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A Mountain Hare Mummy from the Zillertaler Alps

Doris Döppes[1], Heather Gill-Frerking[2], Ulrich Joger[3], Wilfried Rosendahl[1] and Nikolaus Stümpel[3]

Abstract

Natural mummies are preserved by the environment in which they died. Environments that are cold and dry may allow bodies to dry out naturally. A mummified hare from the Zillertaler Alps was investigated. In this paper, the results of the analysis of the medical imaging data, and the results of the species identification by DNA, are presented, and the importance of these results in the field of glacier mummification and the natural history are discussed. Apart from the recent date, the discovery is valuable proof of the propagation of the Alpine Mountain Hare in southern Tyrol. This unusual specimen is a rare example of a preserved and well documented glacier animal mummy from Europe.

Introduction

At a height of 3463 m (11 361 ft), the Hochfernerspitze near Pfitsch (Fig. 1) is one of the highest mountains in the Zillertaler Alps, forming a massif with other mountains over 3000 m altitude that is entirely covered by a glacier above a height of 2500 m (8202 ft, Klier & Klier 1996). The mummified hare (Fig. 2) was discovered by S. Landthaler and E. Zoessmaier at 3000 m (9842 ft) above sea level on the north side of the Hochferner Glacier in 2005, and because they had known about the discovery of »Ötzi, the Iceman«, they handed the mummy over to the state authorities in Bozen/Bolzano, Italy (Rosendahl & Döppes 2010). Immediately after the discovery was announced and before being scientifically analyzed the hare came to be known in the media as "Pfitschi", named after the discovery site, and "Ötzi hare". The animal was kept at the Museum of Natural History in South Tyrol in Bozen/Bolzano but was never displayed. Today, the mummy is in the exhibition "Mummies of the World", which tours the U.S.A. from 2010 to 2014.

The Alps are presently inhabited by two *Lepus* species, the Brown Hare *(Lepus europaeus)* and the Mountain Hare *(Lepus timidus)*. Only the latter colonizes the subalpine and alpine regions of the high Alps (e. g. Grimmberger & Rudloff 2009). *Lepus timidus* is an arcto-alpine species, inhabiting a wide range in northern Palaearctic from the British Isles to East Siberia, and some isolated relict populations in the Alps, Poland and Japan (Angerbjörn & Flux 1995). According to the fossil record it was the most common and most widely distributed hare species in Europe during last glacial periods of the Pleistocene (Lopez-Martinez 1980).

Fig. 1. Location of the discovery site of the Mountain Hare mummy "Pfitschi" from the Zillertaler Alps.

1 Reiss-Engelhorn-Museen, Mannheim, Germany;
E-mail: wilfried.rosendahl@mannheim.de (corresponding author).
2 NTK Services, New Hampshire, USA.
3 State Museum of Natural History, Braunschweig, Germany.

Fig. 2. Glacier mummy of a Mountain Hare discovered in 2005 on the Hochferner Glacier near Pfitsch in South Tyrol. – Photo: W. Rosendahl, rem.

The Alpine Mountain Hare (*Lepus timidus varronis* Miller 1901, Fig. 3) is a glacial relict and inhabits the Alps above ca. 1300 m (4265 ft). This middle-sized herbivore is the smallest subspecies of mountain hares, weighing an average of 2.5 to 3 kg (5.5 to 6.6 lbs) and having an approximate body length of 509 mm (20 in). Males are smaller than females. Even though individual spheres of activity could be higher, the Mountain Hare is found in the Alps at an altitude starting from 1300 m (4265 ft) finding its largest supply of food between 2200–2400 m (7217–7874 ft). The decline of the Alpine Mountain Hare is associated with climate warming and competition with the Brown Hare. The long, splayed hind legs are covered with a hard, bristly fur and act like real snowshoes, providing the evidence that snowy areas are the primary habitat of the Alpine Mountain Hare. Its main enemies are the red fox, golden eagle and eagle owl. The coloring of the fur changes with the photoperiod, from gray in summer to white in winter, and serves first and foremost as camouflage from predators (Spitzenberger 2002, Thulin 2003).

Fig. 3. Male Mountain Hare. – Photo: Wikipedia, Alan Wolfe, June 1992.

Material and methods

Physical appearance and age of the mummy

Medical imaging was used to study the anatomy and extent of preservation of the animal. The mummified hare had shrunk to 350 mm (13.8 in in length) and was conclusively identified as an Alpine Mountain Hare. Not only does the high altitude of his find spot determine this species, but also the longer hindlegs

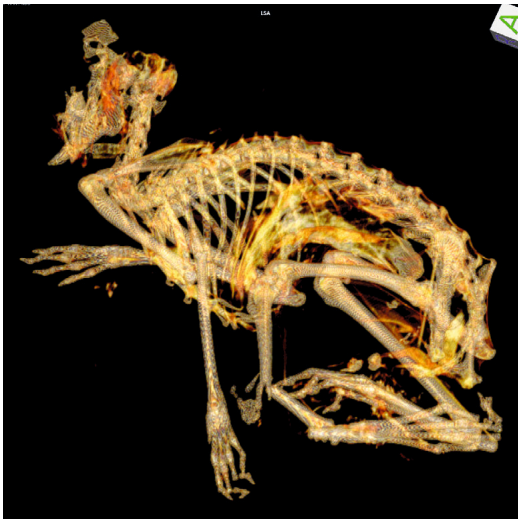

Fig. 4. 3D reconstruction of the skeleton of the hare, left side, from the CT data. – Photo: German Mummy Project, Mannheim.

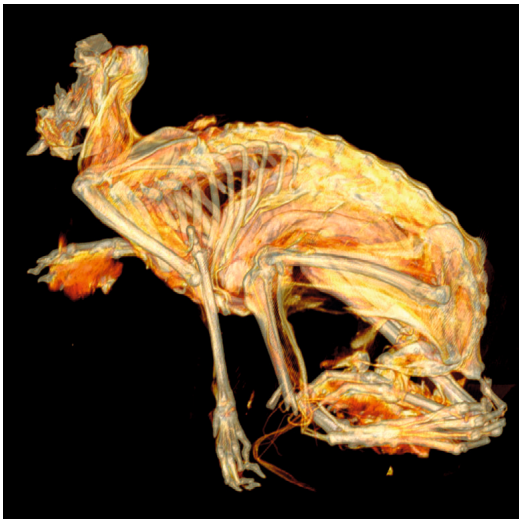

Fig. 5. 3D reconstruction of the soft tissue and skeleton of the hare, from the CT data. – Photo: German Mummy Project, Mannheim.

and shorter forelegs (Fig. 4). Based on the index 59, based on the radius length, expressed in % of the tibia length (Hauser 1921: 102), the hare mummy was clearly determined to be a Mountain Hare. The radius is 67 to 72 % of the tibia in the Mountain Hare, the Brown Hare 73 to 78 %. The index 59 of length of the right radius (L 9.16 cm) of the right tibia (L 13 cm) of the mummy is 70.46 %.

Only small tufts of fur are still present on the back and paws of the mummified Alpine Mountain Hare. There is evidence of preservation of some internal organs, although the specific organs are not identifiable from the medical imaging. There is likely also some muscle, ligament and tendon preserved in the animal mummy (Fig. 5).

A small tissue sample was taken for radiocarbon dating. General interest in the mummified Mountain Hare disappeared a few weeks after the discovery, when it was announced that the hare was more recent than 1950 (ETH-30940, 2005).

Molecular protocols

Ancient DNA is a useful tool for the study of ancient and historic specimens. The research is methodologically hindered, however, by several problems, such as damaged and chemically modified DNA (e. g. Pääbo 1989, Hofreiter et al. 2001, Hansen et al. 2006), contamination from exogenous DNA (e. g. Krause 2010) and PCR inhibition (e. g. Rohland & Hofreiter 2007). To recover genomic DNA from an incisor of the mummified *Lepus*, we used the silica extraction procedure of Rohland & Hofreiter (2007). We strictly followed the procedure of the protocol with few exceptions. The small amount of the species sample enabled us to remove the outer surface of the tooth. After removing dirt with a tissue moistened with HPLC-grade water, we ground the basal part of the incisor in liquid nitrogen with a pestle and mortar, until a fine powder with a total weight of 90 mg was obtained. A small portion (12 mg) was transferred to a 5 ml extraction solution and digested for circa 24 hours. As suggested by Rohland & Hofreiter (2007), we used for DNA binding to silica four times the volume of binding buffer in relation to the volume of extraction solution (20 ml). For resuspension of the aDNA from the dried silica, we doubled the volume of TE buffer to 100 µl.

Strand breaks of DNA molecules are the most common occurrences of damage in most aDNA samples (e. g. Mitchell et al. 2005). In our PCR-based approach we therefore amplified the mitochondrial D-loop (Control region, CR) with totally 506 bp in five small overlapping fragments between 100–188 bp each. For the selective amplification we used two published mammalian control region primers (L15997, H 16498, Gerloff et al. 1999) in combination with six additional newly designed internal primers. PCR was carried

out using the following primer pairs: a) L15997 + R11 5'-CATGTTRATGGGGAAAGGCA-3', b) F2 5'-TC-CAAGTACCTTGTCAC-3' + R9b 5'-CTATGTCCTATTAAGCAGG-3', c) F4 5'-ATTAATGCCTTTCCCCA-3' + R9b, d) Fx 5'-TGCATATAAGCCAGTACATCCC-3' + R4b 5'-AGGATTTGACTTGGATGGTC-3' and e) F10 5'-ccaccttaaccaacatcca-3' + H16498.

Fragments of the mtDNA D-loop were amplified using TaKaRa Ex TaqTM PCR reaction system, containing 2.5 µl 10XBuffer, 2 µl dNTP Mix, 2.5 U enzyme, 0.5 µl of 10 pmol primer each, 3 µl genomic DNA, filled up with dH_2O to 25 µl volume in total. PCR conditions included an initial denaturation step at 95 °C 1 min, followed by 35 cycles of 94 °C 25 s, x °C 30 s, 72 °C 30 s and a final extension at 72 °C for 5 min. Annealing temperatures of the reactions were adjusted to the melting temperatures of primer pairs with: a, 54 °C; b,c, 47.3 °C; d, 53.3 °C; e, 52.4 °C.

Dye terminator cycle sequencing was set up according to suppliers' instructions (DTCS Quick Start Kit, Beckman Coulter) in a three step thermal reaction with 30 cycles of 96 °C 20 s, 50 °C 20 s, 60 °C 4 min. For Dye-terminator removal we used the Agencourt CleanSEQ system, which is based on the solid phase reversible immobilization (SPRI) technology and run the samples on a Beckman Coulter CEQ 8000 sequencing apparatus.

Sequence alignment and phylogenetic analyses

Sequences were edited and assembled using SEQUENCHER (Gene Codes). Recent genetic studies demonstrated that introgression of mt-genes in several Eurasian *Lepus*-species is a not uncommon phenomenon (e. g. Alves et al. 2003, Fredsted et al. 2006, Melo-Ferreira et al. 2007). We therefore initially aligned the D-loop fragment of the mummy against 157 homologous *Lepus* sequences and performed a Bayesian inference analysis (results not shown) for the taxonomic identification of the mummy. D-loop sequences were retrieved from National Center for Biotechnology and Information (http://www.ncbi.nlm.nih.gov/). In a second step we repeated the analysis for a subset of Alpine *Lepus timidus* sequences with known geographic origin. Twenty five *Lepus timidus* sequences were aligned using the software ClustalW (Thompson et al. 1994) implemented in Bioedit 7.0.9 (Hall 1999). To identify the most appropriate models of sequence evolution for the dataset, we used MrModeltest 2.2 (Nylander 2004), and selected the model favoured under the Akaike Information Criterion (AIC). Phylogenetic history was reconstructed under Bayesian inference using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003).

For Bayesian inference (BI) we ran two independent analyses with one cold and three heated chains (MC3) for 10 million generations sampling every 200th generation. Convergence was estimated in Tracer v1.4.1 (Rambaut & Drummond 2007) and observed with the convergence diagnostic parameters (standard deviation of the partition frequencies (Stdev(s)), and the potential scale reduction factor (PSRF)) implemented in MrBayes. To ensure trees were sampled from the posterior distribution after convergence; we discarded conservatively the first 25 % as burn-in.

Result and discussion

Several previous phylogenetic studies (e. g. Wu et al. 2005, Melo-Ferreira et al. 2007) have shown that D-loop (control region, CR) can be a useful marker for determining interspecific relationships and relatively recent evolutionary events. In order to assign the taxonomic status and the phylogeographic history of the ancient *Lepus* mummy we pooled the sequence with additional 157 D-loop traces from different *Lepus* populations retrieved from gene bank, and performed a Bayesian Inference analysis (results not shown). The general BI topology produced distinct clades mostly supported by robust Posterior Probabilities, however several nodes cannot be fully resolved to dichotomies and we found no association between phylogenetic and geographic distances among individuals. Haplotypes of *Lepus timidus* from Europe, Siberia and the Alps were scattered through the tree, a phenomenon already mentioned by Melo-Ferreira et al. (2007). The mt genealogy reveals the *Lepus* mummy from Hochferner Glacier unambiguously as *Lepus timidus* (Mountain Hare). Albeit both species are ecologically separated, reciprocal transfer of mtDNA has been described in Alpine populations (see Melo-Ferreira et al. 2007).

It is not our intention, however, to draw general phylogenetic or phylogeographic considerations, since this has been extensively done before by other authors (e. g. Alves et al. 2003, Wu et al. 2005, Waltari & Cook 2005, Slimen et al. 2006).

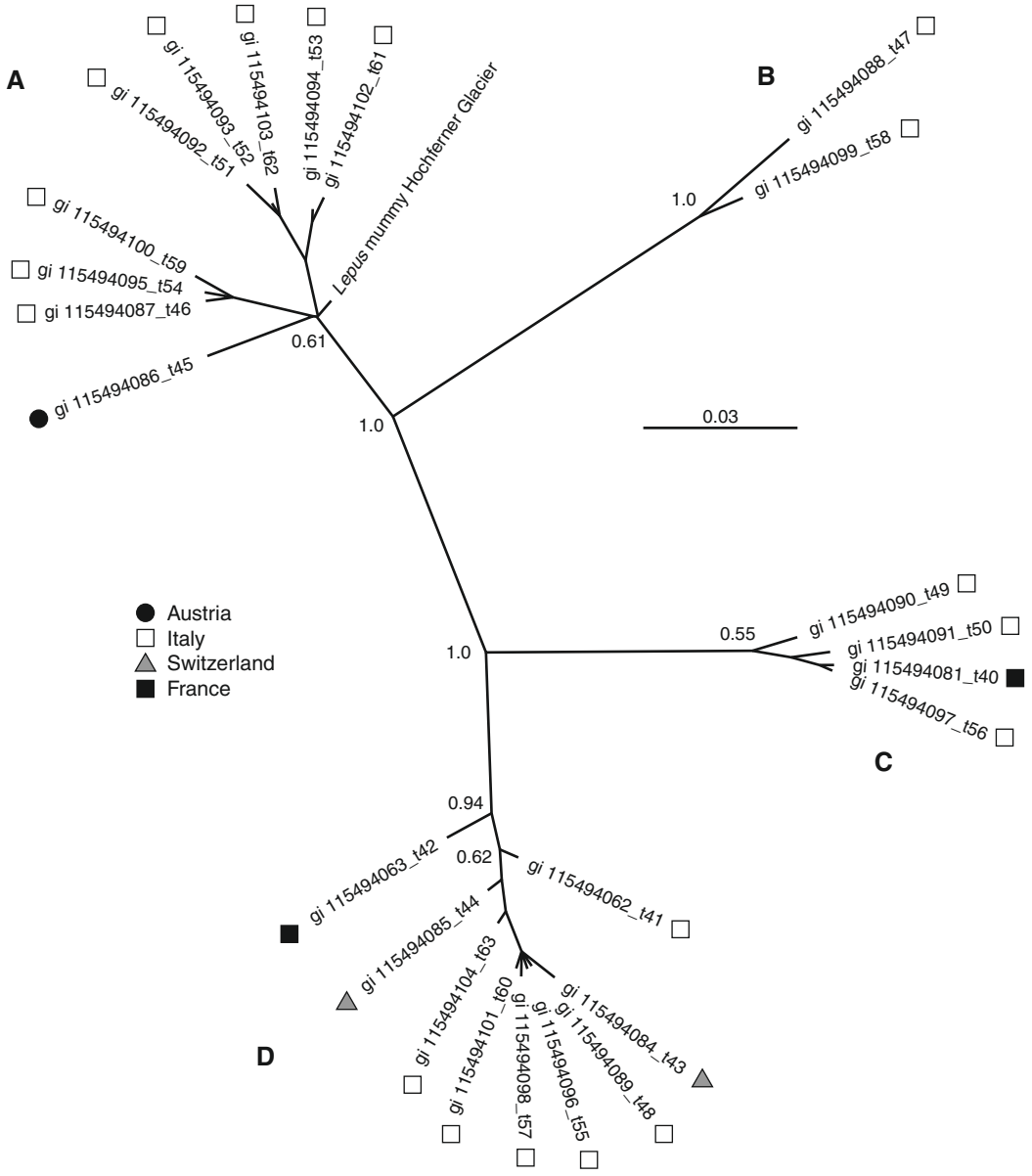

Fig. 6. Unrooted Bayesian 50 % majority-rule consensus tree of the Alpine Mountain Hare *(Lepus timidus)* under the best fit model (HKY+I) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2001) showing the phylogenetic clustering of the *Lepus timidus* mummy from Hochferner Glacier (Italy). Posterior probabilities are indicated for essential clades. Homologous D-loop sequences for the analysis were retrieved from Melo-Ferreira et al. (2007).

Our Bayesian Inference genealogy for a subset of Alpine Mountain Hare populations (see Fig. 6) reveals a high genetic diversity with four distinct haplogroups (A-D), representing fragmented populations with different geographic origins. According to Melo-Ferreira et al. (2007) the genetic architecture was attributed to fragmentation and shrinking of the species range during Pleistocene climate oscillations and partial differentiation by genetic drift.

The D-loop sequence from the mummy is distinct from published homologues and represents a new haplotype, which clusters together with specimens from Italy and Austria in subclade A, although not supported by a robust posterior probability (but the sister group relationship between groups A and B [both mainly Italian] is well supported). The specimen appears in a rather basal position, close to the origin of group A. The features 'basal position', 'short branch in tree', and 'haplotype not found among recent snow hares' would be better compatible with an older radiocarbon age than the 'less than 50 years' found. If the radiocarbon dating was correct, there should be living specimens sharing this particular haplotype with the mummy.

For now, we cannot exclude that the haplotype of the mummy belongs to a yet unstudied recent population. In absence of a regional fine scale sampling the data actually do not allow any significant phylogeographic interpretation. With the observed global warming during the last years this cold-adapted animal has to shift its range to higher altitude. Maybe the mummy came from another warm period of the last 10000 years. Therefore we intend to repeat the age determination with an independently-taken sample, because radiocarbon determined ages can easily be underestimated when the samples are contaminated with recent organic materials.

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