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SHORT COMMUNICATION

A phantom extinction? New insights into extinction dynamics of the Don-hare *Lepus tanaiticus*

S. PROST*[15], M. KNAPP*[25], J. FLEMMIG†, A. K. HUFTHAMMER‡, P. KOSINTSEV§, M. STILLER*[3] & M. HOFREITER*[4]

*Research Group Molecular Ecology, Max-Planck Institute for Evolutionary Anthropology, Leipzig, Germany
†Institute for Medical Physics and Biophysics, Medical Department, University of Leipzig, Leipzig, Germany
‡University of Bergen, Natural History Collections, Bergen, Norway
§Institute of Plant and Animal Ecology RAS, Yekaterinburg, Russia

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Abstract

The Pleistocene to Holocene transition was accompanied by a worldwide extinction event affecting numerous mammalian species. Several species such as the woolly mammoth and the giant deer survived this extinction wave, only to go extinct a few thousand years later during the Holocene. Another example for such a Holocene extinction is the Don-hare, *Lepus tanaiticus*, which inhabited the Russian plains during the late glacial. After being slowly replaced by the extant mountain hare (*Lepus timidus*), it eventually went extinct during the middle Holocene. Here, we report the phylogenetic relationship of *L. tanaiticus* and *L. timidus* based on a 339-basepair (bp) fragment of the mitochondrial D-loop. Phylogenetic tree- and network reconstructions do not support *L. tanaiticus* and *L. timidus* being different species. Rather, we suggest that the two taxa represent different morphotypes of a single species and the extinction of '*L. tanaiticus*' represents the disappearance of a local morphotype rather than the extinction of a species.

Introduction

The extinction of numerous mammals at the Pleistocene to Holocene transition is a very well known and extensively studied phenomenon (Martin & Klein, 1984; Barnosky *et al.*, 2004). Several hypotheses have been suggested as causes of this mass extinction; most prominent among them are climate change and the arrival of modern humans, respectively (Barnosky *et al.*, 2004). While most of the continental large mammals went extinct before the beginning of the Holocene, some like the giant deer (*Megaloceros giganteus*, Stuart *et al.*, 2004), the woolly mammoth (*Mammuthus primigenius*; Vartanyan *et al.*, 1993) or the Caribbean ground sloths (Steadman *et al.*, 2005) survived until the middle Holocene, albeit in restricted areas. Another species that is believed to have survived until the Holocene is the Russian Don-hare (*Lepus tanaiticus*; Kosintsev, 2007). In contrast to other extinct mammals, the Don-hare is a relatively small mammal with a high reproductive rate, whereas most recorded extinctions affected large mammals having low reproductive rates (Stuart, 1991; Johnson *et al.*, 2002).

The Don-hare belongs to the arctic hare group (Genus *Lepus*, subgenus *Lepus*), which consists of three extant species: the arctic hare (*Lepus arcticus*), the Alaskan hare (*Lepus othus*) and the mountain hare (*Lepus timidus*; Wilson & Reeder, 2005). The mountain hare is the only European species of this group, ranging throughout most of northern Europe and Asia. The Alaskan hare is confined to Alaska today, but during the Pleistocene its

Correspondence: Stefan Prost, Research Group Molecular Ecology, Max-Planck Institute for Evolutionary Anthropology, Leipzig, Germany.
Tel.: +43 6649250243; fax: +43 1790449001;
e-mail: stefan.prost@gmi.oeaw.ac.at
[1]Present address: Gregor Mendel Institute of Molecular Plant Biology, Vienna, Austria.
[2]Present address: Department of Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand.
[3]Present address: Department of Biology, Pennsylvania State University, University Park, PA, USA.
[4]Present address: Department of Biology, University of York, York, UK.
[5]These authors contributed equally to the manuscript.

range extended to North East Siberia. Finally, the arctic hare can be found throughout northern North America and in parts of Greenland. The extinct Don-hare (*L. tanaiticus*, Gureev, 1964) is known from Pleistocene to late Holocene layers in Russia including the European part of Russia, the Ural Mountains and Siberia (Gureev, 1964; Hopkins *et al.*, 1982; Averianov, 1998; Kosintsev, 2003; Pitulko *et al.*, 2004; Strukova *et al.*, 2006). During the Holocene, it was slowly replaced by *L. timidus* and went extinct during the subboreal (5–2.5 kya; Bachura & Kosintsev, 2007; Kosintsev, 2007). This replacement took place gradually from the south to the north (Kosintsev, 2007). *Lepus tanaiticus* was common in the Urals but has not been reported from outside Russia, whereas *L. timidus* was the most common hare species in Europe during the last glacial period (Lopez-Martinez, 1980) but has never been found in Pleistocene layers in Russia (Hopkins *et al.*, 1982; Averianov, 1998).

Lepus tanaiticus has been described as an independent species on the basis of it being about 10% larger in size than *L. timidus* as well as based on characteristic deep dental portions in the lower jaw, especially on the p3 (Gureev, 1964). Its independent species status was confirmed by Averianov (1998), who studied remains of this species from seven different Upper Pleistocene localities in the Russian Plain. However, he also pointed out difficulties in assigning fossil hare remains to species. In contrast, Kosintsev (2007) considered the independent species status of the Don-hare as questionable.

Size and dental morphology are generally considered poor markers for species assignment. Body size can vary depending on population density, population growth rate, diet, home range, metabolic and physiological variables and other parameters (Damuth & MacFadden, 1990). Brown bears, for instance, vary substantially in size across their range while still representing a single species (Hilderbrand *et al.*, 1999; Zedrosser *et al.*, 2006). Dental morphology can also be influenced by environmental factors, for example by local nutritional adaptations (e.g. Ungar, 1998). Hence, it remains unclear whether *L. tanaiticus* and *L. timidus* can indeed be considered independent allopatric species or merely represent different local morphotypes of the same species (Kosintsev, 2007). Resolving the phylogenetic status of *L. tanaiticus* and *L. timidus* is therefore essential for our understanding of past morphological and genetic diversity within the arctic hares and of mammalian extinctions during the Holocene.

Ancient DNA techniques provide a tool for evaluating the taxonomic position of extinct species. Using a molecular phylogenetic approach, the phylogenic position of many extinct mammal species such as the woolly mammoth (Rohland *et al.*, 2007), the giant deer (Lister *et al.*, 2005), the cave bear (Krause *et al.*, 2008) as well as some extinct bird species such as the Tasman booby (Steeves *et al.*, 2009) and the dodo (Shapiro *et al.*, 2002) have been unambiguously resolved. Steeves and colleagues used ancient DNA to reject the independent species status of the Tasman booby and thus removed this bird species from the long list of extinct taxa. Other studies used ancient DNA to investigate population relationships in extant and extinct species (e.g. Barnes *et al.*, 2002; Leonard *et al.*, 2007; Knapp *et al.*, 2009). The aim of this study was to shed light on the phylogenetic relationships of the Eurasian late glacial representatives of the arctic hare clade and therefore improve our knowledge of Holocene extinction dynamics.

Materials and methods

Collection site

The site Pymva Shor is situated in the valley stream Pymva Shor (67°10′N; 60°51′E), a small tributary to the Adzva river in the Polar Urals. It is located on a bedrock ledge just under an overhanging limestone rock. At the base of the sequence is a 1-m-thick layer of *in situ* weathered limestone rubble. This bed is covered by a thin layer of sorted sand, most likely of Aeolian origin. On top is a wedge of blocky colluvium of Holocene age. The Don-hare (*L. tanaiticus*) samples were found in layers of limestone rubble (Mangerud *et al.*, 1999; Svendsen *et al.*, 2008). Along with the Don-hare samples bones from arctic fox (*Alopex lagopus*), cave lion (*Panthera leo spelaea*), horse (*Equus* sp.), reindeer (*Rangifer tarandus*), bison (*Bison priscus*), musk ox (*Ovibos moschatus*), narrow-skulled vole (*Microtus gregalis*), collared lemming (*Dicrostonyx gulielmi*), siberian lemming (*Lemmus sibiricus*) and birds were found (Smirnov *et al.*, 1999; Svendsen *et al.*, 2008).

Ancient DNA extraction and amplification

Ancient DNA was extracted from 170 to 260 mg of bone powder originating from six *L. tanaiticus* bones according to the standard silica-based extraction method of Rohland & Hofreiter (2007). Primers for PCR amplification were designed using the web-based tool Primer 3.0 (Rozen & Skaletsky, 2000) adhering mostly to the default parameters. All primer sequences are listed in Table S1. Because of the fragmented nature of ancient DNA (Pääbo *et al.* (2004), Poinar *et al.* (2006)), the maximum amplicon size was chosen not to exceed approximately150 bp (including primers), whereas a minimum fragment size of about 50 bp (including primers) is determined by the length of the primers. The maximum melting temperature difference between forward and reverse primer was reduced to 2 °C. Initial amplifications were performed using a two-step multiplex PCR (Römpler *et al.*, 2006) with a temperature profile for the first and the second step consisting of a 9-min initial Taq activation step at 95 °C, followed by 35 cycles of 94 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s and a final extension for 5 min at 72 °C. Replication of the fragments by a second,

independent PCR was performed using a 60-cycle PCR approach as described in Hofreiter *et al.* (2002) with a 9-min initial Taq activation at 94 °C, followed by 60 cycles of 93 °C for 30 s, 50 °C for 45 s, 72 °C for 45 s and a final extension for 10 min at 72 °C. All PCRs contained 2.5 units Taq Gold, 1× Taq Gold Buffer, 0.625 mM of each dNTP, 0.25 M of each primer, 1 mg mL^{-1} BSA and 4.0 mM $MgCl_2$ in a total reaction volume of 20 μL. In each set of amplifications, three PCR blanks were carried out to monitor potential contamination. All pre-PCR work was carried out in a clean room environment built for handling ancient DNA to avoid external contamination from modern DNA and PCR products. Five of six extracts contained ancient DNA of sufficient quality to amplify fragments larger than 70 bp.

Cloning, sequencing and computational analysis

All amplicons were cloned using the Topo TA cloning Kit (Invitrogen, Gronigen, the Netherlands) and sequenced on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA). A minimum of three clones per PCR were sequenced and analyzed, and consensus sequences were created using Vector NTI AdvanceTM10 (Lu & Moriyama, 2004). Each sequence position was determined from at least two independent amplifications to avoid sequence errors caused by template damage (Hofreiter *et al.*, 2001; Briggs *et al.*, 2007; Brotherton *et al.*, 2007). If consistent differences between two independent amplifications were found, we performed a third PCR to clarify which nucleotide represents the correct sequence. The sequences of *L. tanaiticus* can be accessed via GenBank (Accession numbers: HM126481–HM126485, Table S2). We used sequences from Waltari *et al.* (2004) (Genbank Accessions: AY422231–AY422328) and two sequences of *L. europaeus* from Kasapidis *et al.* (2005) (Genbank Accessions: AY466812 and AY466828) in the hierarchical tree reconstruction and an extended set of only *L. timidus* sequences from Waltari & Cook (2005) and five *L. europaeus* from Kasapidis *et al.* (2005) for the nonhierarchical network reconstruction (see Table S3 online supplementary materials). The latter set was used to investigate whether the *L. tanaiticus* sequences were clustered phylogenetically and/or geographically within the genetic diversity of *L. timidus*. The consensus sequences of all ancient samples as well as the modern sequences were then aligned using the multiple sequence alignment package T-Coffee 5.72 (Notredame *et al.*, 2000).

The best fitting nucleotide substitution model was identified using the software ModelTest 3.7 (Posada & Crandall, 1998). The HKY+I+G model was supported as the best fitting model by the Akaike information criterion (AIC) and subsequently used for all model-based phylogenetic analyses. The phylogenetic relationship between *L. tanaiticus* and the extant arctic hare group members (*L. timidus, L. arcticus* and *L. othus*) was reconstructed using Neighbour-Joining (NJ) and a Markov Chain Monte Carlo (MCMC)-based Bayesian approach. We used two sequences of *L. europeaus* as outgroup in the tree building methods and five sequences in the network analysis (see Table S3 online supplementary materials). For reconstruction of the NJ trees, we used the software Mega 4 (Kumar *et al.*, 2008). NJ bootstrap analyses were performed with the settings described above using 1000 replicates and a 50% majority-rule consensus (M50). The Bayesian analysis was run as implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Four independent runs were performed simultaneously, each with one cold and three heated MCMC chains. The chains were run for 5 million iterations sampling every 100th generation after discarding the first ten per cent as burn-in. The results were checked for convergence using Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer/). To illustrate phylogenetic ambiguity among closely related sequences (Posada & Crandall, 2001; Cassens *et al.*, 2003), we reconstructed a nonhierarchical reduced median network using the software Spectronet (Huber *et al.*, 2002). Furthermore, we estimated the within-species mean genetic distance (GD) and the between-species net mean genetic distance (NMD) using MEGA 4 (Kumar *et al.*, 2008) to calculate the evolutionary distance within and between the studied hare species. These estimates are based on the Maximum Composite Likelihood (MCL) method to estimate evolutionary distances between DNA sequences (Tamura *et al.*, 2004, 2007). The Kimura two-parameter model (Kimura, 1980) was used to correct for multiple substitutions per site.

Results

We successfully sequenced 339 bp of the D-loop from five late glacial *L. tanaiticus* remains from Pymva Shor (Northern Ural) (see Table S2). Sequences were aligned and used to reconstruct the phylogenetic relationship between *L. tanaiticus* and the extant members of the arctic hare group (*L. timidus, L. arcticus* and *L. othus*). *Lepus europeus* was used as outgroup. Both NJ and Bayesian tree reconstructions resulted in a consistent tree topology (Fig. 1). As expected, the members of the arctic hare group cluster together and form a clade that is monophyletic with respect to the *L. europeus* sequences. Within the arctic hare clade, *L. timidus* together with *L. tanaiticus* represent a paraphyletic group in which *L. othus* and *L. arcticus* form monophyletic clades (albeit with moderate support of 0.51 (Bayesian) and 71% (NJ) for *L. othus* and 0.9% and 69% for *L. arcticus,* respectively). More precisely, *L. tanaiticus* sequences were scattered across the range of *L. timidus* without any clustering. Overall, our phylogenetic reconstructions show that the late glacial *L. tanaiticus* mitochondrial DNA sequences fall within the diversity of present day *L. timidus* sequences and unlike *L. othus* and *L. arcticus* do not form any monophyletic cluster.

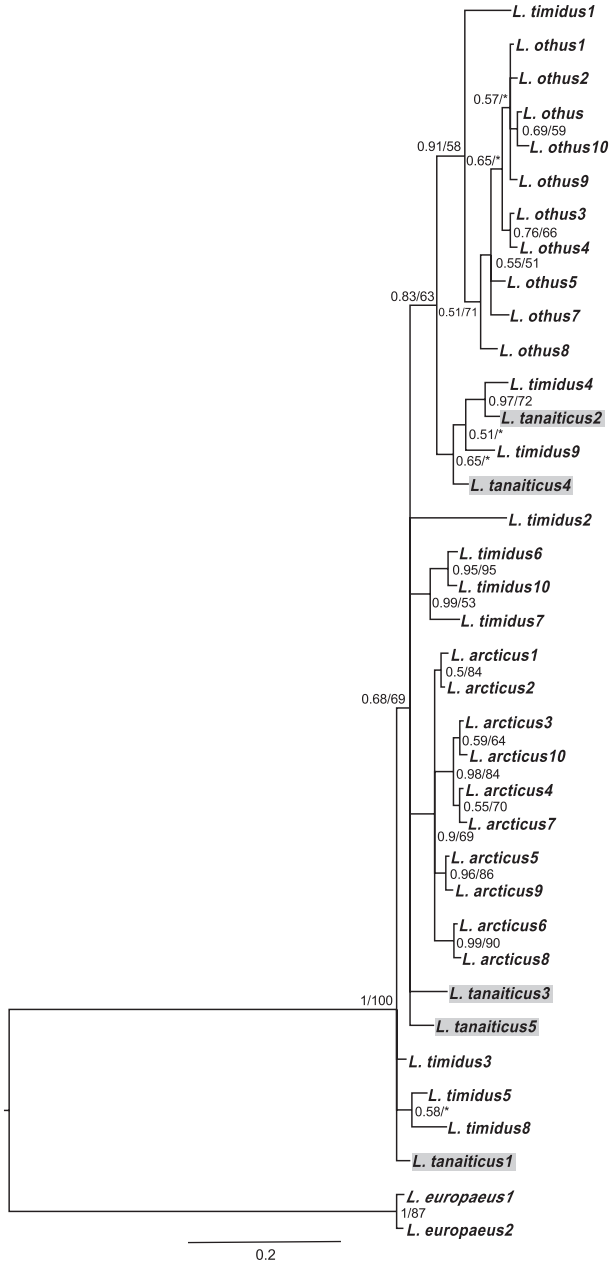

Fig. 1 Fifty per cent majority consensus phylogram as obtained by Bayesian inference. Bayesian posterior probabilities (BPP) and NJ bootstrap values are shown at each branching point (BBP/NJ bootstraps). Bootstrap values were obtained from 1000 replicates. Branches indicated by * are not supported under the 50% majority consensus in the NJ Tree. The tree was reconstructed from 339-bp D-Loop mitochondrial DNA sequences using an MCMC based Bayesian approach as implemented in MrBayes 3.1.2. and NJ as used in Mega 4. Sequences highlighted in grey belong to the ancient *Lepus tanaiticus*. Sequences from *Lepus europaeus* were used as outgroup.

These results were confirmed by the nonhierarchical reduced median network analyses (Fig. 2). In this analysis, we used a different set of *Lepus* sequences (using only *L. timidus* sequences for phylogenetic comparison and *L. europaeus* as outgroup) to survey for potential regional clustering of the *L. tanaiticus* sequences within *L. timidus*. The reduced median network indicates that there is little ambiguity in the data. Two haplotypes of *L. tanaiticus* were identified as ancestral to modern *L. timidus* haplotypes (*L. tanaiticus* two for C.Russia 12 and *L. tanaiticus* one for E.Russia one and E.Russia two, respectively), whereas the remaining three are located at the tips of external branches, respectively. The NMD between the outgroup *L. europeus* and *L. tanaiticus/timidus* is about 13% (Table 1). The negative value for the NMD between *L. timidus* and *L. tanaiticus* indicates that the within-species genetic distance (GD) in *L. tanaiticus* is higher than the GD between *L. tanaiticus* and *L. timidus* (Tables 1 and 2).

Discussion

Both hierarchical and nonhierarchical approaches show that *L. tanaiticus* sequences fall within the current sequence variation of the extant *L. timidus*. Moreover, *L. tanaiticus* sequences do not show phylogenetic associations with either a specific *L. timidus* clade or *L. timidus* sequences from a restricted geographical region. Rather, they cluster with haplotypes from all over the sample range of the mountain hare. Thus, although limited by relatively small sample size, the results from the phylogenetic analyses suggest that *L. tanaiticus* and *L. timidus* represent different populations of the same species. This is further supported by genetic within-species and net mean distances (Tables 1 and 2). The distinct morphological characters of *L. tanaiticus* might therefore only represent a different morphotype within the *L. timidus* complex.

However, although the phylogenetic reconstructions clearly favour conspecificity of the two hare species, this conclusions has to be treated carefully because both hybridization and mtDNA introgression are common among representatives of the arctic hare group (e.g. Thulin *et al.* (1997), Alves *et al.* (2003), Melo-Ferreira *et al.* (2005), Thulin *et al.* (2006), Alves *et al.* (2006), Fredsted *et al.* (2006)). More generally, discrepancies between morphological and molecular phylogenetics (especially when based on mitochondrial DNA alone) are a well-known phenomenon in extant hare species, often because of the introgression of mountain hare mitochondrial DNA into the gene pool of other species (Ben Slimen *et al.*, 2007, 2008; Suchentrunk *et al.*, 2008, 2009). Introgression of mtDNA between hare species was first described in Thulin *et al.* (1997). The authors showed introgression of mtDNA from the mountain hare *L. timidus* into the brown hare *L. europaeus*. Since then it has been widely accepted that phylogenetic conclusions based solely on mtDNA are error-prone when the possibility of introgression is not considered (Alves *et al.*, 2006). Phylogenetic analysis as

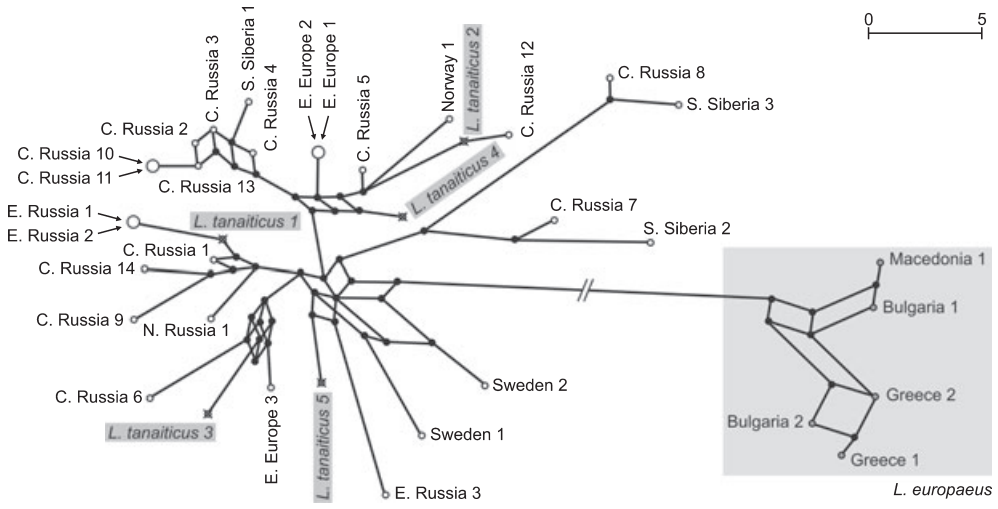

Fig. 2 Reduced median network. Each node represents a haplotype. The size of the nodes is proportional to their haplotype frequency. Missing intermediates are indicated by black dots. Haplotypes indicated by crossed cycles belong to the ancient *Lepus tanaiticus*, white circles belong to modern *Lepus timidus* and grey circles to modern *Lepus europaeus*. Labels in the modern samples refer to the region where the respective specimens were collected [see Waltari *et al.* (2004); Waltari & Cook (2005); Kasapidis *et al.* (2005)]. The lengths of the branches are proportional to the number of substitutions separating the haplotypes (see scale in the upper right corner). The branch between the network body and the outgroup comprises 15 substitutions and was shortened for graphical reasons.

Table 1 Net mean distance as calculated using the Kimura two-parameter model between the *Lepus europaeus*, *Lepus timidus* and *Lepus tanaiticus*, respectively as calculated using MEGA 4 (Kumar *et al.*, 2008). The computed standard errors (1000 replicate bootstraps) are given in italics above the dashes.

	L. europaeus	*L. timidus*	*L. tanaiticus*
L. europaeus	–	*0.020*	*0.021*
L. timidus	0.126	–	*0.001*
L. tanaiticus	0.127	−0.001	–

Table 2 Within-species mean distance (GD) and standard errors (SE) as calculated with MEGA 4 (Kumar *et al.*, 2008) for *Lepus europaeus*, *Lepus timidus* and *Lepus tanaiticus*. Standard errors (SE) were calculated using 1000 bootstrap replicates.

	GD	SE
L. europaeus	0.024	0.006
L. timidus	0.055	0.007
L. tanaiticus	0.050	0.008

those by Waltari *et al.* (2004) and Waltari & Cook (2005) show that although *L. othus* and *L. arcticus* consist of monophyletic clades (two in the case of *L. arcticus* in Waltari & Cook (2005)) species status based solely on mtDNA data is questionable because *L. timidus* is clearly paraphyletic with regard to mtDNA. As introgression is a crucial factor in the interpretation of mtDNA-based phylogenies in hares, there are at least two different scenarios that could explain our results: first conspecificity of *L. timidus* and *L. tanaiticus*, and second introgression of mtDNA between two distinct species.

The assumption of conspecificity would by definition imply that the two late glacial hare species *L. timidus* and *L. tanaiticus* are representatives of one and the same species. Assuming a conspecific genetic relationship, the distinct morphological characters of *L. tanaiticus* would suggest that it represented a distinct ecomorph of *L. timidus*. The two ecomorphs would then have been allopatric during the late Pleistocene, a conclusion supported by the paleontological record. During the Holocene, the *tanaiticus* morphotype would then have been replaced by the *timidus* morphotype, either by *in situ* evolution or by gene flow from regions of the *timidus* morphotype.

In contrast, if our results are explained by mtDNA introgression, *L. tanaiticus* would retain its species status. Introgression of mtDNA from *L. timidus* into other *Lepus* species (e.g. *L. europeaus*) is a well-known phenomenon. As for *L. tanaiticus*, this possibility cannot be ruled out, but given the absence of *L. timidus* in Pleistocene deposits from Russia, it seems unlikely that a complete replacement of *L. tanaiticus* sequences with *L. timidus* sequences would have occurred during the Pleistocene. However, our sample size is small with only five sequences and moreover, introgression could theoretically have happened earlier in the past if the species were not allopatric then (Alves *et al.*, 2003). Thus, although not very well supported by the paleontological record, introgression

cannot as yet be completely discounted as an explanation of our results.

A third possible explanation for the observed phylogenetic pattern would be independent species status of *L. tanaiticus* and *L. timidus* in combination with incomplete lineage sorting. However, given the distribution of *L. tanaiticus* haplotypes across the phylogenetic tree, this explanation appears rather unlikely compared to the aforementioned models.

To unambiguously resolve the taxonomic status of *L. tanaiticus*, studies of nuclear DNA loci would be necessary. Improvements of ancient DNA extraction methods (Rohland & Hofreiter, 2007; Rohland *et al.*, 2009), DNA capturing methods (Briggs *et al.*, 2009) and next generation sequencing technologies have paved the way for studies using ancient nuclear DNA sequences to become more common than in the past. However, retrieval of nuclear DNA strongly depends on the preservation of fossil remains and is therefore still rather the exception than the rule. As more and more nuclear DNA sequences from modern hares become available, it will not be long before nuclear SNPs (single nucleotide polymorphisms) will be available to support studies of interbreeding between different hare species. Because only short sequences need to be amplified and sequenced for such SNP studies, they are ideal for ancient DNA analysis (Svensson *et al.*, 2007, 2008; Ludwig *et al.*, 2009).

Given the currently available sequence data and the fossil record, the most parsimonious explanation for the observed genetic characteristics is that *L. timidus* and *L. tanaiticus* are morphologically distinct groups of one and the same species, the mountain hare (*L. timidus*), although nuclear markers would be necessary to unambiguously address this question. If this interpretation is correct, it would imply that *L. timidus* has previously had a wider morphological range than the modern population. The notion that Pleistocene species may have had a wider morphological range than their modern counterparts has been made before, e.g. for the grey wolf (Leonard *et al.*, 2007), or the genus *Equus* (Orlando *et al.*, 2009). In fact, it has recently been suggested that taxonomic oversplitting because of a failure to appreciate the morphological plasticity of Pleistocene species has led to a general inflation of the number of species described from Pleistocene fauna (Orlando *et al.*, 2009).

As for the history of the Don-hare, our results suggest that this ecological morphotype of the extant mountain hare (*L. timidus*) would have gradually been replaced by the common type rather than going extinct during the Holocene. If these results can be further strengthened by ancient nuclear DNA sequences, the Don-hare should be removed from the list of species that went extinct during the Holocene. This reduction would be congruent with the recent suggestion that the end-Pleistocene loss of taxonomic diversity was smaller on the species level than so far assumed (Orlando *et al.*, 2009). It is interesting that although the effect may be small, this interpretation would remove one of the, if not the, smallest mammalian species from the list of Holocene extinctions. Similar to the late Pleistocene extinctions, this list primarily includes large species with low reproductive rates such as mammoth (Vartanyan *et al.*, 1993), giant deer (Stuart *et al.*, 2004) and Caribbean ground sloth (Steadman *et al.*, 2005), re-emphasizing the question why large-bodied species were disproportionally affected by these extinction waves (Stuart, 1991; Johnson *et al.*, 2002).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primer-oligonucleotide sequences.

Table S2 Lepus tanaiticus samples.

Table S3 Accession numbers and references for the GenBank sequences included in the network reconstruction.

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