k = 2-10 populations and Fig. 7 displays the k = 3 population fractions (Q) for each individual. It indicates that all hares of *L. c. capensis* (i.e., "*L. capensis*" sensu Palacios et al. 2008) have very similar compositions of the three Bayesian population fractions compared to those of most hares of *L. c. centralis* (i.e., "*L. centralis*" sensu Palacios et al. 2008 from Victoria West and Loxton). Most hares from the other localities/regions are less similar to the hares from Victoria West and Loxton or from the Darling region, but some have nevertheless genotype compositions similar to those of the hares from Victoria West and Loxton. Thus, apparently the range of *L. c. centralis* represents a transition zone of the nuclear gene pool linking *L. c. capensis* and *L. c. centralis* on the one hand with *L. c. vernayi*, *L. c. ochropus*, *L. c. kalaharicus*, *L. c. ermeloensis* (as well as *L. c. hartensis* and *L. c. bedfordi*) on the other. There was, however, only a tendency (Mantel test: z = 1589.47; p = 0.053) towards slightly increased numbers of mutation steps in the HV1 sequences (as obtained from the MJ network) with increased CSE distances in pairwise comparisons between individuals within that zone of nuclear gene pool transition. For the set of "less variable loci" we obtained k = 1 as the most likely result of population structuring, and for the set of "highly variable loci" we obtained k = 2. This confirmed our conclusion of no serious influence of homoplasy on our results of genetic differentiation.

The spatial autocorrelation analysis based on pairwise Gregorius (1978) distances yielded slightly lower ($p < 0.001$) distances than expected by chance only in the lowest spatial distance class and the observed overall steady increase of Gregorius distances with increasing spatial distance classes was, however, not significant (distogram and associated p-values not shown). Accordingly, the Mantel test did not reveal a significant (z = 19.68, r = −0.045, p = 0.569) correlation between ln-transformed geographic distances and $F_{ST}/(1\text{-}F_{ST})$ values. Based on the genotypes of all thirteen microsatellite loci, GENECLASS correctly assigned all hares to the respective subspecies with the Bayesian approach of classification, whereas three individuals (one *L. c. vernayi* and two *L. c. ochropus*) were miss-classified to *L. c. kalaharicus* with the distance-based method.

The tests for signals of reduction of effective population size (i.e., genetic bottleneck) in the recent past of the populations yielded the following results for the I.A.M. and the T.P.M.: for *L. c. capensis* p = 0.017 (sig.)/p = 0.170 (n.s.); for *L. c. centralis* p = 0.554 (n.s.)/ p = 0.916 (n.s.); for *L. c. vernayi* p = 0.055 (n.s.)/ p = 0.122 (n.s.); for *L. c. ochropus* p = 0.041 (n.s.)/ p = 0.0633 (n.s.).

The PCO analysis resulted in 23 dimensions necessary to fully explain the structure of the matrix of pairwise CSE distances between individuals. Scattergrams of individual PCO scores are presented for the first eight dimensions in Fig. 8. Only the individual scores for the second PCO-dimension (explaining 10% of the matrix structure) differed significantly between the two subspecies *L. c. capensis* and *L. c. centralis* sensu Roberts (1951); D2: d.f. = 1, F = 35.546, $p < 0.0001$, GLS. For the comparison between "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008), individual PCO scores for the first and second dimensions (explaining together 25.7% of the matrix structure) were significantly different (dim. 1: d.f. = 1, F = 123.04, $p < 0.0001$; dim.2: d.f. = 1, F = 23.2404, $p < 0.0001$, GLS). The Mantel test for the matrices of pairwise CSE distances and mean HKY85 distances between the subspecies did not reveal a significant correspondence (z = 0.871; p = 0.07692).

Discussion

The currently studied South African hares matched either the "*Lepus capensis*" or the "*L. centralis*"-

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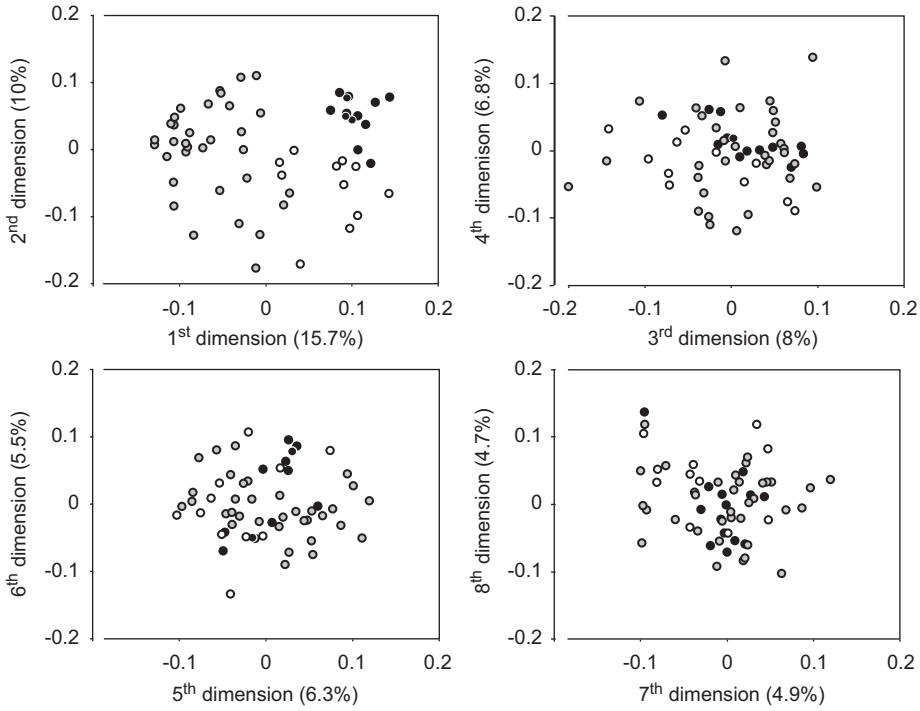

Fig. 8. Bivariate plots of individual principal coordinate scores for the first eight dimensions of the PCO of the CSE chord distance matrix based on microsatellite allele frequencies. White circles = *L. c. capensis* (= *L. capensis* acc. to Palacios et al. 2008), black circles = *L. c. centralis* (= *L. centralis* acc. to Palacios et al. 2008), and grey circles = all other subspecies of *L. capensis* (= *L. centralis* acc. to Palacios et al. 2008).

phenotypes as described by Palacios et al. (2008), as far as we could judge from our somewhat limited phenotypic analyses based on fur patterns (particularly the extension of the ventral white parts), dental, and non-metric skull characters. They were clearly distinct from scrub hares (*L. saxatilis*), which we have also collected by chance in the course of this study, and which were silver-grey with black intermingled on the back and white ventrally, with a relatively sharp demarcation on the flanks. Scrub hares from the Western Cape had also a brownish orange nape compared to the smoky grey of cape hares. As for the two phenotypes "*L. capensis*" and "*L. centralis*" that Palacios et al. (2008) provisionally considered full species, we had the impression that their morphological distinction was not so strict, as we found some few hares with characteristics of both phenotypes, or with single morphological characters of intermediate phenotype (to be published elsewhere). A larger sample of hares particularly from the contact/overlap/transition zones of the two phenotypes (see Palacios et al. 2008) should allow a better conclusion. For some of our currently studied specimens no skulls and skins were available and no phenotypic approval was possible. Those cases, however, concerned specimens from ranges of diverse cape hare subspecies according to Roberts (1951), well away from the range described for "*L. capensis*" sensu Palacios et al. (2008). In two further hares the shape of the enamel groove of the principal upper incisors was somewhat intermediate between those described for *L. capensis* and *L. saxatilis* (Robinson 1986). Nevertheless, the molecular characteristics of all those specimens identified them as *L. capensis*-type hares.

Our molecular data unequivocally demonstrate that all currently studied hares from South Africa belong to a single species, i.e., *Lepus capensis* Linnaeus, 1758 under a (strict or relaxed) Biological Species Concept. Both our mitochondrial (mt) and nuclear (microsatellite) gene pool data indicate relatively close overall gene pool relationships among all of them, and particularly the microsatellite data indicate at least some gene flow between most of the individuals, sampling regions, or subspecies. The comparison of our mtHV1 sequences with a huge number of sequences of different *Lepus* species downloaded from GenBank revealed monophyly for all currently studied cape hares from southern Africa. However, whereas the overall structuring of the not expressing (neutral) nuclear genome – as reflected by microsatellite variation – was not particularly pronounced across the whole study area, the HV1 lineages did show quite a remarkable overall differentiation. But the pattern of sequence differentiation was not concordant with the partitioning into the two morpho-species "*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008). In fact, we did not find a significant partitioning of sequence variation due to the two taxa

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"*L. capensis*" and "*L. centralis*" sensu Palacios et al. (2008), whereas partitioning due to the six *L. capensis* subspecies considered presently is significant. Nevertheless, sequence variation is definitely mostly due to variation within regional populations (i.e., within subspecies). Our results, however, leave open, whether or not there is some assortative mating for certain nuclear genes involved in the expression of the two phenotypes described by Palacios et al. (2008) particularly in the overlap zone where both phenotypes are observed.

Our local *L. c. capensis* (Darling region) and *L. capensis centralis* (Victoria West/Loxton region) samples exhibit some divergence in their mitochondrial gene pool, as indicated by all our three tree-building procedures and the network analysis. No *L. c. capensis* haplotype occurs in the *L. c. centralis* sample and *vice versa*. But the level of divergence in terms of inferred mutation steps separating these two regional phylogroups is relatively low, compared particularly to the haplotype divergence within *L. c. centralis*. And it is of the magnitude of phylogenetic breaks commonly observed within terrestrial mammal species, such as the brown bear (*Ursus arctos*) in Romania (Zachos et al. 2008). An inclusion of samples from localities between the currently studied Darling and Loxton/Victoria West regions might result in an even smaller evolutionary gap between *L. c. capensis* and *L. c. centralis*. On the contrary, a definitely large phylogeographic break is evident between *L. c. capensis* and *L. c. centralis* on the one hand and all other currently studied subspecies on the other. This is concordanly demonstrated by all tree-building procedures and the network analysis. Such a strong mtDNA-based phylogeographic division between cape hares from south-western South Africa on the one hand and from northern/central and north-eastern parts of southern Africa on the other has already been reported by Kryger (2002).

Interestingly, most regions (subspecies) within the central/northern/north-eastern phylogroup reveal hares with both closely and very distantly related haplotypes. This pattern is in line with the spatial autocorrelation analysis of the whole sequence data set. The significant negative Moran's statistic across the whole range of spatial distance classes indicates that dissimilar haplotypes occur not only across larger geographic distances but also to some degree in relatively close vicinity. For the larger geographic distances this can be mainly attributed to increased phylogenetic differentiation. For the shorter dististances it is obviously due to both large phylogenetic divergence within several local populations and the high haplotype idiosyncrasy (see also e.g. Ben Slimen et al. 2008a for one South African *L. capensis* population and Sert et al., in press for *L. europaeus* in Anatolia), because our autocorrelation approach was based on identical vs. non-identical haplotypes. It may suggest a mixture of deep and shallow genealogical structures on the large geographical scale, with some regional partitioning, reflecting either retention of old phylogenetic lineages in regional populations (i.e., shared ancestral polymorphism) or long-distance dispersal of haplotypes over many generations, or both. In fact, this central/north-eastern phylogroup corresponds more or less to a combination of phylogeographical category II for mammals (Avise 2000), namely a deep gene tree with (major) lineages occurring broadly sympatrically, and category III, a shallow gene tree with allopatric lineages. Overall, this pattern can be interpreted as a result of long-term high effective population size and high gene flow (at least during some periods of time) and/or maintenance of shared ancestral polymorphism, and a more recent period of somewhat reduced gene flow with the chance of diversification due to drift or regional selection, particularly in the south-west, the range of *L. c. capensis*. The phylogenetic position of *L. c. capensis* haplotypes together with their significantly lowered level of pairwise HKY85 distances, suggest that this subspecies might represent a phylogenetically relatively young offshoot of a large and widely distributed cape hare gene pool that has invaded the south-westernmost part of South Africa only recently in evolutionary terms. This area encompasses by and large the Fynbos Biome, dominated by small-leaved, evergreen scrubs whose regeneration is intimately related to fire. Before the Late Glacial Maximum (ca. 18.000–20.000 ybp) this area was covered with more extensive woodland and afterwards as well, whereas vegetation has apparently become more open, with more grassland (see eg., Mithen 2003; Mucina and Rutherford 2006) – thus more favourable for cape hares – only later in the Holocene. Particularly the relatively low nucleotide diversity (π) and the significant signal for a bottleneck in the microsatellite data set are not incongruent with a scenario suggesting a relatively recent immigration into the current range of *L. c. capensis*, possibly accompanied by a founder effect (see e.g., Valdiosera et al. 2007 for brown bears in Europe). To substantiate such a phylogeographic hypothesis, a more comprehensive sample is needed, however, specifically from south-western South Africa.

The currently uncovered overall phylogeographic pattern of cape hares is in contrast with that found in Smith's red rock rabbit (*Pronolagus rupestris*), which shows marked divergence into two phylogeographic blocks, separating a north-central range from all other occurrences in South Africa (Matthee and Robinson 1996). It is also not fully congruent with the phylogeographic pattern in scrub hares (*L. saxatilis*) from southern Africa, which constitutes of three phylogeographic assemblages, a northern, a central, and a south-western (Kryger et al. 2004). These incongruities for

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three leporid species in southern Africa might indicate different evolutionary histories due to different ecological requirements of the species in the course of the climate-driven changes of vegetation types in the late Pleistocene (see e.g., Mithen 2003, Mucina and Rutherford 2006 for overview).

The diversity of the (not expressed) nuclear genome is apparently very high in the currently studied cape hares, given the overall high number of alleles found at the thirteen microsatellite loci. But in spite of the concordantly high number of private alleles found, which may be viewed as indicating some (recent) regional differentiation, the overall level of geographical partitioning of the nuclear genome is relatively low. This is indicated by both the F-statistics, which yielded F_{ST} values within the range found e.g., for brown hares (*L. europaeus*) (Ben Slimen et al. 2008b) or among mountain hares (*L. timidus*) (Hamill et al. 2006) across large parts of Europe. It is also indicated by our AMOVA models, which show that only a small portion of relative genetic variability is due to partitioning among subspecies. Correspondingly, none of the six microsatellite loci employed by Kryger (2002) revealed significant differences in allele frequencies between the south-central and northern populations, which she established on the basis of mtDNA sequences; and overall genetic differentiation between those two mitochondrial populations was not very pronounced (Kryger 2002). Accordingly, our coalescence model based migration analysis revealed at least some gene exchange between many pairs of subspecies; albeit with some cases of asymmetric gene flow. The comparison of the results derived from the set of "highly variable loci" with that of "relatively little variation" indicates that our conclusions are not flawed by a serious homoplasy problem for the microsatellite data. This has already been concluded from a very similar set of microsatellite loci in a study on brown and cape hares (*L. europaeus* and *L. capensis*) by Ben Slimen et al. (2008b).

The Basian STRUCTURE analysis conforms in essence with our conclusion of relatively shallow differentiation in the nuclear gene pool of South African cape hares. One result is, however, remarkable, namely that, in discordance with the pattern of mtDNA differentiation, the range of our *L. c. centralis* sample (i.e., Karoo: Loxton and Victoria West) obviously forms to some extent a transition zone in the nuclear gene pool. But levels of nuclear and mitochondrial differentiation between pairs of individual hares in this transition zone are only tentatively positively related. While some hares from this area are very similar in their microsatellite composition to all hares of *L. c. capensis*, some are similar to hares of diverse subspecies of central and north-eastern South Africa. Still some others are intermediate, thus linking *L. c. capensis* and the block of the other subspecies (*L. c. kalaharicus*, *L. c. hartensis*, *L. c. ochropus*, *L. c vernayi*, *L. c. ermeloensis*, *L. c. bedfordi*). Actually, this pattern is concordant with the above stated hypothesis of *L. c. capensis* representing a phylogenetically relatively young offshoot of a big long-term gene pool in more central/north-eastern parts of southern Africa. These STRUCTURE results are obviously not seriously flawed by geographically interdependent samples (see Pritchard and Wen 2003), as we did not find a significant spatial autocorrelation, nor a significant "isolation-by-geographic distance pattern" in the microsatellite data. Hence, in accordance with the perfect Bayesian assignment of all hares to their respective subspecies – which demonstrates the suitability of our marker system to unravel the underlying genetic structure – we are quite confident that our STRUCTURE results do indeed reflect genetic cohesiveness of all the currently studied cape hares via a supposed nuclear transition area in the Karoo (i.e., samples from Victoria West and Loxton).

According to O'Brien and Mayr (1991) "subspecies are groupings of populations, within a species, that share a unique geographic range or habitat and are distinguishable from other subdivisions of the species by multiple, independent, genetically based traits". Our data on nuclear gene pool relationships are not fully consistent with the phenotypic subspecies grouping established by Roberts (1951): e.g., all *L. c. capensis* individuals share a common nuclear gene pool with some of the *L. c. centralis* hares, whereas some hares of *L. c. centralis* share a common gene pool with some indivduals of various subspecies from more central and north-eastern regions in South Africa. Hence, *L. c. centralis* would not be a "good subspecies" in the sense of O'Brien and Mayr (1991), and in the sense of Wiley (1981) and Frost and Hillis (1990), who suggested that the taxonomy of a group should be consistent with its evolutionary history. In terms of phylogeographic-historical aspects, particularly the central and north-eastern subspecies are even more problematic when matching them to particular mitochondrial and nuclear gene pool units. Overall, our results suggest an only superficial correspondence of patterns of phenotypic and molecular divergence for South African cape hares. Apparently, the phenotypically well separated *L. c. capensis* from the Fynbos Biome in the Western Cape shows a relatively shallow divergence from the *L. c. centralis* hares from the Succulent and Nama Karoo. Establishing "syntypes" of *L. capensis* from the skeletal material of *L. c. capensis* by Palacios et al. (2008) to provide a basis for future morphological work on African hares is problematic: as to our understanding of the international rules of zoological taxonomy, establishing "syntypes" is not possible; only a "neotype" could be considered (e.g., Winston 1999; Kraus 2000). We agree that such a possible "neotype" should be chosen from the available material of *L. c. capensis*.

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Any of the ''Interbreeding Species Concepts'' (biological-, genetic-, recognition-, reproductive competition-, generic-, cladistic- or ''Hennigian'' species concepts; see e.g., Lee 2003; Coyne and Orr 2004, Baker and Bradley 2006) delimits the presently studied hares to a single species (i.e., *L. capensis* L., 1758), contrary to a ''Similarity Species Concept'', such as a ''Phenetic Species Concept''. Supposedly, Palacios et al. (2008) have used their morphological distinction together with their chorological data not only in the context of a ''Phenetic Species Concept''; they rather may have used it as a proxy of reproductive isolation between ''*L. capensis*'' and ''*L. centralis*'', which would correspond to an ''Interbreeding Species Concept''. If that was the case, our molecular results indicate that their morphological character system is too fine-grained to refine species divergence in the *L. capensis* complex. It may, however, allow discrimination of major (morphological) populations. But our specimens from the Darling and Loxton/Victoria West regions, which were assigned either to *L. c. capensis* or *L. c. centralis* (or ''*L. capensis*'' and ''*L. centralis*'' sensu Palacios et al. 2008), indicate no particularly pronounced mitochondrial divergence, relative to the divergence from more central, northern, and north-eastern subspecies, and no relevant nuclear divergence as estimated by the microsatellite data set. Hence no good concordance between molecular and morphological divergence patterns seems to exist in this region. Rather, a notable – but also little in absolute terms – nuclear gene pool transition appears to occur in the southwestern Karoo; i.e. within the Loxton/Victoria West area (within *L. capensis centralis*). On the other hand a deep phylogenetic gap in the maternal genome appears to occur in the central Karoo between *L. c. centralis* and *L. c. hartensis*/*L. c. vernayi*, as concluded from the phylogeographic pattern of HV1 haplotypes. This, together with the fact that there is relatively little overall microsatellite differentiation, may suggest a zone of secondary contact of two intraspecific gene pools that had been separated for quite a period of time during the late Pleistocene/early Holocene. Further samples particularly from the southwestern Karoo should allow testing this hypothesis.

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