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Variation of partial transferrin sequences and phylogenetic relationships among hares (*Lepus capensis*, Lagomorpha) from Tunisia

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Abstract North African hares are currently included in cape hares, *Lepus capensis* sensu lato, a taxon that may be considered a superspecies or a complex of closely related species. The existing molecular data, however, are not unequivocal, with mtDNA control region sequences suggesting a separate species status and nuclear loci (allozymes, microsatellites) revealing conspecificity of *L. capensis* and *L. europaeus*. Here, we study sequence variation in the intron 6 (468 bp) of the transferrin nuclear gene, of 105 hares with different coat colour from different regions in Tunisia with respect to genetic diversity and differentiation, as well as their phylogenetic status. Forty-six haplotypes (alleles) were revealed and compared phylogenetically to all available TF haplotypes of various *Lepus* species retrieved from GenBank. Maximum Likelihood, neighbor joining and median joining network analyses concordantly grouped all currently obtained haplotypes together with haplotypes belonging to six different Chinese hare species and the African scrub hare *L. saxatilis*. Moreover, two Tunisian haploypes were shared with *L. capensis*, *L timidus*, *L. sinensis*, *L. yarkandensis*, and *L. hainanus* from China. These results indicated the evolutionary complexity of the genus *Lepus* with the mixing of nuclear gene haplotypes resulting from introgressive hybridization or/and shared ancestral polymorphism. We report the presence of shared ancestral polymorphism between North African and Chinese hares. This has not been detected earlier in the mtDNA sequences of the same individuals. Genetic diversity of the TF sequences from the Tunisian populations was relatively high compared to other hare populations. However, genetic differentiation and gene flow analyses (AMOVA, F_{ST}, Nm) indicated little divergence with the absence of geographically meaningful phylogroups and lack of clustering with coat colour types. These results confirm the presence of a single hare species in Tunisia, but a sound inference on its phylogenetic position would require additional nuclear markers and numerous geographically meaningful samples from Africa and Eurasia.

Keywords *Lepus* · Tunisia · Transferrin · Phylogenetics · Genetic diversity · Coat colour

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Introduction

Hares (genus *Lepus*) from Africa display a bewildering number of external phenotypes, particularly in terms of body size and coat colour, even within relatively small ranges. Such phenotypical characteristics have traditionally been used in systematics and taxonomy along with skull and dental characters, but due mainly to large intraspecific variation and often little interspecific differences such characters have resulted in problematic systematic classifications. Even despite quite intensive molecular phylogenetic analyses of hares (e.g., Halanych and Robinson 1999; Pierpaoli et al. 1999; Wu et al. 2000; Alves et al. 2003; Yamada et al. 2002; Waltari et al. 2004; Kasapidis et al.

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2005; Waltari and Cook 2005; Wu et al. 2005; Alves et al. 2003, 2006; Ben Slimen et al. 2006, 2008a, b; Melo-Ferreira et al. 2005, 2009, 2012), evolutionary relationships among many African forms are still unclear. All hares from Africa are currently considered belonging to only a few species (e.g., Angermann 1965, 1983; Flux 1983; Flux and Angermann 1990; see also Wilson and Reeder 1993; Pérez-Suárez et al. 1994; Nowak 1999; Hoffmann and Smith 2005).

In North Africa, hares are conventionally considered as forms of the geographically widespread and phenotypically very variable cape hare, *L. capensis* L., 1758, that has been described originally from the Cape of Good Hope in South Africa (e.g., Flux and Angermann 1990, see also Ben Slimen et al. 2005, 2006, 2008a, b; Suchentrunk et al. 2009). However, based on mitochondrial (mt) DNA sequences, hares from some parts of North Africa and Sardinia were tentatively considered a separate species ("*L. mediterraneus*") (Pierpaoli et al. 1999; Alves et al. 2003; Ben Slimen et al. 2007; Ben Slimen 2008). Contrary, allozyme, mtDNA PCR–RFLP and microsatellite data (Ben Slimen et al. 2005, 2006, 2008a, b) of hares from Tunisia, northern Morocco and Egypt suggested conspecificity with nominal cape hares from the South African Cape Province. Moreover, those latter results suggested close nuclear gene pool (allozymes, microsatellites) relationships with central European brown hares (*L. europaeus* Pallas, 1778), and supported Petter's (1959, 1961) hypothesis of all North African forms (except for one isolated occurrence of *L. victoriae* in NW Algeria; see also Flux and Angermann 1990) and brown hares belonging to *L. capensis* L., 1758 (see also Petter 1972). The current taxonomic uncertainties within the genus *Lepus* might result from complex evolutionary scenarios for many hare taxa, such as occasional (or multiple) episodes of introgressive hybridization and/or shared ancestral polymorphism. Indeed, recent studies (Melo-Ferreira et al. 2011, 2012; Liu et al. 2011; Alves et al. 2003, 2008) have reported introgression among European, North American, and Chinese hare species. Although introgression concerned mainly mtDNA, signals of reticulate evolution have also been demonstrated to occur in the nuclear genome of various hare species (e.g., Thulin et al. 2006; Melo-Ferreira et al. 2014a), including African taxa (Gebremariam 2013).

In Tunisia, hares occupy the entire country including the islands of Djerba and Kerkennah (Cheniti 1995). From the northern humid to the southern arid regions, hares are characterized by phenotypic variation in body size, pelage colour and ear length (Petter 1961; Ben Slimen et al. 2005, 2006, 2008a). In spite of being protected by strict hunting regulations, this species is increasingly threatened by poaching, extensive monocropping, and overgrazing. Notably, except for their occurrence in protected areas, no

other plans have so far been taken by authorities to preserve this game species; there are for instance no restocking operations using hares from breeding stations and to our knowledge introduction of allochthonous hares into the wild has never occurred in Tunisia.

The analysis of mt control region (CR-1) sequences of Tunisian hares (Ben Slimen et al. 2007; Ben Slimen 2008) revealed a monophyletic pattern for the obtained haplotypes, with the absence of sequences at unexpected phylogenetic positions that would otherwise have indicated introgressive hybridization and/or shared ancestral polymorphism. The hypothesis of a separate species status of the hares from Tunisia (North Africa) different from *Lepus capensis* sensu lato rests exclusively on mtDNA data (Ben Slimen 2008; see also Suchentrunk et al. 2008), whereas allozyme and microsatellite data are not incongruent with the hypothesis of conspecificity with the cape hare.

In this work, we investigated the genetic diversity and the population structuring of nuclear transferrin (TF) sequences in hares from Tunisia and intended to assess the phylogenetic relationships of Tunisian hares with different *Lepus* species. We were particularly interested in the geographical or phenotypical structuring of nuclear sequence variation, and addressed the following questions: (1) do the phylogenetic relationships of these nuclear sequences with those of other hare species confirm the earlier results obtained from mtDNA sequences, or is the phylogenetic position of Tunisian hares as obtained by the TF sequences rather congruent with other nuclear markers (allozymes, microsatellites)? and (2) is there any evidence of introgressive hybridization or shared ancestral polymorphism in the TF sequences of hares from Tunisia?

Materials and methods

Samples

A total of 105 hares were collected by hunters between 2003 and 2006 at eleven locations in Tunisia across a distance of ca. 450 km between the northern Mediterranean seaboard with Mediterranean climate and high annual rainfall (ca. 800 mm) and the arid northern parts of the Sahara desert with less than 100 mm annual rainfall. Localities and sample size of these specimens are shown in Fig. 1. All hares matched in essence the description of forms conventionally considered belonging to *Lepus capensis* L., 1758, according to their external phenotypes, morphometric skull characters, and dental traits (see e.g., Petter 1959, 1961; Osborn and Helmy 1980; Robinson 1986; Flux and Angermann 1990; Suchentrunk and Davidovic 2004). The analyzed hares displayed four colour types: grayish brown (GB) in northern and central Tunisia;

Fig. 1 Sample locations of hares from Tunisia. Sample sizes are given in *parentheses*. *STH* Sidi Thabet, *BEJ* Beja, *NAD* Nadhour, *WES* Weslatia, *KAL* Kalâa, *BKL* Bekalta, *CHE* Cherarda, *SND* Sned, *DOU* Douz, *TAT* Tataouine, *BGD* Ben Guerdène
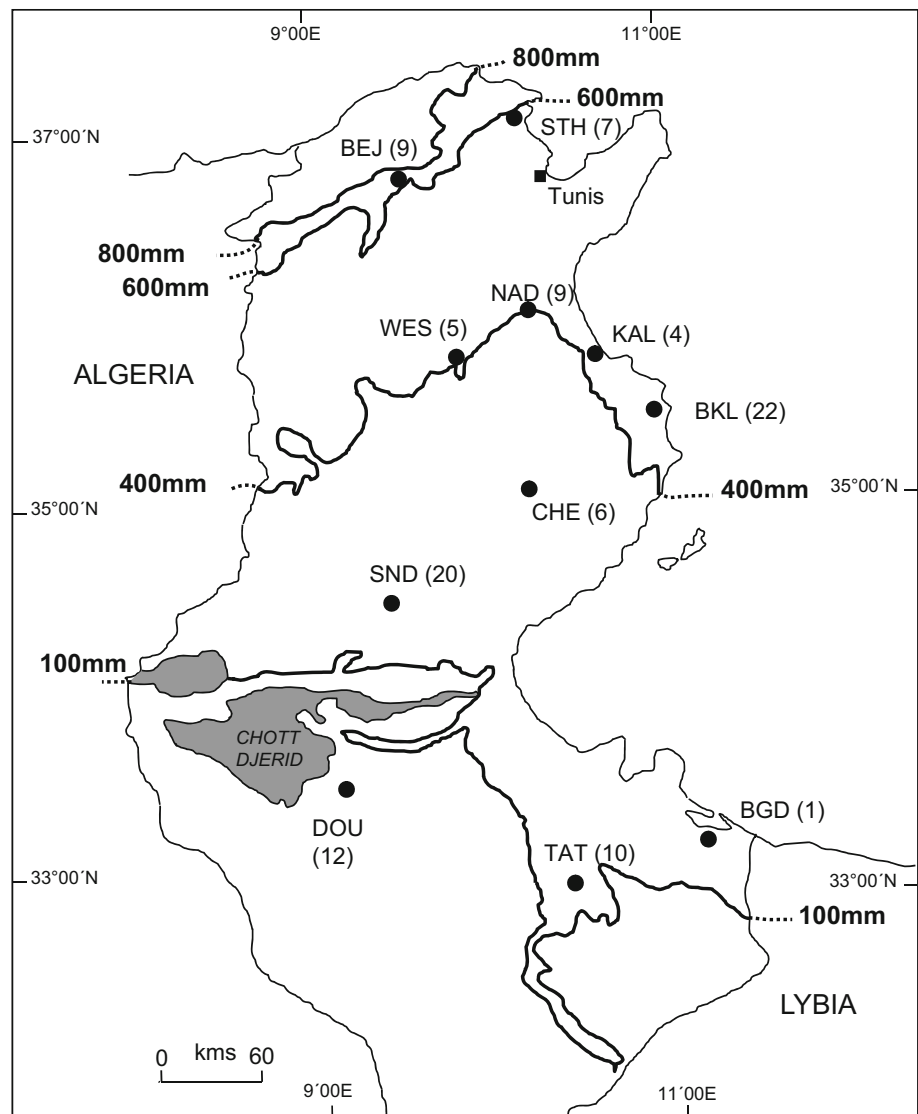
yellowish brown (YB) and yellowish (Y) in central Tunisia or yellowish pale (YP) in southern Tunisia. Collected hares were grouped into three populations: the North (N) population with samples from BEJ and STH; the Central (C) population with samples from NAD, WES, KAL, BKL, CHE and SND; and the south (S) population with samples from DOU, TAT, and BGD.

DNA amplification and sequencing

Total DNA was extracted from diverse tissues (liver, skeletal muscles, ear cartilage, tongue) using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Polymerase Chain Reaction (PCR) and direct sequencing of forward and reverse strands of a 474 bp segment of the transferrin (TF) gene (between exons 6 and 7) were performed for all 105 specimens from Tunisia following Alves et al. (2003).

Data analysis

Nuclear DNA polymorphism

Sequences were visually inspected and aligned using the BioEdit 7.2.5 program (Hall 1999). We used Phase 2.1.1 (Stephens et al. 2001; Stephens and Donnelly 2003) to reconstruct haplotypes (alleles) for the nuclear sequences. Five replicate runs of 1000 generations after 1000 generations of burn-in were performed.

The program DnaSP version 4.1 (Librado and Rozas 2009) was used to estimate the following indices of DNA polymorphism for the different populations: haplotype

diversity (h), nucleotide diversity (π), and mean number of pairwise differences (k).

Partitioning of genetic variation and population differentiation

The amount of variation due to partitioning into the presently considered populations was calculated by an AMOVA model using Arlequin 3.5 (Schneider et al. 2000). A second AMOVA model was used to test for significant partitioning of sequence variability associated with the four colour types. Due to low sample size in some sampling localities, a second AMOVA with "reduced data set 1" (including only the following sampling localities: BEJ, NAD, BKL, SND, DOU and TAT) was also conducted for the two models described above. The significance of these statistics was tested using 10,000 randomized permutations. In addition, we calculated overall and pairwise F_{ST} values for the geographic and the colour populations.

Coalescent theory based maximum likelihood estimates for migration rates (Beerli and Felsenstein 2001) among populations were calculated with the program MIGRATE Version 3.3 (Beerli 2006) that applies a Markov chain Monte Carlo approach. MIGRATE calculates a maximum likelihood estimate of Mm between populations using a coalescent approach, relaxing the traditional assumption of symmetrical migration. In a second approach, the pairwise number of migrants was estimated by $Nm = (1 - F_{ST})/4*F_{ST}$ (Wright 1969) with the program GENETIX v. 4 (Belkhir 1999). Estimates of Nm for the islands model are based on the assumptions that all populations were initially colonized independently from a common source population, that gene flow is random among populations, and that populations have reached a gene flow-drift equilibrium.

Mismatch distribution

We examined the historical demography of the Tunisian hares by calculating the "mismatch distribution" of the sequences, which represents the frequency distribution of pairwise differences among all haplotypes, and tested its fit to a Poisson distribution by Monte Carlo simulations of 10,000 random samples using Arlequin 3.5 (Schneider et al. 2000). The observed distribution of pairwise genetic differences between haplotypes within a population allows distinction between different demographic histories. Stable or slowly declining populations at demographic equilibrium generate a multimodal distribution reflecting the stochastic shape of the underlying gene trees. Populations that went through a recent sudden bottleneck or expansion produce a unimodal distribution (Rogers and Harpending 1992).

Phylogenetic analysis

Phylogenetic analysis of new sequence data requires the inclusion of all (reliable) sequences available for alignment. This is particularly necessary in the genus *Lepus*, due to its relatively recent radiation, the shallow evolutionary lineage divergence, and the high likelihood of reticulate evolution. Thus, for the phylogenetic analyses, we included all TF sequences available from GenBank that could be aligned to our sequences, encompassing 391 bp of the TF gene. However, sequences with considerable number of ambiguous nucleotide positions "N" were not used. Accession numbers of all sequences used downloaded from GenBank, associated taxonomic information and geographic origin are listed in Table S1 (Supplemental Material). Sequences with more than one heterozygous position were analyzed with Phase 2.1.1 (Stephens et al. 2001; Stephens and Donnelly 2003) to reconstruct haplotypes (alleles). All our analyses were based on resolved haplotypes.

The fit of our locus alignment to 88 models of sequence evolution was assessed using the computer program jModelTest v0.1 (Posada 2008) and the Akaike information criterion. The best fit model was the GTR + I+G (shape = 0.6510). The neighbor joining (NJ) and the maximum likelihood (ML) analysis were performed with the option cited above using Mega 6 (Tamura et al. 2013). Statistical support of internal nodes was calculated with 1000 bootstrap repetitions.

Since variation within the nuclear locus was low, a second phylogenetic analysis was performed using the species-tree inference methodology (Heled and Drummond 2010), as implemented in BEAST v1.6.1 (Drummond and Rambaut 2007).

In BEAST, the assignment of specimens to taxa must be given as a prior for the analysis. The input file for BEAST was created using the application BEAUti, part of the package, and partitions and models were edited by hand to fit the models determined by jModeltest. Posterior phylogenies were determined in BEAST using an uncorrelated lognormal relaxed clock (Drummond et al. 2006) and the Yule tree prior. Three replicate runs of 50 million generations were performed, sampling trees and parameter estimates every 5000 generations.

As alternative phylogenetic approach, relationships between haplotypes were determined using Median-Joining networks (Bandelt et al. 1999). Contrary to tree-based inference, this approach enables to uncover possible alternative phylogenetic pathways for haplotypes (i.e., depiction of evolutionary relationships among haplotypes are not reduced to bifurcating events); this may facilitate inference on the presence of shared ancestral polymorphism or recent introgression, particularly for clusters with

low bootstrap support. Such networks may be particularly useful to infer intraspecific evolutionary patterns, and if reticulate evolutionary scenarios are anticipated (Moulton and Huber 2009; Hall 2011; Morrison 2012). In these analyses, rabbit (*Oryctolagus cuniculus*) and Eastern cottontail (*Sylvilagus floridanus*) sequences (Supplemental Table S1) were used as outgroups.

In a second analysis, only the Tunisian sequences were considered and one *L. granatensis* sequence (AY176249) and a single *L. europaeus* sequence (AY176259) were included as outgroup taxa. The best-fit model for this analysis was TPM2uf + I. The NJ and ML analyses were performed with this model using PAUP (Swofford 2001). The ML analysis was run with heuristic ML searches using TBR branch swapping and nodal support estimates by using non-parametric bootstrapping and restriction to 100 pseudo-replicates.

Results

Polymorphism of nuclear DNA

The TF sequences produced an alignment of 468 bp with 31 positions (6.6 %) being polymorphic of which 20 (4.27 %) were singletons. Among all sequences, 8 were homozygous and 9 were heterozygous at single position. Among the 88 heterozygous sequences at more than one position, the allelic pairs were determined with 100 % of probability in 75 % of all cases (Supplemental Table S2). The lowest probabilities ranged between 50 and 60 % in four cases. In each of these heterozygous cases, one allele was present more than once with a second unique allele. Rare alleles constituted an important component of the molecular signature used to estimate several population genetic parameters, and so omitting genotypes in which rare alleles resided could affect phylogeographic analyses (Garrick et al. 2010). Therefore, we opted to keep the complete data set, including some low-probability phase calls. Moreover, Garrick et al. (2010) suggested that lowering the PHASE threshold to 60 % have little or no increase in false positives. Harrigan et al. (2008) suggested also that phase probability of 50 % were associated with unique polymorphisms and not with uncertainty, and that the PHASE method may outperform cloning at least when the latter is based on a limited number of clones.

A total of 46 different phased haplotypes (alleles) were detected in the 105 analyzed hares (Table 1). All phased haplotypes obtained in this study were deposited in GenBank (accession numbers KX146863–KX146908). The currently detected high allele number has led to high haplotype diversity (h = 0.955, Table 2) and a high level of population heterozygosity, which was 87.5 % in the N,

93.94 % in the C and 91.3 % in the S populations. Neutrality statistics were consistently non-significant (Table 2), confirming the assumption of selective neutrality of the investigated portion of the TF (intron) sequences.

Genetic diversity parameters were similar in the three considered geographic populations (Table 2). Overall haplotypic diversity was high (h = 0.955 ± 0.007), however nucleotide diversity ($\pi = 0.0073 \pm 0.0009$) and pairwise mean differences (k = 3.41,536) were relatively low.

In order to detect a possible effect of low phased sequences in our results, we decided to delete haplotypes obtained from these sequences and run, with this new "reduced data set 2", all analyses performed in this study. In total 14 individual sequences (six in N, five in C, and three in S) with phasing probabilities lower than 90 % were removed. The deletion of these individuals resulted in the loss of the following eleven unique haplotypes: TN20, TN25, TN29, TN30, TN34, TN38, TN39, TN40, TN41, TN43, TN46. In all cases, genetic diversity parameters estimated with the "reduced data set 2" were very similar to those reported for the data set including all haplotypes (Table 2), suggesting that in the present study haplotypes with ambiguous phase determination did not change inferences from our analyses.

Partitioning of genetic variation and population differentiation

Despite significant differentiation among populations, AMOVA models showed that variation occurred mainly within populations (Table 3). The results of the first AMOVA model, where populations were categorized according to their geographic origin, yielded a low contribution (1.31 %, not significant) of the among populations variance towards the total variance; this underscored the absence of differentiation among populations. Similarly, the second AMOVA model indicated a low degree of partitioning when the populations consisted of four colour types, with 1.6 % of the total variance being recorded among them. Similar AMOVA results were observed for the "reduced data set 1 and 2" (Table 3).

Overall F_{ST} between the three populations (0.017, SD 0.016) and the four colour morpho-types (0.016, SD 0.015) were not significant indicating absence of a genetic structure. Similarly, low pairwise F_{ST} values (Table 4) indicated relatively low genetic structure, although most values were significant, between the three geographic populations (F_{ST} range between 0.010 and 0.039). Low genetic structure was also recorded for the four morpho-types with F_{ST} values ranging from 0.007 between the GB and YP hares, and 0.056 between Y and YP hares. These findings were further corroborated by the estimates of pairwise numbers of

Table 1 Accession numbers, haplotype names, frequencies of the phased Tunisian TF sequences, and associated sampling localities and sample sizes (in parentheses) (see Fig. 1)

Accession numbers	Haplotype names	Frequencies	Sampling localities and sample size
KX146863	TN1	11	NAD (3), BKL (2), BEJ (1), SND (3), STH (1), TAT (1)
KX146864	TN2	5	NAD (1), KAL (1), BKL (1), STH (1), TAT (1)
KX146865	TN3	12	CHE (2), BKL (3), BEJ (6), SND (1)
KX146866	TN4	23	BEJ (1), BKL (6), NAD (2), CHE (4), WES (3), KAL (2), SND (2), STH (1), DOU (1), TAT (1)
KX146867	TN5	2	BKL
KX146868	TN6	3	BKL (2), SND (1)
KX146869	TN7	11	BKL (3), NAD (3), SND (2), KAL (2), BEJ (1)
KX146870	TN8	3	STH (1), SND (1), NAD (1)
KX146871	TN9	2	SND (1), DOU (1)
KX146872	TN10	7	WES (1), SND (5), DOU (1)
KX146873	TN11	14	STH (2), NAD (5), KAL (2), WES (2), BKL (3)
KX146874	TN12	17	BKL (4), SND (2), BEJ (1), BGD (1), DOU (9)
KX146875	TN13	13	NAD (2), BKL (2), BEJ (2), WES (1), SND (2), STH (1), DOU (1), TAT (2)
KX146876	TN14	13	CHE (2), SND (5), DOU (3), TAT (3)
KX146877	TN15	4	BKL
KX146878	TN16	3	STH (1), CHE (1), DOU (1)
KX146879	TN17	4	CHE (2), DOU (1), TAT (1)
KX146880	TN18	10	BKL (2), WES (2), SND (2), STH (1), BGD (1), TAT (1), DOU (1)
KX146881	TN19	5	BKL (2), SND (3)
KX146882	TN20	2	BEJ (1), KAL (1)
KX146883	TN21	6	BKL (2), SND (1), CHE (1), DOU (1), TAT (1)
KX146884	TN22	1	BKL
KX146885	TN23	5	BKL (1), DOU (1), TAT (3)
KX146886	TN24	1	BKL
KX146887	TN25	1	BKL
KX146888	TN26	1	BEJ
KX146889	TN27	8	BEJ (1), STH (2), BKL (1), NAD (1), SND (1), DOU (1), TAT (1)
KX146890	TN28	3	BKL (1), WES (1), SND (1)
KX146891	TN29	1	BEJ
KX146892	TN30	1	STH
KX146893	TN31	1	SND
KX146894	TN32	1	SND
KX146895	TN33	1	SND
KX146896	TN34	1	SND
KX146897	TN35	2	DOU (1), TAT (1)
KX146898	TN36	2	SND
KX146899	TN37	1	STH
KX146900	TN38	1	SND
KX146901	TN39	1	STH
KX146902	TN40	1	BEJ
KX146903	TN41	1	BEJ
KX146904	TN42	1	DOU
KX146905	TN43	1	TAT
KX146906	TN44	1	TAT
KX146907	TN45	1	TAT
KX146908	TN46	1	TAT

Table 2 Measures of genetic diversity in the Tunisian hare populations (N—North, C—Central, S—South): haplotype diversity (h), nucleotide diversity (π), mean number of pairwise differences (k). Results of neutrality tests

Populations (sample size)	Diversity indices			Neutrality tests		
	h	π	k	Tajima's D	Fu & Li's D*	Fu & Li's F*
N[1] (n = 16)	0.952	0.0067	3.131	−1.019	−0.482	−0.770
N[2] (n = 10)	0.921	0.0068	3.174	−0.717	−0.667	−0.791
C[1] (n = 66)	0.948	0.0073	3.399	−0.913	0.764	0.118
C[2] (n = 61)	0.945	0.0073	3.430	−0.851	0.727	0.129
S[1] (n = 23)	0.926	0.0070	3.276	−1.022	0.654	0.094
S[2] (n = 20)	0.924	0.0072	3.345	−0.955	0.628	0.117
Total[1] (n = 105)	0.955	0.0073	3.415	−1.102	0.222	−0.404
Total[2] (n = 91)	0.950	0.0074	3.457	−0.811	1.154	0.421

[1] Results are based on all phased haplotypes

[2] Results are based on phased haplotypes with probability >90 %

Table 3 Hierarchical analysis of molecular variance (AMOVA) of TF sequences among (A) Tunisian hare populations, as well as among (B) external phenotypes (coat colour types) for all and the "reduced data sets" (see Materials and methods); ns—not significant

	Hierarchy	df	% total variance	ϕ Statistic	P value
Geographic populations					
All data set	Among populations	2	1.31	ϕ_{CT} 0.013	ns
	Among sampling locations within populations	10	1.81	ϕ_{SC} 0.018	<0.05
	Within sampling locations	209	96.88	ϕ_{ST} 0.031	<0.001
"Reduced data set 1"	Among populations	2	1.81	ϕ_{CT} 0.018	ns
	Among sampling locations within populations	5	1.32	ϕ_{SC} 0.013	ns
	Within sampling locations	163	96.87	ϕ_{ST} 0.031	<0.001
"Reduced data set 2"	Among populations	2	1.51	ϕ_{CT} 0.015	<0.05
	Among sampling locations within populations	10	1.82	ϕ_{SC} 0.018	<0.05
	Within sampling locations	181	96.67	ϕ_{ST} 0.033	<0.001
Coat colour types					
All data set	Among coat colour types	3	1.60	ϕ_{CT} 0.016	ns
	Among sampling locations within colour types	13	1.72	ϕ_{SC} 0.017	<0.01
	Within sampling regions	209	96.69	ϕ_{ST} 0.031	<0.001
"Reduced data set 1"	Among coat colour types	2	0.76	ϕ_{CT} 0.008	ns
	Among sampling locations within colour types	5	2.37	ϕ_{SC} 0.024	<0.01
	Within sampling regions	163	96.86	ϕ_{ST} 0.031	<0.001
"Reduced data set 2"	Among coat colour types	3	4.27	ϕ_{CT} 0.043	<0.01
	Among sampling locations within colour types	13	1.6	ϕ_{SC} 0.017	ns
	Within sampling regions	181	94.13	ϕ_{ST} 0.059	<0.001

migrants (Nm) between geographic populations (Table 4), ranging between 6.51 (S–N) and 26.75 (N–C) individuals per generation. Similarly, the MIGRATE results showed cohesion of the nuclear gene pool between the N and C populations, with maximum likelihood estimates of individuals migrating per generation showing high bidirectional gene flow. The S population showed unidirectional gene flow with the absence of individuals migrating out of this population (Table 4). For F_{ST} values and Nm estimated with both methods similar results were obtained for the whole data set and the "reduced data set 2" (Table 4).

Mismatch distribution

Overall and single population observed mismatch distributions were unimodal and corresponded to the expected

Table 4 Pairwise F_{ST} values among Tunisian hare populations (upper diagonal left part of the table). Pairwise numbers of migrants according to Wright (1969) are shown in the left part of the table (lower diagonal). Numbers of migrating individuals as calculated by the MIGRATE program for either direction (the source population of migration is indicated in the first column and respective receiving populations are indicated in the line with the population acronyms N, C, S); values based in all haplotypes and reduced data set 2 are in upper and lower lines, respectively; * P < 0.01

	F_{ST} and Nm (Wright 1969)			Nm Likelihood method		
	N	C	S	N	C	S
N	–	0.010	0.039*	–	5980	2630
		0.015	0.040*		3590	1230
C	26.75	–	0.028*	4630	–	3370
	18.87		0.026*	7210		3880
S	6.51	8.83	–	0.000	0.000	–
	5.97	9.29		711.45	300	

Fig. 2 Maximum likelihood (ML) dendrogram of Tunisian haplotypes with all downloaded from GenBank (see supplemental Table S1). For better overview, only taxa names for clades are given. The *gray area* shows the Tunisian clade where names are indicated only for non-Tunisian haplotypes. Percentage of bootstrap values (1000 pseudo-replications) are given only for major clades for both NJ/ML analyses, if above 50 %

distributions generated under the sudden expansion model (Supplemental Fig. S1). However, goodness-of-fit statistics (sum of squared deviation SSD = 0.029, P < 0.001; Harpending's raggedness index R = 0.077, P < 0.001) was significant and allowed to reject the null hypothesis of a sudden population size expansion.

Phylogenetic analysis

The NJ, ML trees (Fig. 2), and the median-joining network (Fig. 3) of the Tunisian TF haplotypes plus all those downloaded from GenBank showed that Tunisian haplotypes are not monophyletic. Indeed, haplotypes belonging to seven different hare species from different regions around the world were grouped together with Tunisian hares. These species inlcude *L. capensis*, *L. hainanus*, *L. yarkandensis*, *L. sinensis*, *L. mandshuricus* and *L. timidus* from China and *L. saxatilis* from Mozambique (if savanna hares from Mozambique are considered conspecific with *L. saxatilis*). Haplotypes of these species do not show only similarity but some are identical to those detected in hares from Tunisia. Indeed, the TN8 haplotype is present in *L. yarkandensis*, *L. timidus* and *L. hainanus* and the haplotype TN9 is present in hares from China (*L. capensis* and *L. sinensis*). In addition, the haplotypes TN8, TN11 and TN24 are also found in cape hares from Morocco. However, the tree topologies were supported only by low bootstrap values.

The median-joining network (Fig. 3) showed that the maximum distance between Tunisian haplotypes was seven mutation steps, which was similar to intraspecific divergence recorded in *L. americanus* (eight mutation steps). Notably, these divergences were similar to the interspecific

distance recorded between *L. europaeus* and *L. timidus* (maximum of 9 mutation steps), or higher than that recorded between *L. sinensis* and *L. mandshuricus* (maximum of 4 mutation steps). The maximum distance between any two species was recorded between *L. californicus* and *L. americanus*, *L. europaeus*, or *L. granatensis* (21 mutation steps).

The haplotype phylogeny was confirmed by the species tree (Fig. 4), based on a Bayesian approach that grouped the north African cape hares (Tunisia and Morocco) with five Chinese species: *L. capensis* sensu lato, *L. sinensis*, *L. hainanus*, *L. yarkandensis* and *L. mandshuricus* and one African species, the scrub hare, *L. saxatilis* (if *L. victoriae* is considered conspecific with *L. saxatilis*; for the correct nomenclature of African savanna hares see Angermann and Feiler 1988, see also Suchentrunk et al. 2007, 2008) from Mozambique. Similarly, posterior probabilities values were low in most cases. When using the "reduced data set 2" with haplotypes showing high phasing probabilities similar to those obtained with the whole data set were observed for the network (Supplemental Fig. S3), the NJ, the ML, and the Bayesian species trees (not shown). The NJ and ML phylogenetic trees (Supplemental Fig. S2) of the Tunisian TF sequences revealed a lack of geographically meaningful phylogroups and no clustering concordant with coat colour types.

Discussion

The deletion of haplotypes with low phasing probabilities affected only marginally our results of genetic diversity and differentiation as well as the phylogenetic analysis. These results suggest that in our study the used phasing approach was efficient enough to produce reliable haplotypes and genotypes for the TF sequences. Therefore, our discussion and conclusions are based on the analyses of all currently detected haplotypes and the ones downloaded from GenBank.

Polymorphism and population history

With an average haplotype diversity of 95.5 % Tunisian hares exhibit a high degree of genetic polymorphism
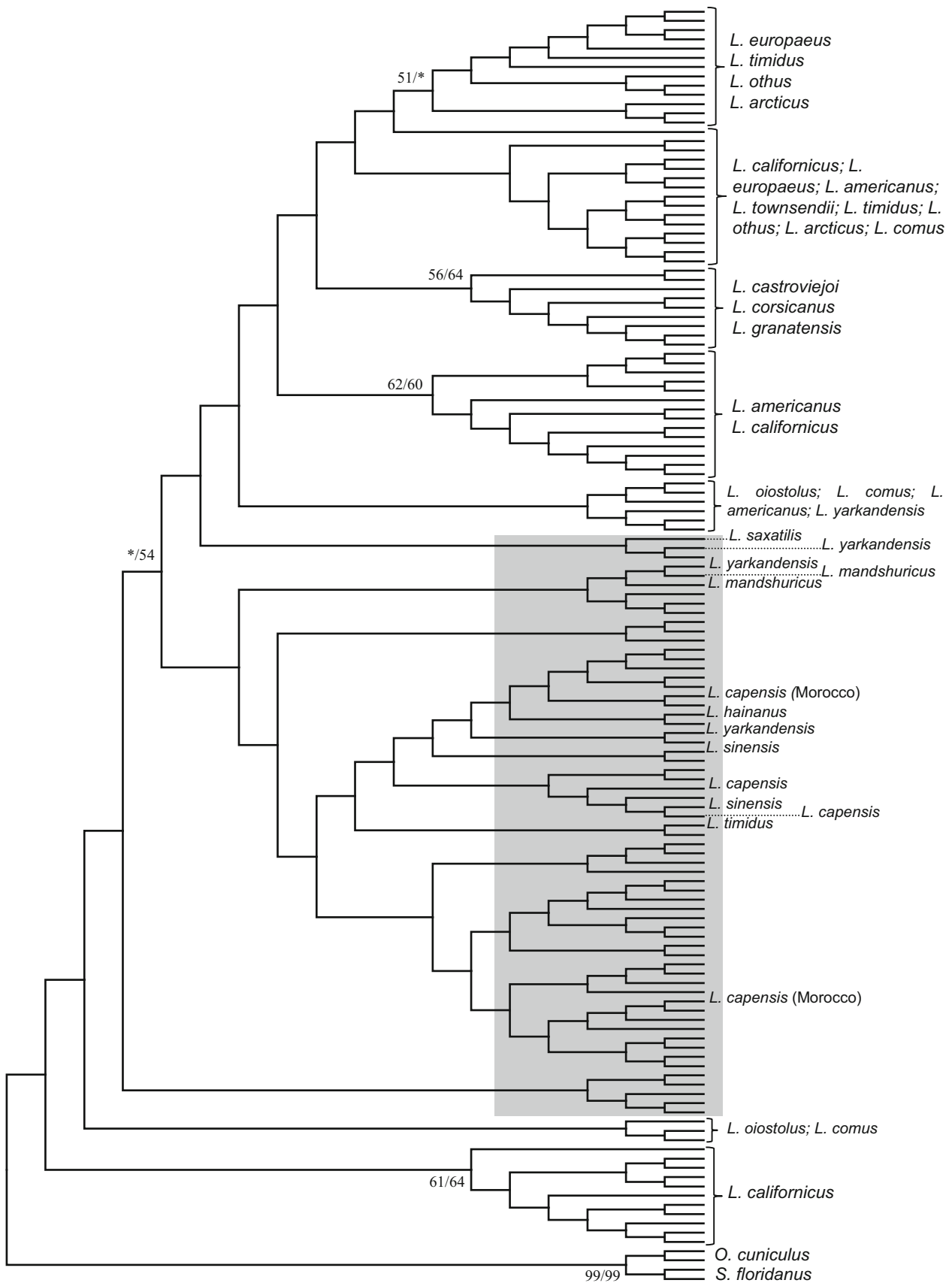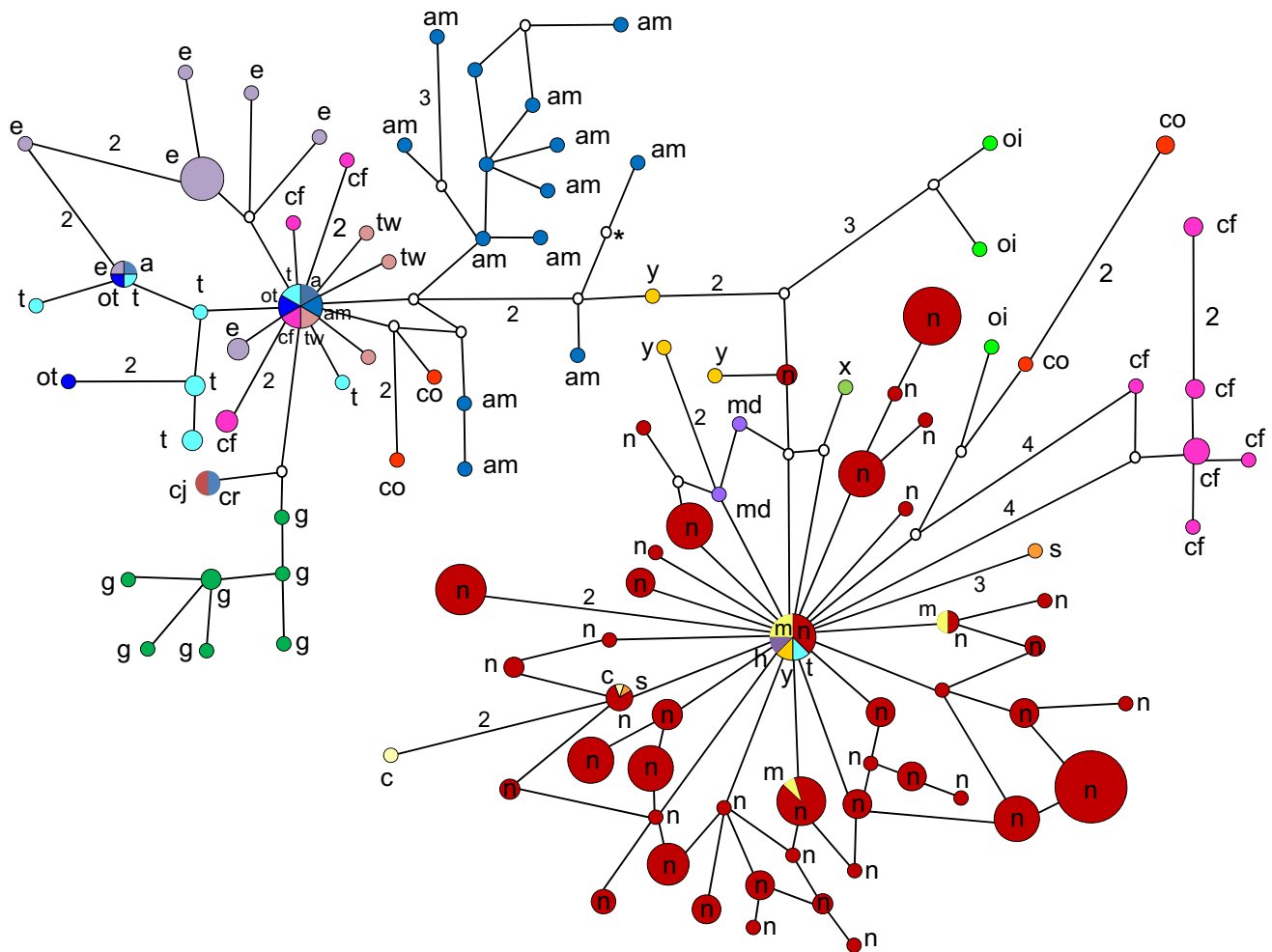
Fig. 3 Median joining network of the Tunisian TF haplotypes with all other haplotypes downloaded from GenBank (see Supplemental Table S1). Haplotypes (pies) are proportional to the total sample number. Small white circles indicate inferred haplotypes, not revealed presently, numbers associated with lines give numbers of substitutions between any two haplotypes, if more than one; single mutational steps between any two haplotypes are not indicated. *Asterisks* denote haplotype linked to those of outgroups. Taxa acronyms: c—*Lepus capensis* China, m—*L. capensis* Morocco, n—*L. capensis* Tunisia, x—*L. saxatilis*, co—*L. comus*, h—*L. hainanus*, md—*L. mandshuricus*, s—*L. sinensis*, oi—*L. oiostolus*, y—*L. yarkandensis*, a—*L. arcticus*, am—*L. americanus*, cf—*L. californicus*, cj—*L. castroviejoi*, cr—*L. corsicanus*, e—*L. europaeus*, g—*L. granatensis*, ot—*L. othus*, t—*L. timidus*, tw—*L. twonsendii*

comparable to that detected in the mitochondrial d-loop sequences of the same individuals (h = 0.966, Ben Slimen 2008) or in d-loop sequences of hares from two species from southern Africa (h = 0.969 in *L. saxatilis* and h = 0.974 in *L. capensis*; Kryger 2002). This diversity is clearly higher than that estimated for mtDNA of different European brown hare (*L. europaeus*) populations from Austria (Hartl et al. 1993: h = 85.8 %) and Germany (Faber et al. 1997: h = 75.8 %; Fickel et al. 2005, h = 90 %). However, the nucleotide diversities for our overall Tunisian hare sample and the three populations separately were very low compared to other nuclear (π = 0.079 for HBA1 and π = 0.044 for HBA2; Campos et al. 2012) or mtDNA sequences (π = 0.027 in hares from Tunisia, Ben Slimen 2008; π = 0.031 in *L. saxatilis* and

π = 0.059 in *L. capensis*, Kryger 2002; π = 0.65 in *L. europaeus*, Fickel et al. 2005) reported for brown hares. Low nucleotide diversity in combination with high haplotype diversities indicating shallow evolutionary divergences is considered a proof of rapid population growth from bottlenecked ancestral populations (Avise 2000). Even if our test statistics (SSD and R for the mismatch distribution) does not confirm this result, signatures of sudden population size expansions were also reflected by the results of the unimodal mismatch distribution, the negative signs of Tajima's D, and Fu & Li's F* statistics (Tajima 1989; Fu and Li 1993; Simonsen et al. 1995), and the starlike phylogeny of Tunisian hares depicted in the network (Fig. 3). Notably, a recent expansion event was also suggested by a significant bimodal distribution of the
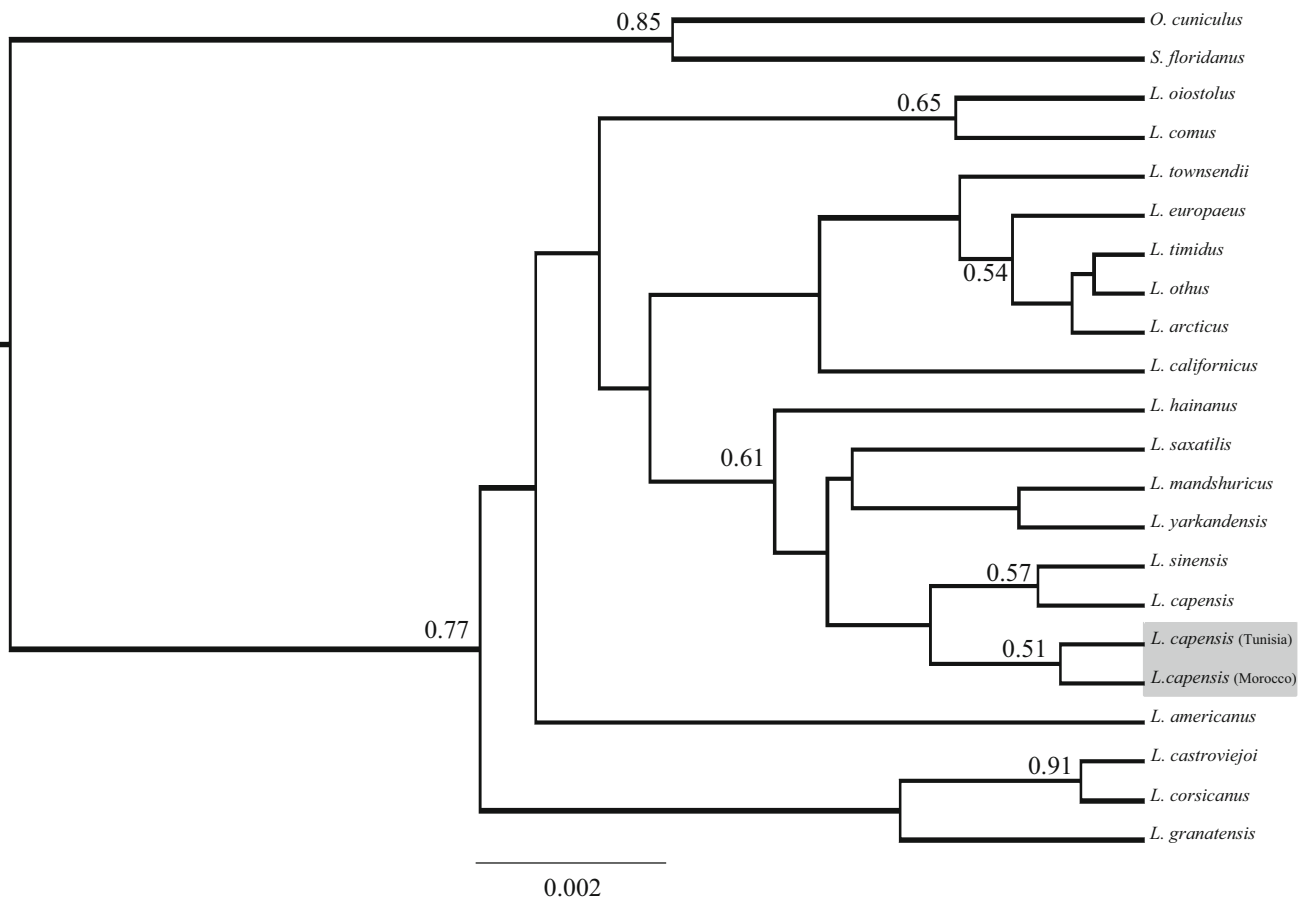
Fig. 4 Transferrin sequences consensus tree generated from the *BEAST species-tree inference output (the posterior probability of each clade is depicted in front of *each node*)

mt CR-1 haplotypes (Ben Slimen 2008). Such an expansion scenario could have happened during various periods of significant climatic changes in the recent past of North Africa. The most recent major climatic change across large parts of northern Africa occurred between ca. 12 000-4000 BP and brought a significant increase of humidity and precipitation after a hyper-arid period in the Maghreb, with widely expanding deserts or semi-desert (Klein 1984; Jamet 1991). Those climatic changes were accompanied by oscillating habitat expansions and contractions that most likely have had an influence on the ranges of hares in North Africa and on the levels of gene flow among their populations; for instance subfossil hare remains (considered belonging to *L. capensis*) have been recovered at various locations across the Sahara, which was a dry savanna with water courses during the early Holocene (e.g., Pachur and Altmann 2006). However, genetic data from modern hare populations across larger parts of northern Africa, which could help to interpret the geographic pattern of transferrin sequences in Tunisia, are not available.

Genetic structure

The genetic structure was relatively shallow, as indicated by our AMOVA, F_{ST} and Nm results. The phylogeographic structure was shallow as well, in spite of the presence of a high number of unique haplotypes; the latter finding suggested that newly arisen mutations have not yet had enough time to spread through the entire distribution range, compared to ancestral haplotypes that are likely more common and geographically more widespread. For the four coat colour phenotypes, little TF sequence divergence was also detected, although the most southern population with YP coat colour showed somewhat higher sequence differentiation to the other three colour types. However, the haplotype distribution suggests that 60 % (12 out of 20) of the haplotypes are shared with the other phenotypes. Therefore, the observed differentiation seems to reflect rather the demographic history of the populations and the complex gene flow profiles that might recently have been influenced by the presence of geographic barriers, such as the Chott el

Jerid Lake between the South and the other parts of the country. Moreover, similar patterns of genetic differentiation were observed for genotypes at 14 microsatellite loci (Ben Slimen 2008), which indicated that the highest F_{ST} was recorded between the Northern and Southern populations ($F_{ST} = 0.028$), and between the Y and YP colour phenotypes ($F_{ST} = 0.037$). However, results based on mtDNA CR-1 sequences (Ben Slimen 2008) were different to the results presented here and those previously reported for microsatellites. In this study, the Northern and Southern populations showed the lowest F_{ST} (0.035) value among all pairwise comparisons and the highest F_{ST} value for the colour phenotypes between YB and Y ($F_{ST} = 0.101$). Moreover, a higher level of differentiation was detected in mtDNA CR-1 sequences (Ben Slimen 2008), with the presence of four different phylogroups compared to the cohesive gene pool and lower level of differentiation in the studied TF sequences. According to Templeton (2006) among others, differences between nuclear and mitochondrial markers are expected to occur as both markers give information on demography and dispersal that take place during different time periods, i.e., the most recent past between 4Ne and Ne generations for mtDNA, and the distant past before 4Ne generations for nuclear DNA.

Phylogenetic relationships

Current taxonomy for North African hares (i.e., Egypt and the Maghreb including Libya) considers all the forms cited by Ellerman and Morrison-Scott (1951) belonging to *L. capensis* (Flux and Angermann 1990; Wilson and Reeder 1993; Nowak 1999; see also Ben Slimen et al. 2007; Suchentrunk et al. 2008). However, Hoffmann and Smith (2005) argue that hares traditionally considered cape hares from north of southern Africa may represent a different species, but this could not be confirmed by microsatellite data (Ben Slimen et al. 2008a, b). Our present phylogenetic analysis of TF sequences revealed two major findings: first, the studied Tunisian hares are closely related to the Moroccan hares; second, Tunisian hares are also closely related to Chinese hares belonging to a different species, with the presence of two shared haplotypes. Furthermore, both species are well differentiated from hares considered belonging to brown hares *L. europaeus* from central Europe. Our first finding suggests close nuclear DNA relationships among hares across large parts of North Africa. This is corroborated by mtDNA PCR–RFLP, mtDNA control region sequences and microsatellites data that demonstrate close phylogenetic relationships between Tunisian, Egyptian, and Moroccan hares (Ben Slimen et al. 2006, 2007, 2008a, b). Regarding the second point, our results do not permit a conclusion concerning the taxonomic position of the Tunisian hares in the wider phylogenetic context. It rather confirms earlier findings based on nuclear sequences in the genus *Lepus* that suggested frequent signals of shared ancestral polymorphism and/or ancestral or modern introgressive hybridization at various stages (Thulin et al. 2006; Alves et al. 2008; Melo-Ferreira et al. 2012; Liu et al. 2011). Indeed, our phylogenetic models of the transferrin sequences do not corroborate Petter's (1959) hypotheses (see Ben Slimen et al. 2008b) that all North African hares (except for a small isolated occurrence of *L. victoriae* in NW Algeria; see Flux and Angermann 1990) and *L. europaeus* belong to *L. capensis*, nor Pierpaoli's et al. (1999) hypothesis of a separate species status of (some) North African forms (e.g., "*L. mediterraneus*").

The trans-specific polymorphism detected in Tunisian hares and other hare species, strongly indicated the occurrence of nuclear introgression or/and shared ancestral polymorphism. Several studies (e.g., Alves et al. 2008; Melo-Ferreira et al. 2012, 2014b; Liu et al. 2011) have already reported introgression events between several "good hare species" both in mtDNA and nuclear genes. For the latter, Liu et al. (2011) considered that the trans-specific polymorphism could be interpreted as a result of introgressive hybridization as evidenced by bidirectional and recent introgression between different Chinese species. However, Melo-Ferreira et al. (2011) suggested incomplete lineage sorting as an explanation of the detected trans-specific polymorphism in their study based on an isolation-with-migration model that showed a very limited nuclear gene flow. For Tunisian hares, analysis of the mtDNA CR-1 sequences failed to detect introgression in the mitochondrial genome (Ben Slimen et al. 2007; Ben Slimen 2008) where all haplotypes were monophyletic and clearly distinct from all other taxa (including sequences of the Chinese hare species analyzed here). Moreover, Gebremariam (2013) found that one Chinese *Lepus timidus* TF haplotype was identical to one of the Tunisian cape hares and an ancestral haplotype from east African *L. starcki* and *L. habessinicus*. This haplotype could be an ancestral one shared among different species related to east African hares. It is also possible that *L. timidus* (or other Chinese hares) could have been introgressed by Chinese *L. capensis* (that belongs to *L. capensis* sensu lato), as Liu et al. (2011) suggested mtDNA introgression from *L. capensis* into *L. timidus*. Finally, the finding of identical TF haplotypes in *L. habessinicus* and Tunisian hares by Gebremariam (2013) could have resulted from a historical or ancestral introgression of the east African *L. habessinicus* into north African cape hares, when earlier distributional ranges were larger than those observed today (e.g., during the early Holocene, when today's Sahara was a dry savanna). Unpublished data of one of the present authors (FS) suggest even conspecificity of *L. habessinicus* and *L. capensis*

(including cape hares from North Africa). Overall, phylogenetic relationships among hares from Africa are still far from being clearly resolved, and only geographically meaningful, comprehensive morphological and population genetic data, in addition to traditional molecular phylogenetic results obtained from both mtDNA and multiple nuclear loci, will help to understand evolutionary relationships and species delineation.

In conclusion, the high number of detected haplotypes in hares from Tunisia with a relatively high level of divergence in terms of mutation steps suggests that, in order to draw a comprehensive picture of phylogenetic relationships, any phylogenetic analysis of the genus *Lepus* should include a reasonable number of specimens representing the overall distributional range of the species/taxa in question. Moreover, the presently found close similarity of TF haplotypes together with earlier mtDNA results (Ben Slimen et al. 2007) among different coat colour types suggest that variation in coat colour has been previously overestimated for resolving the systematics of the genus *Lepus*, particularly *Lepus capensis* sensu lato. Local differences in these traits are a common phenomenon in many small mammals and seem to result from adaptation to climate and habitat variation rather than from historical phylogenetic differences. Avise et al. (1987) strongly opposed subspecies delineations based on traits whose genetic underpinnings are unknown and Zink and Avise (1990) cautioned that intraspecific taxonomies based on morphological characters that exhibit phenotypic plasticity or variability tend to reflect patterns of ecological associations rather than evolutionary relationships. Finally, the phylogeny obtained here for the TF sequences was different to the one based on mtDNA CR-1 sequences of the same specimens (Ben Slimen et al. 2007; Ben Slimen, 2008) and both are also different to those obtained with other markers (allozymes, microsatellites: Ben Slimen et al. 2005, 2008b). Several studies have emphasized that gene trees are not necessarily congruent with species trees (e.g., Brower et al. 1996; Maddison 1997; Walker and Avise 1998; Nichols 2001) and that single gene divergences do not necessarily coincide with the actual species divergences. The genealogy of a single nuclear gene may well be inconsistent with the phylogeny of the species under investigation (Avise 1989; Wakeley 2000). Moore (1995) suggested that a mitochondrial gene tree would be more likely to be congruent with the species tree than a nuclear-gene tree, due to the fact that the population genetics of the mtDNA is determined by an effective population size (Ne) that is one-fourth as large as that of a nuclear-autosomal gene. In any case, the strong indications of occurrence of (ancestral or modern) mitochondrial and nuclear introgression or/and shared ancestral polymorphism for various hare taxa, and the evidence for such evolutionary scenarios published earlier for several hare species (e.g., Suchentrunk et al. 1999; Thulin et al. 1997a, b, 2003; Alves et al. 2003; Melo-Ferreira et al. 2005), points on the one hand to the generally shallow evolutionary divergence pattern, the reticulate evolutionary mode among hare species, and the difficulty to infer their phylogenetic relationships. The present results represent one further step to understand evolutionary relationships among hares from a region in northern Africa, where different external phenotypes are occurring along a steep ecological gradient (from the Mediterranean northern seaboard to the arid Sahara). They corroborate earlier findings of nuclear allozyme, microsatellite polymorphism, as well as mitochondrial d-loop sequences that suggested the presence of only one species, despite marked phenotypic variation (including body size and weight). Understanding the phylogenetic status of these hares, particularly the relationship with other hares from Africa traditionally considered *Lepus capensis*, however, needs a comprehensive sampling in terms of specimens and molecular markers from many areas in Africa (including also *L. habessinicus*).

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