This is a post-peer-review, pre-copyedit version of an article published in Heredity. The final authenticated version is available online at https://doi.org/10.1038/s41437-019-0229-8

The evolutionary history of the Cape hare (*Lepus capensis sensu lato*): insights for systematics and biogeography

Sara Lado[1,2,*], Paulo C. Alves[1,2,3], M. Zafarul Islam[4,5], José C. Brito[1,2], José Melo-Ferreira[1,2]

[1]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Universidade do Porto, Campus de Vairão, 4485-661 Vairão, Portugal

[2]Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal

[3]Wildlife Biology Program, University of Montana, Missoula, MT 59812, USA

[4]National Wildlife Research Center (NWRC), Post Box 1086, Taif, Saudi Arabia

[5]Ilia State University, Tbilisi 0162, Georgia

[*]Current affiliation: Research Institute of Wildlife Ecology, Vetmeduni Vienna, Vienna, Austria

Corresponding author: José Melo-Ferreira, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Universidade do Porto, Campus de Vairão, 4485-661 Vairão, Portugal, tel: +351 252 660 411, jmeloferreira@cibio.up.pt

Running title: Phylogeography and systematics of the Cape hare

Word count: 6626

Abstract

Inferring the phylogeography of species with large distributions helps deciphering major diversification patterns that may occur in parallel across taxa. Here, we infer the evolutionary history of the Cape hare, *Lepus capensis sensu lato*, a species distributed from southern Africa to Asia, by analysing variation at 18 microsatellites and 9 DNA (1 mitochondrial and 8 nuclear) sequenced loci, from field and museum-collected samples. Using a combination of assignment and coalescent-based methods, we show that the Cape hare is composed of five evolutionary lineages, distributed in distinct biogeographic regions – north-western Africa, eastern Africa, southern Africa, the Near East and the Arabian Peninsula. A deep phylogenetic break possibly dating to the Early Pleistocene was inferred between the African and Asian *L. capensis* groups, and the latter appear more closely related to other Eurasian hare species than to African Cape hares. The inferred phylogeographic structure is shared by numerous taxa distributed across the studied range, suggesting that environmental changes, such as the progressive aridification of the Saharo-Arabian desert and the fluctuations of savannah habitats in Sub-Saharan Africa, had comparable impacts across species. Fine-scale analyses of the western Sahara-Sahel populations showed rich fragmentation patterns for mitochondrial DNA but not for microsatellites, compatible with the environmental heterogeneity of the region and female philopatry. The complex evolutionary history of *L. capensis sensu lato*, which possibly includes interspecific gene flow, is not reflected by taxonomy. Integrating evolutionary inference contributes to an improved characterization of biodiversity, which is fundamental to foster the conservation of relevant evolutionary units.

Key Words: African Mammals, Biogeography, Conservation Genetics, Lagomorphs, Phylogeography, Species-tree Inference, Taxonomy

Introduction

Characterizing the evolutionary history of species and the geographic structure of genetic diversity and lineages (phylogeography) provides valuable and powerful information to understand the processes that influenced organism diversification (Avise 2009). Similar diversification patterns across co-distributed species, in time and space, can be used to explain the general biogeographic processes underlying such patterns (Gutiérrez-García and Vázquez-Domínguez 2011). The analysis of multi-locus sequence datasets in coalescent frameworks, taking into account the variance of lineage sorting, has become an important tool to assist the taxonomic classification of organisms (Fujita et al. 2012), particularly when morphological distinctiveness is unclear or cryptic (Bickford et al. 2007). Even if molecular-based methods primarily detect genetic structure, without distinguishing intra from interspecific processes (Sukumaran and Knowles 2017), the information that molecular inferences generate about the evolutionary processes behind lineage diversification is central to modern integrative taxonomy (Fujita et al. 2012), with direct implications for the definition of appropriate conservation units (Scheffers et al. 2012; Adams et al. 2014).

The taxonomy of the Cape hare (*Lepus capensis*) is historically controversial. Current classifications consider that *L. capensis sensu lato* exhibits a disjunct distribution in Africa, with no gene flow between southern and northern range margins, and that its distribution extends to the Middle East (Schai-Brown and Hackländer 2018) and possibly to Iran and Pakistan (Drew et al. 2008). Although earlier assessments indicated that the distribution of *L. capensis* could extend to China, Mongolia and Russia, the formal recognition of *Lepus tolai* and *Lepus tibetanus* as valid species removes *Lepus capensis* from these regions, and possibly also from Iran and Pakistan (Schai-Brown and Hackländer 2018). The wide phenotypic dissimilarity of *L. capensis sensu lato* over its range, such as in fur colour, body size or ear length (Ben Slimen et al. 2007; Schai-Brown and Hackländer 2018), resulted in a variable number of classified subspecies over time (see Flux and Angermann 1990; Schai-

79 Brown and Hackländer 2018). However, it remains uncertain whether this morphological
80 diversity reflects gradients of local adaptation or deep evolutionary divergence. Hoffman &
81 Smith (2005) informally divided the species into four major geographic partitions – southern
82 Africa, eastern Africa, Arabia and north-western Africa – that could merit specific
83 classification, but the validation of these partitions awaits additional data. Moreover, the
84 relationships of the Cape hare with neighbouring hare species is historically controversial
85 (Ben Slimen et al. 2008b; Schai-Brown and Hackländer 2018 and references therein). In
86 addition to *L. capensis sensu lato*, five other species are currently classified in Africa (*L.*
87 *saxatilis*, *L. victoriae*, *L. habessinicus*, *L. fagani* and *L. starcki*), distinguished by
88 morphological characters (Kingdon 2013). However, their evolutionary history, genetic
89 differentiation and distribution remain poorly understood, despite recent progresses in the
90 *habessinicus-fagani-starcki* group (Tolesa et al. 2017). Another difficulty is posed by the
91 frequent sharing of mitochondrial DNA (mtDNA) variation and discordant differentiation
92 patterns with nuclear markers (Ben Slimen et al. 2006, 2008a), which may result from a
93 tendency of hare species to hybridize and to exchange genetic variation over the contact
94 zones (Alves et al. 2006; Melo-Ferreira et al. 2012, 2014b). Therefore, a formal revision of
95 the taxonomy of *L. capensis sensu lato* awaits a more integrative and precise understanding
96 of population structure and evolutionary history of the species over its range.
97
98 The Cape hare, as currently classified, is distributed across a wide geographical area,
99 including the savannah and dry desert regions of Southern Africa, the dry savannah regions
100 of Central, West and North Africa, and parts of the Saharo-Arabian region (Hoffmann and
101 Smith 2005; Drew et al. 2008; Schai-Brown and Hackländer 2018) (Fig. 1). Comparative
102 studies have identified common phylogeographic patterns of organisms associated with
103 these habitats. In sub-Saharan Africa, a north-south divide has been shown to occur across
104 a diverse array of savannah-dwelling taxa, which has been explained by the periodic
105 population confinement on either side of Central Africa tropical forests (e.g. Lorenzen et al.
106 2012; Bertola et al. 2016). East Africa has also been shown to harbour divergent evolutionary

lineages across taxa, which may be related with regional spatial and temporal heterogeneity and rifting promoting isolation and divergence (Lorenzen et al. 2012; Aghová et al. 2017). In North Africa, phylogeographic patterns are usually associated with barriers and dispersal corridors created by the cyclic expansions and contractions of the Saharo-Arabian desert and changes in hydrologic networks (Brito et al. 2014; Mairal et al. 2017; Stewart et al. 2017).

In this work, we use a multi-locus genetics approach and the widest sampling of the species to date to i) infer population structure of *L. capensis sensu lato* and relate genetic differentiation to geography, ii) evaluate relationships and divergence among evolutionary lineages, and iii) discuss the inferred evolutionary history according to known biogeographic patterns across taxa. We test the validity of the putative subdivision of *L. capensis sensu lato* in four geographic explicit groups (Hoffmann and Smith 2005), and assess whether major bioclimatic and physiographic factors that affected phylogeographic patterns across savannah-associated species also influenced the population history of the Cape hare.

Materials and Methods

Sampling and DNA extraction

In total, 162 *Lepus capensis sensu lato* samples from Africa and the Near East were collected (Tables S1 and S2). Part of the used samples (72) resulted from road-kill specimens found during fieldwork along Northwest Africa. The remaining samples were kindly provided by other researchers (75) and museums (15) (see complete museum sampling information in Table S3). Although samples were classified as *L. capensis* based on geographic location and morphology, no *a priori* assignment to a given population or lineage was considered for the analyses, given the controversies on the ranges of distinct African hares (e.g. Moores et al. 2012).

135

136 Genomic DNA from field-collected samples was extracted from preserved tissues (liver or
137 ear) using the JETQUICK Tissue DNA Kit (Genomed). The museum samples (dry skins)
138 were extracted following the protocol described by Bi et al. (2013), in an isolated and
139 autonomous room with devoted sterilized equipment, in order to prevent contamination with
140 modern DNA.

141

142 *Genotyping of microsatellites and DNA sequencing*

143

144 Microsatellite loci for African *L. capensis sensu lato* were newly developed in this work by
145 Genoscreen (http://www.genoscreen.fr/), using pooled high-quality DNA of 12 samples from
146 north-west Africa (see Table S1). Genomic DNA libraries were enriched for microsatellites
147 with microprobes, and sequenced via high throughput Titanium pyrosequencing on a 454-
148 GsFLX® sequencer (Roche Diagnostics). AUTODIMER (Vallone and Butler 2004) was used
149 to predict and avoid hairpin structures and dimers within primer multiplexes. Initially, 38
150 microsatellite loci were selected to optimize polymerase chain reactions, evaluate genotyping
151 success (in a ABI3130xl Genetic Analyzer; Applied Biosystems), and test their compatibility
152 in multiplexed reactions. Variability in a selected set of 19 loci was then assessed in 50
153 specimens from north-west Africa, southern Africa and the Near East (Table S1). Eighteen
154 variable microsatellite loci were finally selected, organized in three multiplexes with
155 fluorescent marked primers (M13 tails), and genotyped (see details in Table S4).
156 GENEMAPPER v4.0 (Applied Biosystems) was used to read and score the genotyping
157 results, followed by visual inspection. Museum samples were amplified four times and 30%
158 of field-collected samples were re-amplified to ensure consistency of genotype
159 determination. The final dataset included the 162 *L. capensis sensu lato* specimens.

160

161 Partial mitochondrial cytochrome *b* was sequenced in 139 *L. capensis sensu lato* samples
162 (three from Melo-Ferreira et al. 2012). This dataset was complemented with 41 sequences

from putative *L. capensis* samples from China and four additional species (*L. saxatilis*, *L. timidus*, *L. granatensis* and *L. europaeus*), obtained from previous studies (Halanych et al. 1999; Matthee et al. 2004; Melo-Ferreira et al. 2007, 2011, 2012; Ramírez-Silva et al. 2010; Liu et al. 2011b). Eight nuclear DNA loci (DARC – Duffy blood group, chemokine receptor; HPX – hemopexin; SPTBN1 – spectrin, beta, non-erythrocytic 1; TF – transferrin; OXA1L – oxidase assembly 1-Like; TG – thyroglobulin; TSHB – thyroyd stimulating hormone beta; UCP2 – uncoupling protein 2) were sequenced in 61 *L. capensis sensu lato* specimens (58 newly sequenced, and 3 from Melo-Ferreira et al. 2012). These samples represented the population clusters identified with microsatellites (except eastern Africa for which only museum samples were obtained, and which could not be amplified). This dataset was also complemented with available sequences from putative *L. capensis* from China and three other hare species (*L. granatensis*, *L. europaeus* and *L. timidus*) retrieved from GenBank (Alves *et al* 2003, 2006; Matthee et al. 2004; Melo-Ferreira *et al* 2009, 2011, 2012; Liu *et al* 2011a, unpublished). Table S2 provides a detailed description of the final sequence datasets, including missing information.

Fragments for newly sequenced specimens were amplified using described primers (Wallner et al. 2001; Matthee et al. 2004; Melo-Ferreira et al. 2009). For 15 museum samples, only a smaller cytochrome *b* portion could be amplified, using a second primer pair designed in this work (two replicates were done to ensure absence of contamination from exogenous DNA). Detailed primer information and PCR conditions are shown in Table S5. Purified PCR products were sequenced using the standard Sanger sequencing protocol at Macrogen Inc. (Netherlands), using both forward and reverse primers.

Population genetics analyses

Genepop (Raymond and Rousset 1995) was used to test for deviations to Hardy-Weinberg and linkage equilibria, which could be indicative of allele dropout, non-independence among

loci and other biases. Markov chain parameters for exact tests were set at 10,000 dememorizations and 100 batches, with 5,000 iterations per batch, and the Bonferroni correction for multiple tests was applied. Given the *a priori* uncertainties about population structure, which could cause spurious deviations from equilibrium, we arbitrarily divided the dataset according to the countries of origin of the samples, and performed the tests in these subsamples. For low sample sizes, samples from neighbouring countries were pooled for a minimum sample size of 7 (Table S6).

The Bayesian assignment method implemented in STRUCTURE v.2.3.3 (Pritchard et al. 2000; Falush et al. 2003) was used to infer population structure, without prior assignment of specimens to populations. Given that the model considering correlated allele frequencies performs better in separate analyses of divergent populations (Evanno et al. 2005 and STRUCTURE documentation), we performed a hierarchical structure analysis (Coulon et al. 2008; Cheng et al. 2014; Pisa et al. 2015). At each round of the analysis, the dataset was split according to the best K number of groups and re-run independently until no structure was found (i.e. no sorting of specimens per K clusters with high probability). Individuals with probability of assignment lower than 80%, i.e. potentially admixed specimens, could not be unequivocally attributed to a cluster and were discarded for the following rounds. For each K number of clusters (K=1 to K=10), three independent replicates of 1,000,000 generations of burn-in followed by 1,000,000 MCMC generations were run using the admixture model with correlated allele frequencies. The best number of K populations for each dataset was determined using the ΔK method (Evanno et al. 2005), as implemented in STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt 2012).

An additional Bayesian clustering analysis was performed using TESS v2.3.1 (Chen et al. 2007; Durand et al. 2009), which takes into account the spatial distribution of samples. An admixture analysis using the conditional autoregressive (CAR) Gaussian model was performed with a linear trend degree. Ten independent replicates of 130,0000 sweeps from

K=2 to 10 were performed, discarding the first 30,000 sweeps as burn-in. The best number of K clusters was determined based on the stabilization of average DIC (deviance information criterion) with K_{max}. An additional 140 replicates (total 150) were performed for the best K_{max}. Results were summarized from the 10% of replicates with the lowest DIC values, using CLUMPP (Jakobsson and Rosenberg 2007). F_{ST} between pairs of clusters estimated with Structure was calculated using FSTAT (Goudet 1995) and significance was determined through genotype randomization (correcting the p=0.05 significance threshold using the Bonferroni correction for multiple tests).

Genealogical relationships among mtDNA haplotypes were determined using the Median-Joining (MJ) algorithm with software POPART v.1.7 (Leigh and Bryant 2015). In addition, spatial clustering of individuals was performed for the mtDNA dataset using the Bayesian approach implemented in BAPS 6 (Corander et al. 2008; Cheng et al. 2013). Prior upper values for the number of clusters was specified with a maximum of K = 10 and five independent runs.

Phylogenetic analyses

The cytochrome *b* phylogeny was determined using both Bayesian (BI) and Maximum Likelihood (ML) inference methods, with BEAST v1.8.1 (Drummond and Rambaut 2007) and Garli v1.0 (Zwickl 2006) respectively. The European wild rabbit, *Oryctolagus cuniculus* was used as an outgroup. The best-fit model of evolution was chosen using jModeltest among 88 possible models and the AICc criterion (Darriba et al. 2012). In Garli v1.0, five independent search replicate runs were performed, specifying the optimal mutation model but not fixing the model parameters. No starting topology was defined and the program was set to run until no significantly better scoring topology was found after 50,000,000 generations. Tree support was estimated using 500 bootstrap replicates. BI was performed using BEAST v1.8.1, setting as prior the determined mutation model, or the next-most parameterized model available in

BEAST when the best-fit model was not implemented. Posterior probabilities were determined using the Yule tree prior and the uncorrelated lognormal relaxed clock (Drummond et al. 2006), with three replicate runs of 100,000,000 generations, sampling trees and parameter estimates every 10,000 generations. Convergence was assessed in Tracer v1.7 (Rambaut et al. 2018).

Phylogenetic relationships among species and evolutionary groups (inferred by microsatellite analyses) were inferred from nuclear DNA sequences using the multilocus coalescent-based method *BEAST (Heled and Drummond 2010), implemented in BEAST v1.8.1. First, PHASE v2.1.2 (Stephens et al. 2001) was used to determine the phase of alleles, running 1,000 generations after 1,000 generations of burn-in, with a thinning interval of 1. *BEAST assumes no gene flow among the determined groups, and we minimized violation of this assumption using specimens that were determined as non-admixed by the microsatellite analyses (thus used as proxy of genomic admixture levels). Also, mtDNA sequences were not included given the suspicion of secondary mtDNA introgression (data not shown), and the widespread occurrence of this phenomenon in hares (Thulin et al. 2006; Liu et al. 2011b; Melo-Ferreira et al. 2012, 2014b; Levänen et al. 2018). Finally, we opted to maintain the full alignments, as it has been shown that recombination has minimal impacts in species-tree determination (Lanier and Knowles 2012). Specimens were assigned to distinct populations considering i) the determined microsatellite population clusters, ii) the putative *L. capensis* sequences from China, and iii) three other species: *L. granatensis*, *L. europaeus* and *L. timidus*. Outgroup sequences were not included, as the method combines estimates of the root of each gene tree using the multispecies coalescent (Heled and Drummond 2010). Phylogenies were inferred from the full sequence dataset, including a string of Ns with the same length of the alignment in few cases where a locus was not available for a population (southern Africa and China for 2 and 5 loci respectively). A separate analysis that excluded the Chinese and southern Africa populations was performed to assess the robustness of the topology of the remaining populations/species.

275

276 Nucleotide substitution and relaxed clock models were set for each locus as described
277 above. A Yule tree prior was used, considering each terminal branch of the tree as an
278 independent evolutionary unit. Three independent runs of 500,000,000 generations sampling
279 every 80,000 generations were performed. Node dating was performed using a rate of 3.17 x
280 10^{-3} substitutions per site per Ma for the reference locus TF (Melo-Ferreira et al. 2012),
281 estimated from a calibrated divergence between the European rabbit and hares of 11.8 Ma
282 (Matthee et al. 2004).

283

284 *Isolation-with-migration and coalescent simulations*

285

286 The divergence history among distinct evolutionary entities was further explored using the
287 isolation-with-migration (IM) framework (Hey 2010) and the multilocus nuclear DNA
288 sequence dataset. This analysis was applied in three instances. First, to re-assess the
289 divergence parameters between entities representing the deepest phylogeographic divide
290 inferred in our phylogenetic analysis – Arabian Peninsula and north-western Africa
291 populations. Second, to re-estimate the divergence between southern and northern African *L.*
292 *capensis*. Third, to clarify the apparent discordance between nuclear and mtDNA divergence
293 between the Near East *L. capensis* population and *L. europaeus* – close mtDNA relationship
294 but deep nuclear divergence. IMa2 (Hey 2010) was used to infer effective population sizes
295 (populations 1, 2 and ancestral), divergence time and gene flow rates (Hey and Nielsen
296 2004) in the three pairwise analyses. Datasets were reduced to the largest non-recombining
297 blocks using IMgc (Woerner et al. 2007), which has been shown to reduce biases in the final
298 IM estimates (Strasburg and Rieseberg 2010). Three independent runs were performed,
299 varying the parameters' upper bound priors and the starting seeds and using the HKY
300 mutation model (Hasegawa et al. 1985). A likelihood ratio test was applied to assess whether
301 migration was significantly different from zero (Nosil et al. 2009). The locus-specific mutation
302 rates estimated by Melo-Ferreira *et al* (2012) were used, except for the TG locus, for which it

303 was newly determined using the same methodology.

304

305 To investigate whether the cyto-nuclear discordance inferred for the Near East *L. capensis*
306 population may have resulted from incomplete lineage sorting alone, coalescent simulations
307 were done with SIMCOAL V2.1.2 (Laval and Excoffier 2004). 10,000 cytochrome *b* datasets
308 were simulated under a coalescent model with no gene flow, using the divergence time (*t*)
309 and effective population sizes (Ne) modelled with IMa2 for Near East and *L. europaeus*, and
310 the mutation rate determined by Melo-Ferreira *et al* (2012) for cytochrome *b*. An ancestral
311 haploid population of size $Ne_A/2$ was simulated to split into two descendant populations of
312 sizes $Ne_1/2$ and $Ne_2/2$, *t* generations ago, with no gene flow occurring after the split. For each
313 simulation, the minimum uncorrected sequence divergence (*Dxy*) between descendant
314 populations was calculated and used to build the distribution of minimum expected distances.

315

316 **Results**

317

318 *Population structure*

319

320 The 18 newly discovered microsatellite loci were successfully genotyped in 162 *L. capensis*
321 *sensu lato* samples. The number of alleles per locus varied between 8 and 28 (see Table
322 S4). Considering nine data partitions based on geographic proximity, 3.7% and 0.6% of the
323 tests rejected conformation to Hardy-Weinberg and linkage equilibria respectively. Given that
324 significant deviations were sporadic across loci and not consistent across partitions, all loci
325 were retained for subsequent analyses.

326

327 The STRUCTURE clustering analyses supported the subdivision in two clusters (K=2) at the
328 uppermost hierarchical level: a predominantly north-western population (hereafter NW
329 Africa), and a second cluster with the remaining individuals (Fig. 1 and Figures S1 and S2).
330 The following round divided the second group in 4 sub-clusters (K=4) – East Africa (E Africa),

331 the Near East, Arabia and South Africa (S Africa) (Fig. 1 and Figures S1 and S2). In the NW
332 Africa group, vast mixed assignment of individuals to K clusters with no geographic partition
333 was found, and thus no further subdivision was considered (Fig. 1 and Figures S1 and S2).
334 After both rounds of hierarchical analysis, 140 specimens were attributed to a single cluster,
335 while 22 showed mixed assignment probability to distinct clusters (i.e. <0.8 probability)
336 (Table S2).
337
338 The spatial Bayesian clustering analysis suggested K=5 as the best number of clusters
339 (Figure S2), which however corresponded to 4 partitions with proportions of individual
340 assignment >0.8 (the fifth cluster had no assigned individuals). Results were consistent
341 across replicate runs, from K_{max}=4 to 10. Spatial clustering of African samples was
342 compatible with STRUCTURE results, suggesting clusters in NW Africa, E Africa and S
343 Africa, but placing the Near East and Arabia specimens in a single group. A separate
344 analysis with these specimens and K_{max}=2, confirmed the STRUCTURE separation in Near
345 East and Arabia (Figure S1). No signs of error-driven batch effects for museum samples
346 were found, as these were assigned logically according to geography.
347
348 Pairwise F_{ST} among the five inferred clusters varied between 0.11 (Near East vs. NW Africa)
349 and 0.27 (S Africa vs. E Africa) (Table S7). Differentiation was generally significant, except
350 between Arabia and East Africa (note that the significance of F_{ST} involving southern Africa
351 could not be assessed due to low sample size).
352
353 The BAPS analysis of mtDNA structure suggested six evolutionary groups for *L. capensis*
354 *sensu lato*: three groups in NW Africa, S Africa, Near East/East Africa and Arabia) (Figure
355 S3). The mtDNA haplotype network (Figure S4) nevertheless showed genealogical
356 separation of Near East and East Africa haplotypes and of the haplotype from Mali (which
357 represents a fourth lineage from NW Africa). We therefore mapped the distribution of eight
358 maternal haplogroups – NWA-I to NWA-IV, S Africa, E Africa, Near East, Arabia (Fig. 1).

These eight mtDNA haplogroups coincide with the evolutionary units identified using microsatellites, with the exception of the NW Africa, for which mtDNA suggested four different, yet closely related, sublineages (NW Africa I-IV; Fig. 1 and Figure S4).

Phylogenetic analyses

Eight nuclear and one mitochondrial DNA loci were sequenced (GenBank accession numbers MK775981-MK776533) and aligned together with sequences retrieved from GenBank (Table S2), for a total of 5164 bp (see Table S5). The inclusion of sequences from other *Lepus* species in the cytochrome *b* phylogeny showed that the eight lineages sampled in *L. capensis sensu lato* (Fig. 1) do not form a monophyletic group (Fig. 2 and Figure S5): the Near East and E Africa lineages are closely related to *L. europaeus*, and the putative *L. capensis* from China to *L. timidus*. The mtDNA phylogeny excluding museum samples and using the longer DNA fragment showed a similar topology (but removed the East Africa haplotypes) (Figure S5).

In the nuclear loci, 86% of the sequence length corresponded to introns. The multi-locus coalescent-based phylogeny inferred with the nuclear DNA dataset suggested a major phylogenetic divide that splits African from Asian and European *Lepus* lineages around 1 million years ago (Ma) (Fig. 3). The Near East and Arabian populations were found to share a very recent common ancestor, appearing more closely related to putative *L. capensis* from China and the Eurasian hare species (*L. europaeus*, *L. granatensis* and *L. timidus*), than to the remaining African *L. capensis* lineages (NW Africa and S Africa; note that E Africa specimens could not be included in this analysis). Even if posterior probabilities are not high for some nodes, the grouping of non-African lineages of *L. capensis sensu lato* with other hare species was inferred with high probability (0.98; Fig. 3a). This phylogenetic pattern remains when removing the lineages for which not all loci were sequenced (*L. capensis* populations from China and S Africa) (Fig. 3b).

Isolation-with-migration and coalescent simulations

Estimates retrieved from the isolation-with-migration analyses were consistent across replicate runs (Table 1). Keeping the non-recombining blocks reduced the dataset to 8-18% of the alignment length. Among the analysed pairs of populations, nuclear gene flow was not significantly different from zero. Even if in some instances the posterior density curves did not allow to estimate the 95% confidence intervals of the divergence times, point estimates were consistent with the inferences based on the multi-locus species tree (Fig. 3).

In order to determine whether the low divergence between Near East *L. capensis* and *L. europaeus* inferred for mtDNA was compatible with divergence history inferred from the nuclear DNA, we used these parameters to simulate the expected Cytb divergence under a model with no gene flow. The empirical pairwise mtDNA divergence was found to be smaller than the 5^{th} percentile of the simulated minimum distances in all instances (Fig. 4), suggesting that incomplete lineage sorting does not explain the close mtDNA relationship between European *L. europaeus* and Near East *L. capensis* in our dataset (see Melo-Ferreira et al. 2012, 2014b).

Discussion

Population structure with deep divergence in Lepus capensis sensu lato

Our microsatellite analyses suggest that *L. capensis sensu lato* is composed of five evolutionary units with geographic structure – north-western Africa, southern Africa, eastern Africa, Arabia and the Near East (Fig. 1). These results are concordant with the insights of Hoffmann and Smith (2005), who restricted *L. capensis* to Southern Africa, and informally suggested three species-level division for the remaining *capensis*-type hares – Northwest

Africa, East Africa and Arabia-Near East. Given the gaps in our sampling scheme, we cannot completely exclude that smoother transitions of genetic structure could occur between evolutionary units. However, our multilocus coalescent-based nuclear DNA phylogeny supports two major *L. capensis sensu lato* clades, and does not retrieve the species as monophyletic when including other *Lepus* lineages, as the Near East and Arabian Peninsula specimens appear more closely related to Eurasian hare species (Fig. 3). This major phylogenetic/phylogeographic divide could not be created by spurious structure from spatial correlation of our gapped opportunistic sampling scheme. These results therefore show that the Asian *L. capensis* groups have an independent evolutionary origin from their African counterparts (Fig. 3). The relevant uncertainties of molecular dating calibrations (Ho et al. 2015) indicate caution is needed when interpreting the absolute inferred ages of the divergence events. Still, our analyses suggest that the African-Asian split is the oldest divergence event, and possibly occurred during the Early Pleistocene (Fig. 3). We could not, however, include East African samples in our nuclear DNA phylogeny. The microsatellite analysis and mtDNA phylogeny suggest that this is a separate group, and potentially more closely related with the Near East haplotypes (Figs. 1 and 2). If that is the case, the major phylogenetic divide in *L. capensis sensu lato* may therefore run across East Africa. Regardless, our results confirm that the classification of the Asian evolutionary units as *L. capensis* is controversial (Hoffmann and Smith 2005), and indicate that a reassessment of the relationships of these populations with other currently classified Asian hare species, such as *L. tolai* or *L. tibetanus* (to which the sequences obtained from specimens from China recovered here may belong to) is needed. Our work further suggests the distinction of the Near East and Arabian evolutionary units, in line with the reported morphological uniqueness of the Arabian hares (Drew 2000; Zubair et al. 2011). Whether this would merit taxonomic recognition requires further investigation of the biology, ecology and genetics of these groups.

In Africa, the Cape hare has a disjunct northern and southern distribution and our analyses recover these as separate evolutionary units (Figs. 1 and 3). This is supported by absence of admixture in all instances of our analyses – microsatellites (Figure S1), mtDNA (Figure S4) and IM model applied to the multilocus dataset (Table 1). The split between northern and southern Africa *L. capensis* may have occurred during the Middle Pleistocene, in a period of strong climate oscillations (Brown et al. 2007). However, given the small sample size of the Southern population, further analyses with larger sample sets are needed to clarify its degree of differentiation and divergence.

In northern Africa, the microsatellite analyses of our comprehensive sampling did not show geographic substructure (Fig. 1; Figure S1). This result is surprising given the environmental and topographic heterogeneity of the range occupied by the species in the region. Indeed, the analyses of mtDNA variation suggest subdivision into several geographically structured haplogroups (Fig. 1 and 2). While the haploid nature and uniparental transmission of mtDNA can contribute to faster lineage sorting at local geographic scales, mtDNA structure could have been reinforced by female philopatry and male-mediated dispersal. The female-linked marker can thus mark the regional historical structure of the species, which was homogenized at biparentally transmitted loci by recent male-mediated gene flow (Fahey et al. 2014). Female philopatry has been suggested in several hare species (Fickel et al. 2005; Hamill et al. 2007; Melo-Ferreira et al. 2014a) including in southern Africa *L. capensis* and *L. saxatilis* (Kryger et al. 2002). In addition, we cannot exclude that some of these clades may have introgressed from an unsampled neighbouring species.

Secondary mitochondrial DNA introgression

Our results suggest that the mtDNA haplotypes sampled in the Near East and East Africa regions were more closely related to the European brown hare (*Lepus europaeus*) than expected considering the inferred model of divergence of the species (Figs 2-4). The Cape

hare specimens from the Near East are close to the possible contact area between *L. capensis* and *L. europaeus*, and our simulations suggest that the mtDNA similarity may result from introgression (Fig. 4). This suspicion adds to numerous works showing that mtDNA introgression is a pervasive phenomenon among hare species, both in current and historical contacts (e.g. Melo-Ferreira et al. 2005, 2014b). However, understanding whether these results reflect a smoother local transition with gene flow between the currently classified species in the region (see Ben Slimen et al. 2008b) demands a detailed analysis of regional genetic variation, with precise estimates of differentiation and divergence at the nuclear and mtDNA levels. Another instance of cyto-nuclear discordance of phylogenetic patterns concerns the relatedness of putative *L. capensis* from China to *L. timidus* for mtDNA. Studies based on mtDNA have reported this similarity (Yu 2004; Wu et al. 2005) which could result from mtDNA introgression (Alves et al. 2006, 2008), regardless of the most appropriate taxonomic classification of the specimens from China (Hoffmann and Smith 2005; Liu et al. 2011b; Cheng et al. 2012). Even though we could not formally explore this, our analyses show a discordance of mtDNA and nuclear phylogenies (Fig. 2 and 3), which may result from mtDNA introgression.

Biogeographic insights from comparative phylogeography

Our multilocus nuclear DNA phylogeny of *L. capensis sensu lato* suggested that the most ancient phylogenetic divide separates the African (north-western and southern) from the Asian (Near East, Arabian) populations (Fig. 3). The transition from Africa to Asia has been shown to be an important phylogeographic barrier for several animals, with estimates of segregation time varying from the Pliocene to the late Pleistocene, for example in jackals (Koepfli et al. 2015), cheetahs (Charruau et al. 2011), African desert jerboas (Ben Faleh et al. 2012) or geckos (Metallinou et al. 2012). The progressive aridification of the Saharo-Arabian region following the desert onset after the Late Miocene-Pliocene likely imposed strong dispersal barriers (Pokorny et al. 2015; Mairal et al. 2017). The alternation between

498 arid and humid conditions during the Pleistocene possibly allowed dispersal events between
499 north-eastern Africa and the Arabian Peninsula (Stewart et al. 2017), which may explain the
500 time-frame of the divergence inferred here for *L. capensis* (~1 Ma). For some groups, the
501 Asian clade is closely related to the eastern Africa one, such as for the helmeted terrapin
502 (Wong et al. 2010), or Dorcas gazelles (Lerp et al. 2011). Our lack of sampling from the East
503 Africa group for multilocus phylogenetic analyses does not allow clarifying the possible link
504 between East African and the Near East, but the mtDNA data seems to point in that direction
505 (Fig. 2), notwithstanding the possibility of mtDNA introgression from *L. europaeus* (see
506 above). The maintenance of dispersal corridors along the Nile and the Red Sea mountains
507 may have maintained North-South ecological corridors (Metallinou et al. 2012; Brito et al.
508 2014) (Fig. 1). The inferred admixture of our Niger sample in the microsatellite analysis and
509 its inclusion in the eastern Africa mtDNA clade may support East-West dispersal corridors of
510 savannah found immediately south of the desert, as suggested by vicariance-expansion
511 cycles inferred for murid rodents (Brouat et al. 2009; Dobigny et al. 2013).
512
513 In Africa, we found three major evolutionary units for the Cape hare – North, East and South
514 (Fig. 1). These phylogeographic divides are remarkably similar to those inferred for species
515 associated with African savannah, such as ungulates (Lorenzen et al. 2012), lions (Bertola et
516 al. 2016), giraffes (Brown et al. 2007; Fennessy et al. 2016) or rodents (Granjon et al. 2012;
517 Bryja et al. 2014). This suggests species persistence in refugia created by the expansion and
518 contraction of tropical forests and consequent savannah fragmentation, mostly associated
519 with changes in precipitation regimes along the glacial cycles (Dupont 2011). Interestingly,
520 despite similar geographic structures, estimated times of diversification vary among taxa,
521 from the Pliocene to Pleistocene. This suggests repeated contraction-expansions along Plio-
522 Pleistocene, possibly resulting in deeper divergence in less mobile species (such as rodents)
523 and more recent divergence times for more mobile mammals (such as hares or ungulates).
524

525 The marked phylogeographic structure we found for mtDNA in North Africa (Fig. 1) provides
526 important insights on the complexity of Sahara-Sahel biogeographical patterns, even though
527 this structure appeared diluted at biparentally inherited markers (Fig. 1; Figure S1). The
528 largest mtDNA haplogroup, NWA - I (Fig. 1 and 2), covers most of the Maghreb, and
529 expands throughout Tunisia and Libya. This suggests that the Atlas Mountains are
530 permeable to gene flow in hares, which contrasts with animals with low dispersal abilities
531 (Brown et al. 2002). Mitochondrial DNA clade NWA - II (Fig. 1 and 2) appears endemic to the
532 Atlantic Coastal Sahara, from the southern slopes of the Atlas Mountains to the Tagant
533 Mountain of Mauritania. Though delimited by the desert, this region benefits from humid
534 winds, and is prone to harbour endemic forms for several species, such as lizards (Velo-
535 Anton et al. 2018) and snakes (Gonçalves et al. 2018). The transition between clades II and
536 III does not coincide with apparent landscape barriers to gene flow (terrain is mostly flat and
537 the river present in the area, Lakra, is dry most of the year) and may represent a secondary
538 contact from populations temporarily restricted to refugia. A similar pattern was found in the
539 Schokari sand racer (Gonçalves et al. 2018). Clade NW-IV is represented by a single
540 haplotype in our work (Figs. 1 and 3), sampled in Central Sahel, Mali, and may represent a
541 distinct lineage delimited by the Niger river (e.g. Dobigny et al. 2013), as suggested in other
542 river systems (e.g. Brouat et al. 2009). Further sampling is needed to clarify this distinction.
543 To summarize, the complex physiography of the Sahara-Sahel with its mountain chains, river
544 systems and Atlantic and Mediterranean influence, seems to have promoted differentiation,
545 generating a heterogeneous gene pool that may have been homogenized at the nuclear
546 DNA level by male-mediated dispersal, likely along the Atlantic Sahara corridor (Fig. 1).
547
548 *Conclusions*
549
550 The inferred evolutionary history of *L. capensis sensu lato* over its range is complex, with
551 differentiation, deep divergence of African and Asian populations, and instances of
552 interspecific gene flow, possibly more pronounced at mtDNA. The phylogeographic structure

553 of the Cape hare finds parallel in other taxa, particularly those associated with African open
554 savannah habitats, with distinct lineages found in North, East and South Africa. Furthermore,
555 it suggests that the complex environmental heterogeneity in coastal areas of North Africa and
556 of the Sahara-Sahel region promoted differentiation, which appears to have been eroded at
557 the nuclear DNA possibly by male-biased dispersal. In Northeast Africa, the deep
558 phylogeographic break may be associated with cyclic barriers created by the aridification of
559 the Saharo-Arabian desert. Our results suggest that *L. capensis sensu lato* is not
560 monophyletic and current taxonomy does not reflect the complexity of its evolutionary history.
561 This study provides valuable information to guide future taxonomic revisions, which will be
562 important to foster the conservation of the evolutionary groups. Filling sampling gaps in
563 future studies, including neighbouring species, will provide a deeper understanding of the
564 transition between population groups, and allow a powerful quantification of the magnitude of
565 genetic isolation barriers that may persist among lineages. This study shows that assessing
566 the evolutionary history of species using multilocus approaches allows precise inferences of
567 population divergence, taking into account and measuring gene flow. Such inferences
568 contribute to an improved quantification and characterization of biodiversity, and to the
569 definition of appropriate conservation units.
570
571 **Acknowledgments**
572
573 This work was financially supported by Portuguese national funds through the Fundação
574 para a Ciência e a Tecnologia (FCT; project HybridAdapt, FCT-ANR/BIA-EVF/0250/2012).
575 J.C.B. and J.M.F. were supported by Programa Operacional Potencial Humano-Quadro de
576 Referência Estratégico Nacional (POPH-QREN) funds from the European Social Fund and
577 Portuguese Ministério da Ciência, Tecnologia e Ensino Superior through FCT
578 (IF/00459/2013 and IF/00033/2014 Investigador FCT research contracts, respectively). All
579 samples used in this work were collected before the Nagoya Protocol entered into force.
580 Museum samples from the Natural History Museum in Berlin were collected under the

financial support provided by SYNTHESYS grant DE-TAF-4131 (funded by the European Union under FP7 grant agreement 226506). North African field samples were collected with funds from National Geographic Society (CRE-7629-04, CRE-8412-08), Mohammed bin Zayed Species Conservation Fund (11052709, 11052707, 11052499, 13257467), Fundação para a Ciência e Tecnologia (PTDC/BIA-BEC/099934/2008, PTDC/BIA-BIC/2903/2012), and FEDER through COMPETE-Operational Programme for Competitiveness Factors (FCOMP-01-0124-FEDER-008917, -028276) to JCB. Additional support was obtained from project NORTE-01-0145-FEDER-000007 supported by the Norte Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). We thank MS Shah for sampling in Saudi Arabia. Franz Suchentrunk, Conrad Matthee, João Maia, Raquel Vasconcelos, Teresa Luísa Silva, Lahoussine Ouragh, the Natural History Museum in Berlin and the Natural History Museum in Vienna kindly contributed with samples for this study (voucher codes for the museum specimens are in Table S3). We thank the curators of these Natural History Museums for support. We also thank James Harris for his kind revision the manuscript. Author contributions: JMF and PCA devised the study with insights from JCB. SL and PCA sampled in the Natural History Museums. JCB led field expeditions to North Africa. MZI led sampling in Saudi Arabia. SL performed all laboratory work and analyses under supervision of JMF. SL and JMF wrote the paper. All authors discussed and interpreted the results, and read, revised and approved the final version of the manuscript.

Conflict of interests

The authors declare no conflict of interests.

Data Archiving

608 Newly obtained DNA sequences were deposited in GenBank with accession numbers
609 MK775981-MK776533. The microsatellite genotypes and DNA alignments were deposited in
610 Dryad (doi:####).

611

612 **References**

613 Adams M, Raadik TA, Burridge CP, Georges A (2014). Global Biodiversity Assessment and
614 Hyper-Cryptic Species Complexes: More Than One Species of Elephant in the Room? *Syst*
615 *Biol* **63**: 518-533.

616 Aghová T, Šumbera R, Piálek L, Mikula O, McDonough MM, Lavrenchenko LA et al. (2017)
617 Multilocus phylogeny of East African gerbils (Rodentia, Gerbilliscus) illuminates the history of
618 the Somali-Masai savanna. *J Biogeogr* **44**: 2295-2307.

619 Alves PC, Ferrand N, Suchentrunk F, Harris DJ (2003). Ancient introgression of *Lepus*
620 *timidus* mtDNA into *L. granatensis* and *L. europaeus* in the Iberian Peninsula. *Mol*
621 *Phylogenet Evol* **27**: 70-80.

622 Alves PC, Harris DJ, Melo-Ferreira J, Branco M, Ferrand N, Suchentrunk F et al. (2006).
623 Hares on thin ice: Introgression of mitochondrial DNA in hares and its implications for recent
624 phylogenetic analyses. *Mol Phylogenet Evol* **40**: 640-641.

625 Alves PC, Melo-Ferreira J, Freitas H, Boursot P (2008). The ubiquitous mountain hare
626 mitochondria: multiple introgressive hybridization in hares, genus *Lepus*. *Philos Trans Royal*
627 *Soc B* **363**: 2831-2839.

628 Avise JC (2009). Phylogeography: retrospect and prospect. *J Biogeogr* **36**: 3-15.

629 Ben Faleh A, Granjon L, Tatard C, BoratyŃSki Z, Cosson JF, Said K (2012). Phylogeography
630 of two cryptic species of African desert jerboas (Dipodidae: Jaculus). *Biol J Linn Soc* **107**: 27-
631 38.

632 Ben Slimen H, Suchentrunk F, Ben Ammar Elgaaied A (2008a). On shortcomings of using
633 mtDNA sequence divergence for the systematics of hares (genus Lepus): An example from
634 cape hares. *Mamm Biol* **73**: 25-32.

635 Ben Slimen H, Suchentrunk F, Memmi A, Sert H, Kryger U, Alves PC et al. (2006).
636 Evolutionary relationships among hares from North Africa (*Lepus* sp or *Lepus* spp.), cape
637 hares (*L. capensis*) from South Africa, and brown hares (*L. europaeus*), as inferred from
638 mtDNA PCR-RFLP and allozyme data. *J Zool Syst Evol Res* **44**: 88-99.

639 Ben Slimen H, Suchentrunk F, Shahin AB, Ben Ammar Elgaaied A (2007). Phylogenetic
640 analysis of mtCR-1 sequences of Tunisian and Egyptian hares (*Lepus* sp. or spp.,
641 Lagomorpha) with different coat colours. *Mamm Biol* **72**: 224-239.

642 Ben Slimen H, Suchentrunk F, Stamatis C, Mamuris Z, Sert H, Alves PC et al. (2008b).
643 Population genetics of cape and brown hares (*Lepus capensis* and *L. europaeus*): A test of
644 Petter's hypothesis of conspecificity. *Biochem Syst Ecol* **36**: 22-39.

Bertola LD, Jongbloed H, van der Gaag KJ, de Knijff P, Yamaguchi N, Hooghiemstra H et al. (2016). Phylogeographic Patterns in Africa and High Resolution Delineation of Genetic Clades in the Lion (*Panthera leo*). *Sci Rep* **6**: 30807.

Bi K, Linderoth T, Vanderpool D, Good JM, Nielsen R, Moritz C (2013). Unlocking the vault: next-generation museum population genomics. *Mol Ecol* **22**: 6018-6032.

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K et al. (2007). Cryptic species as a window on diversity and conservation. *Trends Ecol Evol* **22**: 148-155.

Brito JC, Godinho R, Martinez-Freiria F, Pleguezuelos JM, Rebelo H, Santos X et al. (2014). Unravelling biodiversity, evolution and threats to conservation in the Sahara-Sahel. *Biol Rev* **89**: 215-231.

Brouat C, Tatard C, Bâ K, Cosson J-F, Dobigny G, Fichet-Calvet E et al. (2009). Phylogeography of the Guinea multimammate mouse (*Mastomys erythroleucus*): a case study for Sahelian species in West Africa. *J Biogeogr* **36**: 2237-2250.

Brown DM, Brenneman RA, Koepfli KP, Pollinger JP, Mila B, Georgiadis NJ et al. (2007). Extensive population genetic structure in the giraffe. *BMC Biol* **5**: 57.

Brown RP, Suárez NM, Pestano J (2002). The Atlas mountains as a biogeographical divide in North–West Africa: evidence from mtDNA evolution in the Agamid lizard Agama impalearis. *Mol Phylogenet Evol* **24**: 324-332.

Bryja J, Mikula O, Šumbera R, Meheretu Y, Aghová T, Lavrenchenko LA et al. (2014). Pan-African phylogeny of *Mus* (subgenus *Nannomys*) reveals one of the most successful mammal radiations in Africa. *BMC Evol Biol* **14**: 256.

Charruau P, Fernandes C, Orozco-Terwengel P, Peters J, Hunter L, Ziaie H et al. (2011). Phylogeography, genetic structure and population divergence time of cheetahs in Africa and Asia: evidence for long-term geographic isolates. *Mol Ecol* **20**: 706-724.

Chen C, Durand E, Forbes F, François O (2007). Bayesian clustering algorithms ascertaining spatial population structure: a new computer program and a comparison study. *Mol Ecol Notes* **7**: 747-756.

Cheng C, Ge D, Xia L, Zhou C, Yang Q (2012). Morphometrics study on the so called "Cape hare" (Lagomorpha:Leporidae:*Lepus*) in China. *Acta Theriol Sinica* **32**: 275-286.

Cheng E, Hodges KE, Melo-Ferreira J, Alves PC, Scott Mills L (2014). Conservation implications of the evolutionary history and genetic diversity hotspots of the snowshoe hare. *Mol Ecol* **23**: 2929-2942.

Cheng YT, Liu J, Yang LQ, Sun C, Kong QP (2013). Mitochondrial DNA Content Contributes to Climate Adaptation Using Chinese Populations as a Model. *PLoS One* **8**.

Corander J, Marttinen P, Siren J, Tang J (2008). Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics* **9**: 539.

Coulon A, Fitzpatrick JW, Bowman R, Stith BM, Makarewich CA, Stenzler LM et al. (2008). Congruent population structure inferred from dispersal behaviour and intensive genetic surveys of the threatened Florida scrub-jay (Aphelocoma coerulescens). *Mol Ecol* **17**: 1685-1701.

685 Darriba D, Taboada GL, Doallo R, Posada D (2012). jModelTest 2: more models, new
686 heuristics and parallel computing. *Nat Methods* **9**: 772.

687 Dobigny G, Tatard C, Gauthier P, Ba K, Duplantier JM, Granjon L et al. (2013). Mitochondrial
688 and nuclear genes-based phylogeography of *Arvicanthis niloticus* (Murinae) and sub-
689 Saharan open habitats pleistocene history. *PLoS One* **8**: e77815.

690 Drew C (2000). The distribution of the Cape Hare, *Lepus capensis*, in Abu Dhabi Emirate,
691 United Arab Emirates. *Zool Middle East* **20**: 15-20.

692 Drew C, O'Donovan D, Simkins G, Al Dosary M, Al Khaldi AM, Mohammed OB et al. (2008).
693 *Lepus capensis*. *The IUCN Red List of Threatened Species* **2008**: e.T41277A10429185.

694 Drummond AJ, Ho SY, Phillips MJ, Rambaut A (2006). Relaxed phylogenetics and dating
695 with confidence. *PLoS Biol* **4**: e88.

696 Drummond AJ, Rambaut A (2007). BEAST: Bayesian evolutionary analysis by sampling
697 trees. *BMC Evol Biol* **7**: 214.

698 Dupont LM (2011). Orbital scale vegetation change in Africa. *Quaternary Sci Rev* **30**: 3589–
699 3602.

700 Durand E, Jay F, Gaggiotti OE, Francois O (2009). Spatial inference of admixture proportions
701 and secondary contact zones. *Mol Biol Evol* **26**: 1963-1973.

702 Earl DA, vonHoldt BM (2012). STRUCTURE HARVESTER: a website and program for
703 visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet*
704 *Resour* **4**: 359-361.

705 Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using
706 the software STRUCTURE: a simulation study. *Mol Ecol* **14**: 2611-2620.

707 Fahey AL, Ricklefs RE, Dewoody JA (2014). DNA-based approaches for evaluating historical
708 demography in terrestrial vertebrates. *Biol J Linn Soc* **112**: 367-386.

709 Falush D, Stephens M, Pritchard JK (2003). Inference of population structure using
710 multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**: 1567-
711 1587.

712 Fennessy J, Bidon T, Reuss F, Kumar V, Elkan P, Nilsson MA et al. (2016). Multi-locus
713 Analyses Reveal Four Giraffe Species Instead of One. *Curr Biol* **26**: 2543-2549.

714 Fickel J, Schmidt A, Putze M, Spittler H, Ludwig A, Streich WJ et al. (2005). Genetic
715 structure of populations of European brown hare: Implications for management. *J Wildlife*
716 *Manage* **69**: 760-770.

717 Flux JEC, Angermann R (1990). The hares and jackrabbits. In: A. CJ and Flux JEC (eds)
718 *Rabbits, Hares and Pikas. Status Survey and Conservation Action Plan.* IUCN: Switzerland.

719 Fujita MK, Leaché AD, Burbrink FT, McGuire JA, Moritz C (2012). Coalescent-based species
720 delimitation in an integrative taxonomy. *Trends Ecol Evol* **27**: 480-488.

721 Goncalves DV, Martinez-Freiria F, Crochet PA, Geniez P, Carranza S, Brito JC (2018). The
722 role of climatic cycles and trans-Saharan migration corridors in species diversification:
723 Biogeography of Psammophis schokari group in North Africa. *Mol Phylogenet Evol* **118**: 64-
724 74.

725 Goudet J (1995). FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *J*
726 *Hered* **86**: 485-486.

727 Granjon L, Colangelo P, Tatard C, Colyn M, Dobigny G, Nicolas V (2012) Intrageneric
728 relationships within *Gerbilliscus* (Rodentia, Muridae, Gerbillinae), with characterization of an
729 additional West African species. *Zootaxa* **3325**: 1-25.

730 Gutiérrez-García TA, Vázquez-Domínguez E (2011). Comparative Phylogeography:
731 Designing Studies while Surviving the Process. *BioScience* **61**: 857-868.

732 Halanych KM, Demboski JR, van Vuuren BJ, Klein DR, Cook JA (1999). Cytochrome b
733 phylogeny of North American hares and jackrabbits (*Lepus*, Lagomorpha) and the effects of
734 saturation in outgroup taxa. *Mol Phylogenet Evol* **11**: 213-221.

735 Hamill RM, Doyle D, Duke EJ (2007). Microsatellite analysis of mountain hares (*Lepus*
736 *timidus hibernicus*): Low genetic differentiation and possible sex-bias in dispersal. *J Mammal*
737 **88**.

738 Hasegawa M, Kishino H, Yano T (1985). Dating of the human-ape splitting by a molecular
739 clock of mitochondrial DNA. *J Mol Evol* **22**: 160-174.

740 Heled J, Drummond AJ (2010). Bayesian inference of species trees from multilocus data.
741 *Mol Biol Evol* **27**: 570-580.

742 Hey J (2010). Isolation with migration models for more than two populations. *Mol Biol Evol*
743 **27**: 905-920.

744 Hey J, Nielsen R (2004). Multilocus methods for estimating population sizes, migration rates
745 and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and
746 *D. persimilis*. *Genetics* **167**: 747-760.

747 Hoffmann RS, Smith A (2005). Order Lagomorpha. In: Wilson DE, Reeder DM (eds) *Mammal*
748 *Species of the World (third edition)*. Johns Hopkins University Press: Baltimore.

749 Jakobsson M, Rosenberg NA (2007). CLUMPP: a cluster matching and permutation program
750 for dealing with label switching and multimodality in analysis of population structure.
751 *Bioinformatics* **23**: 1801-1806.

752 Kingdon J (2013). Mammalian evolution in Africa. In: Kingdon J, Butynski TM, Happold DCD,
753 Happold M, Hoffmann M (eds) *The Mammals of Africa, Introductory chapters and Afrotheria*.
754 Bloomsbury: Amsterdam.

755 Koepfli KP, Pollinger J, Godinho R, Robinson J, Lea A, Hendricks S et al. (2015). Genome-
756 wide Evidence Reveals that African and Eurasian Golden Jackals Are Distinct Species. *Curr*
757 *Biol* **25**: 2158-2165.

758 Kryger U, Robinson TJ, Bloomer P (2002). Isolation and characterization of six polymorphic
759 microsatellite loci in South African hares (*Lepus saxatilis* F. Cuvier, 1823 and *Lepus capensis*
760 Linnaeus, 1758). *Mol Ecol Notes* **2**: 422-424.

761 Lanier HC, Knowles LL (2012). Is Recombination a Problem for Species-Tree Analyses?
762 *Syst Biol* **61**: 691-701.

763 Laval G, Excoffier L (2004). SIMCOAL 2.0: a program to simulate genomic diversity over
764 large recombining regions in a subdivided population with a complex history. *Bioinformatics*
765 **20**: 2485-2487.

766 Leigh JW, Bryant D (2015). popart: full-feature software for haplotype network construction.
767 *Methods Ecol Evol* **6**: 1110-1116.

768 Lerp H, Wronski T, Pfenninger M, Plath M (2011). A phylogeographic framework for the
769 conservation of Saharan and Arabian Dorcas gazelles (Artiodactyla: Bovidae). *Org Divers*
770 *Evol* **11**: 317.

771 Levänen R, Thulin C-G, Spong G, Pohjoismäki JLO (2018). Widespread introgression of
772 mountain hare genes into Fennoscandian brown hare populations. *PLoS One* **13**: e0191790.

773 Liu J, Chen P, Yu L, Wu SF, Zhang YP, Jiang XL (2011a). The taxonomic status of *Lepus*
774 *melainus* (Lagomorpha: Leporidae) based on nuclear DNA and morphological analyses.
775 *Zootaxa*: 47-57.

776 Liu J, Yu L, Arnold ML, Wu CH, Wu SF, Lu X et al. (2011b). Reticulate evolution: frequent
777 introgressive hybridization among chinese hares (genus *Lepus*) revealed by analyses of
778 multiple mitochondrial and nuclear DNA loci. *BMC Evol Biol* **11**.

779 Lorenzen ED, Heller R, Siegismund HR (2012). Comparative phylogeography of African
780 savannah ungulates. *Mol Ecol* **21**: 3656-3670.

781 Mairal M, Sanmartín I, Pellissier L (2017). Lineage-specific climatic niche drives the tempo of
782 vicariance in the Rand Flora. *J Biogeogr* **44**: 911-923.

783 Matthee CA, van Vuuren BJ, Bell D, Robinson TJ (2004). A molecular supermatrix of the
784 rabbits and hares (Leporidae) allows for the identification of five intercontinental exchanges
785 during the Miocene. *Syst Biol* **53**: 433-447.

786 Melo-Ferreira J, Alves PC, Freitas H, Ferrand N, Boursot P (2009). The genomic legacy from
787 the extinct *Lepus timidus* to the three hare species of Iberia: contrast between mtDNA, sex
788 chromosomes and autosomes. *Mol Ecol* **18**: 2643–2658.

789 Melo-Ferreira J, Alves PC, Rocha J, Ferrand N, Boursot P (2011). Interspecific X-
790 Chromosome and Mitochondrial DNA Introgression in the Iberian hare: Selection or Allele
791 Surfing? *Evolution* **65**: 1956-1968.

792 Melo-Ferreira J, Boursot P, Carneiro M, Esteves PJ, Farelo L, Alves PC (2012). Recurrent
793 Introgression of Mitochondrial DNA Among Hares (*Lepus* spp.) revealed by Species-tree
794 Inference and Coalescent Simulations. *Syst Biol* **61**: 367-381.

795 Melo-Ferreira J, Boursot P, Randi E, Kryukov A, Suchentrunk F, Ferrand N et al. (2007). The
796 rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion
797 and retreat with hybridization in the Iberian Peninsula. *Mol Ecol* **16**: 605-618.

798 Melo-Ferreira J, Boursot P, Suchentrunk F, Ferrand N, Alves PC (2005). Invasion from the
799 cold past: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into
800 three other hare species in northern Iberia. *Mol Ecol* **14**: 2459-2464.

801 Melo-Ferreira J, Farelo L, Freitas H, Suchentrunk F, Boursot P, Alves PC (2014a). Home-
802 loving boreal hare mitochondria survived several invasions in Iberia: the relative roles of
803 recurrent hybridisation and allele surfing. *Heredity* **112**: 265-273.

804 Melo-Ferreira J, Seixas FA, Cheng E, Mills LS, Alves PC (2014b). The hidden history of the
805 snowshoe hare, *Lepus americanus*: extensive mitochondrial DNA introgression inferred from
806 multilocus genetic variation. *Mol Ecol* **23**: 4617-4630.

807 Metallinou M, Arnold EN, Crochet P-A, Geniez P, Brito JC, Lymberakis P et al. (2012).
808 Conquering the Sahara and Arabian deserts: systematics and biogeography of
809 *Stenodactylus* geckos (Reptilia: Gekkonidae). *BMC Evol Biol* **12**: 258.

810 Moores R, Brown D, Martin R, Lees AC (2012). Status and identification of hares *Lepus* sp.
811 in Western Sahara and Southern Marocco. *Go-South Bulletin* **9**: 126-130.

812 Nosil P, Harmon LJ, Seehausen O (2009). Ecological explanations for (incomplete)
813 speciation. *Trends Ecol Evol*. **24**: 145-156.

814 Pisa G, Orioli V, Spilotros G, Fabbri E, Randi E, Bani L (2015). Detecting a hierarchical
815 genetic population structure: the case study of the Fire Salamander (*Salamandra*
816 *salamandra*) in Northern Italy. *Ecol Evol* **5**: 743-758.

817 Pokorny L, Riina R, Mairal M, Meseguer AS, Culshaw V, Cendoya J et al. (2015). Living on
818 the edge: timing of Rand Flora disjunctions congruent with ongoing aridification in Africa.
819 *Front Genet* **6**: 154.

820 Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using
821 multilocus genotype data. *Genetics* **155**: 945-959.

822 Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarisation in
823 Bayesian phylogenetics using Tracer 1.7. *Syst Biol* **67**: 901-904.

824 Ramírez-Silva JP, González-Cózatl FX, Vázquez-Domínguez E, Cervantes FA (2010).
825 Phylogenetic position of Mexican jackrabbits within the genus Lepus (Mammalia:
826 Lagomorpha): a molecular perspective. *Revista Mexicana de Biodiversidad* **81**: 721-731.

827 Raymond M, Rousset F (1995). GENEPOP (version 1.2): a population genetics software for
828 exact tests and ecumenicism. *J Hered* **86**: 248-249.

829 Schai-Brown S, Hackländer K (2018). Cape hare, *Lepus capensis* Linnaeus 1758. In: Smith
830 AT, Johnston CH, Alves PC and Hackländer K (eds) *Lagomorphs: Pikas, Rabbits and Hares*
831 *of the World*. John Hopkins University Press: Baltimore, USA.

832 Scheffers BR, Joppa LN, Pimm SL, Laurance WF (2012). What we know and don't know
833 about Earth's missing biodiversity. *Trends Ecol Evol* **27**: 501-510.

834 Stephens M, Smith NJ, Donnelly P (2001). A new statistical method for haplotype
835 reconstruction from population data. *Am J Hum Genet* **68**: 978-989.

836 Stewart M, Louys J, Price GJ, Drake NA, Groucutt HS, Petraglia MD (2017). Middle and Late
837 Pleistocene mammal fossils of Arabia and surrounding regions: Implications for
838 biogeography and hominin dispersals. *Quatern Int*.

839 Strasburg JL, Rieseberg LH (2010). How robust are "Isolation with Migration" analyses to
840 violations of the IM model? A simulation study. *Mol Biol Evol* **27**: 297-310.

841 Sukumaran J, Knowles LL (2017). Multispecies coalescent delimits structure, not species. *P*
842 *Natl Acad Sci USA* **114**: 1607.

843 Thulin CG, Fang M, Averianov AO (2006). Introgression from *Lepus europaeus* to *L. timidus*
844 in Russia revealed by mitochondrial single nucleotide polymorphisms and nuclear
845 microsatellites. *Hereditas* **143**: 68-76.

Tolesa Z, Bekele E, Tesfaye K, Ben Slimen H, Valqui J, Getahun A et al. (2017). Mitochondrial and nuclear DNA reveals reticulate evolution in hares (*Lepus* spp., Lagomorpha, Mammalia) from Ethiopia. *PLoS One* **12**: e0180137.

Vallone PM, Butler JM (2004). AutoDimer: a screening tool for primer-dimer and hairpin structures. *Biotechniques* **37**: 226-231.

Velo-Anton G, Martinez-Freiria F, Pereira P, Crochet PA, Brito JC (2018). Living on the edge: Ecological and genetic connectivity of the spiny-footed lizard, *Acanthodactylus aureus*, confirms the Atlantic Sahara desert as a biogeographic corridor and centre of lineage diversification. *J Biogeogr* **45**: 1031-1042.

Wallner B, Huber S, Achmann R (2001). Non-invasive PCR sexing of rabbits (*Oryctolagus cuniculus*) and hares (*Lepus europaeus*). *Mamm Biol* **66**: 190-192.

Woerner AE, Cox MP, Hammer MF (2007). Recombination-filtered genomic datasets by information maximization. *Bioinformatics* **23**: 1851-1853.

Wong RA, Fong JJ, Papenfuss TJ (2010). Phylogeography of the African Helmeted Terrapin, *Pelomedusa subrufa*: Genetic Structure, Dispersal, and Human Introduction. *Proc Calif Acad Sci* **61**: 575–585.

Wu C, Wu J, Bunch TD, Li Q, Wang Y, Zhang YP (2005). Molecular phylogenetics and biogeography of *Lepus* in Eastern Asia based on mitochondrial DNA sequences. *Mol Phylogenet Evolution* **37**: 45-61.

Yu X (2004). Molecular Systematics of the Genus Lepus in China. Institute of Zoology, Chinese Academy of Sciences, Beijing.

Zubair M, Shukkur EAA, Azeez PA, Jayson EA (2011). Feeding behaviour of three species of falcons in the wild in united arab emirates. *Millenium Zoology* **12**: 13-19.

Zwickl DJ (2006). Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. University of Texas, Austin.

Tables

Table 1: Isolation-with-migration maximum likelihood estimates (95% posterior density intervals in parentheses, when estimated) of demographic parameters obtained with IMa2 between three pairs of populations.

Pop.1	Pop.2	*Ne1*[†]	*Ne2*[†]	*NeA*[†]	*t*[‡]	2Nm1→2[§]	2Nm2→1[§]
NW Africa	Arabia	1,080,674	177,353	63,847	716,508	0.02 (n. s.)	0.00 (n. s.)
		(830,014; 1,411,733)	(96,953; 190,859)	-	(489,495; 1,000,746)	(0.00; 0.81)	-
S Africa	NW Africa	218,983	1,107,427	68,823	767,482	0.01 (n. s.)	0.15 (n. s.)
		-	(798,766; 1,548,868)	-	(433,794; 1,190,153)	-	-
Near East	*L. europaeus*	352,933	161,392	221,691	860,755	0.01 (n. s.)	0.06 (n. s.)
		(207,503; 686,358)	(83,356; 278,445)	-	-	-	-

[†]Effective population size of population 1 (Ne1), 2 (Ne2) and the ancestral population (NeA); [‡]Time in years since species 1 and 2 split; [§]Population migration rate into population 1 (2Nm2→1) and population 2 (2Nm1→2), n.s. – not significant.

882 **Figure Legends**

883

884 Figure 1: Geographic distribution of *Lepus capensis* and samples unambiguously attributed
885 to a microsatellite cluster. Grey background indicates the distribution of *L. capensis*
886 according to IUCN Red List (www.redlist.org). Symbols indicate sampling localities: shapes
887 indicate the eight mtDNA lineages (according to the BAPS and network analyses, which
888 agrees with the phylogenetic inference shown in Fig. 2) and their grey shades depict the five
889 evolutionary groups inferred from microsatellites (see Figure S1). Symbols are not
890 proportional to the number of individuals samples in the locality and more than one specimen
891 may be represented in each geographical point. Arrows indicate hypothesized dispersal
892 corridors in the Sahara-Sahel – Atlantic Sahara, Nile River and Red Sea mountains (Brito et
893 al. 2014).

894

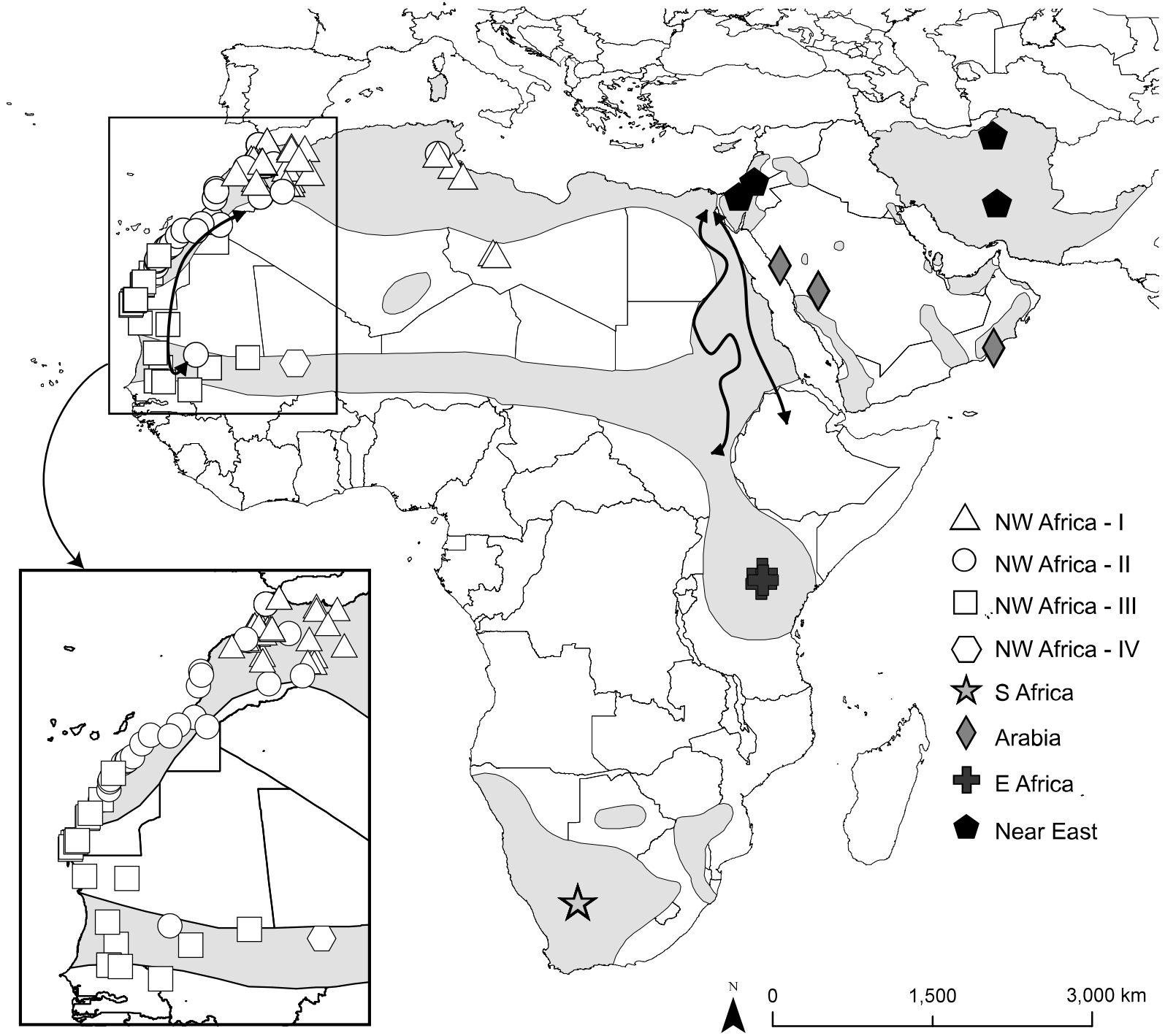
895 Figure 2: Bayesian inference mtDNA phylogeny of *L. capensis*, *L. saxatilis* and Eurasian hare
896 species (*L. timidus*, *L. europaeus* and *L. granatensis*), rooted by a rabbit (*Oryctolagus*
897 *cuniculus*) haplotype. Bayesian posterior probabilities and maximum likelihood bootstrap
898 supports (scaled from 0 to 1) are shown next to nodes when the first is above 0.50
899 (Bayesian/Maximum likelihood inferences). *L. capensis* clades agree with those depicted in
900 Fig. 1.

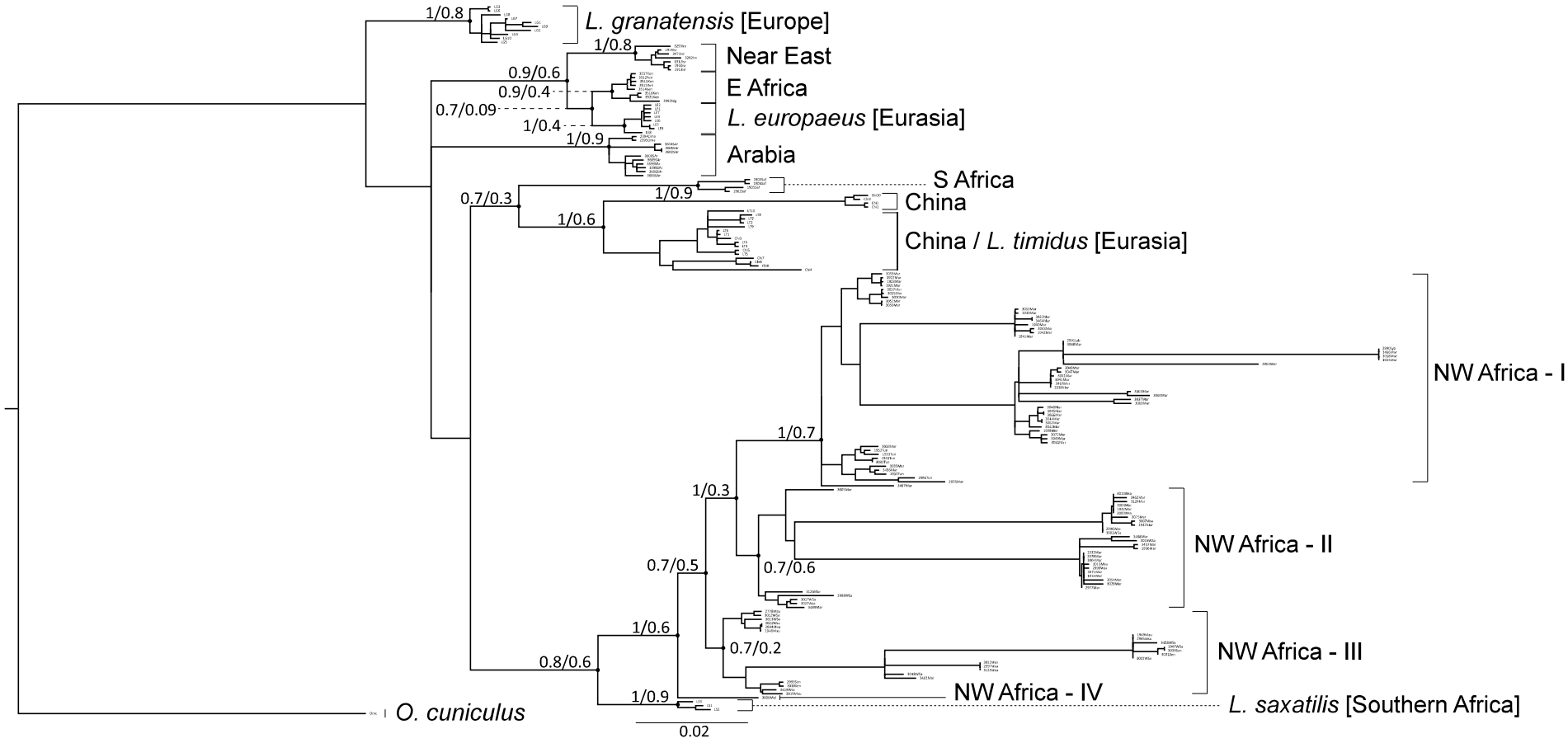
901

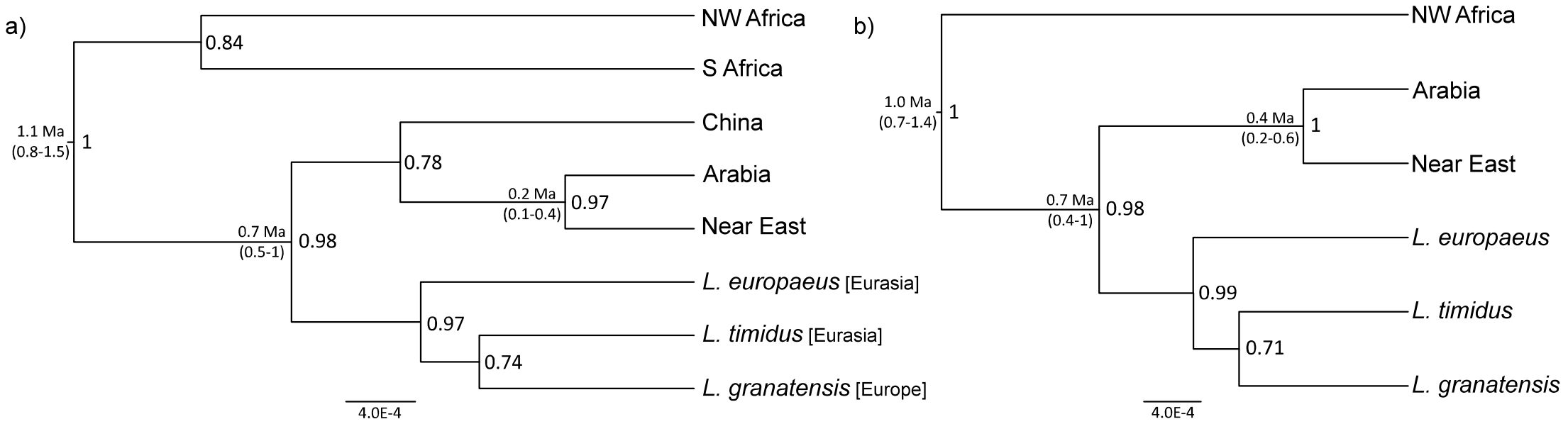
902 Figure 3: Nuclear DNA species tree inferred from eight nuclear loci (posterior probabilities
903 are shown on the right of each node), including (a) African and non-African populations of *L.*
904 *capensis* and Eurasian *Lepus* species and (b) excluding South African and Chinese
905 populations, for which not all loci were sequenced. Estimates of split times in units of million
906 years (Ma) are indicated for *L. capensis sensu lato* nodes supported by posterior
907 probabilities above 0.95 (95% confidence intervals shown in brackets).

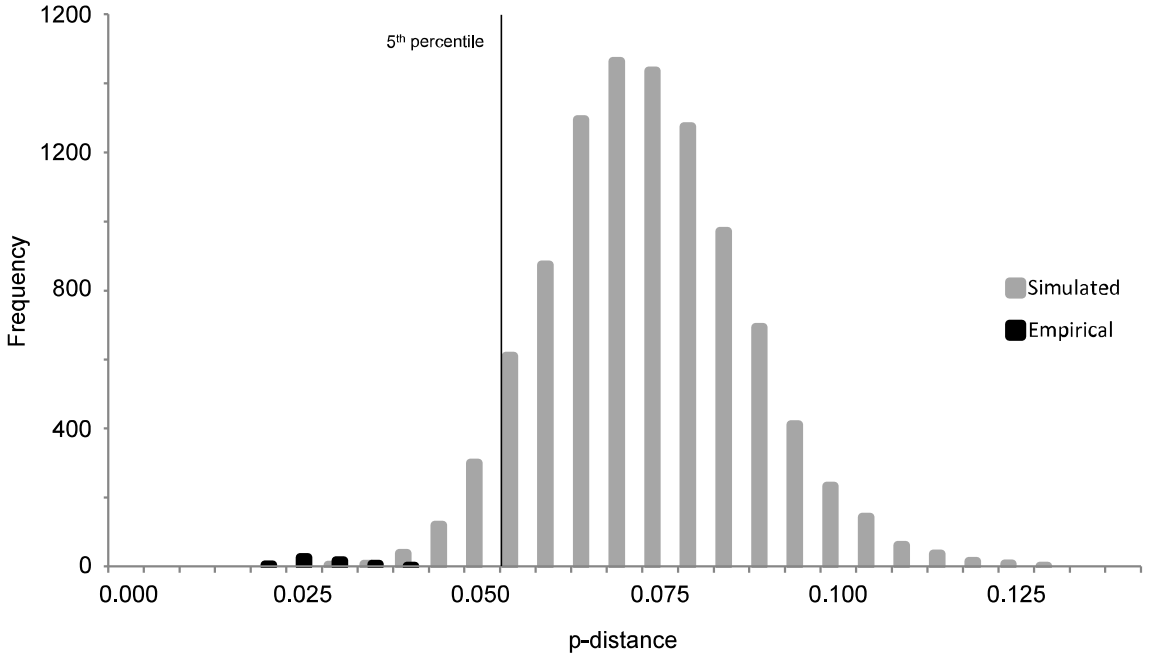
908

909 Figure 4: Distribution of empirical and simulated uncorrected p-distances between European
910 *L. europaeus* and the Near East *L. capensis* population. Simulations were conducted based
911 on population parameters estimated with the isolation-with-migration multi-locus analysis.
912 Grey bars show the distribution of minimum pairwise uncorrected p-distances per simulation
913 between *L. europaeus* and the Near East *L. capensis* population (the vertical line indicates
914 the 5^{th} percentile) and black bars depict the empirical pairwise p-distances between the same
915 populations.


NW Africa - I
NW Africa - II
NW Africa - III
NW Africa - IV
S Africa
Arabia
E Africa
Near East
N
0
1,500
3,000 km


1/0.8
L. granatensis [Europe]
1/0.8
Near East
0.9/0.6
0.9/0.4
E Africa
0.7/0.09
L. europaeus [Eurasia]
1/0.4
1/0.9
Arabia
S Africa
0.7/0.3
1/0.9
China
1/0.6
China / L. timidus [Eurasia]
NW Africa - I
1/0.7
1/0.3
NW Africa - II
0.7/0.5
0.7/0.6
1/0.6
NW Africa - III
0.7/0.2
0.8/0.6
NW Africa - IV
1/0.9
L. saxatilis [Southern Africa]
O. cuniculus
0.02


a)
NW Africa
0.84
S Africa
1.1 Ma
(0.8-1.5)
1
China
0.78
Arabia
0.2 Ma
(0.1-0.4)
0.97
Near East
0.7 Ma
(0.5-1)
0.98
L. europaeus [Eurasia]
0.97
L. timidus [Eurasia]
0.74
L. granatensis [Europe]
4.0E-4
b)
NW Africa
1.0 Ma
(0.7-1.4)
1
Arabia
0.4 Ma
(0.2-0.6)
1
Near East
0.7 Ma
(0.4-1)
0.98
L. europaeus
0.99
L. timidus
0.71
L. granatensis
4.0E-4


1200
1200
800
400
0
Frequency
5th percentile
Simulated
Empirical
0.000
0.025
0.050
0.075
0.100
0.125
p-distance