Ötzi's last meals: DNA analysis of the intestinal content of the Neolithic glacier mummy from the Alps

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Samples of the intestinal content were collected from the ileum and colon of the Neolithic glacier mummy popularly known as the Tyrolean Iceman, or Ötzi. DNA was extracted from the samples and PCR amplified, using a variety of primer pairs designed to bind to different genes (mammal mitochondrial 12S ribosomal RNA gene, plant/fungal nuclear 18S ribosomal RNA gene, plant chloroplast ribulose bisphosphate carboxylase large subunit gene). This made it possible to distinguish between animal and plant food residues (macroremains) and pollen (microremains). According to the DNA reconstruction, the man's last meal was composed of red deer (*Cervus elaphus*) meat, and, possibly, cereals; this meal had been preceded by another one based on ibex (*Capra ibex*), different species of dicots, and cereals. The DNA spectrum corresponding to pollen residues in the colon, on the other hand, fits with the hypothesis that the last journey of the Neolithic hunter/warrior was made through a subalpine coniferous forest to the site at over 3,200 m above sea level, where his mummified body was to be discovered 5,000 years later.

Iceman | *Cervus elaphus* | *Capra ibex*

The so-called Tyrolean Iceman (also popularly known as the Similaun Man, or Ötzi) is a naturally mummified male corpse found on the Alps on September 19th, 1991. The presence, near the body, of leather and hide clothing and equipment, including, among other items, a bow, a quiver full of arrows, and a copper axe, led the archaeologists to suppose that the man could have been a hunter or even a prehistoric warrior of high rank. The age of the mummy, 5,350–5,100 years before present, as assessed by radiocarbon dating, has offered scientists a unique opportunity to investigate the life and health status of a Late Neolithic human (1, 2). During the last 10 years, the body and equipment have been the object of a number of anthropological, paleopathological, and ethnological investigations. In particular, samples of the colon content were analyzed by paleobotanists (3, 4). Very recently, the scientific world and the media were thrilled by the discovery of a stone arrowhead still stuck in the left shoulder of the mummy, a find that led to a rethinking of the possible cause of death of the hunter/warrior, previously attributed to freezing. On September 25th, 2000, the corpse, presently kept in cold storage at the South Tyrol Archaeological Museum in Bolzano, Italy, was fully defrosted for the first time (5). This made it possible to collect high-quality samples for the analyses. Previous studies had shown that the analysis of DNA in paleofeces was a promising way to identify the diet of ancient animals and men (6, 7). To obtain further insight into the food the Iceman ate in his last hours of life, we investigated the DNA preservation and composition in samples of the intestinal content taken from the large and small bowel of the mummy.

Materials and Methods

Sample Collection. Samples of the intestinal content were collected during the September 25th, 2000, survey of the mummy by Eduard Egarter-Vigl of the Regional Hospital of Bolzano. The sample from the colon was collected through the anal orifice, taking care to separate the outermost portion of the content from the rest, whereas the sample from the ileum was obtained by incising the lower tract of the small intestine and squeezing out the content. All of the operations were performed by using sterile instrumentation inside the sterile facility annexed to the Iceman's cold storage room at the South Tyrol Museum of Archaeology.

DNA Extraction. Two specimens (58 mg from the colon and 68 mg from the ileum) were resuspended in 350 μl of a medium containing 50 mM Na_2EDTA, 50 mM Tris·HCl (pH 8.0), 1% (wt/vol) SDS, and 6% (vol/vol) water-saturated phenol. After imbibition, the samples were left overnight at 4°C. The next morning, the samples were transferred into sterile mortars and homogenized with pestles. During the milling phase, 350 μl of the above-described medium was added to each sample. The homogenates were collected in Eppendorf tubes, taking care to rinse the mortar and pestle with a further 350 μl of extraction medium, then extracted sequentially by using equal volumes of phenol, phenol/chloroform/isoamilic alcohol (25:24:1), and ether. The DNA fraction was precipitated from the final supernatant by centrifugation at 13,500 $\times$ *g* for 5 min after the addition of 1/10 volume of 2 M sodium acetate and 2.5 volumes of cold (−20°C) ethanol. The DNA precipitates were resuspended in 20 μl of sterile distilled water and stored at −25°C until use. Contrarily to what was reported in previous papers on the analysis of DNA from coprolites (6, 7), no further purification was needed. This is attributable to the fact that we were dealing with food residues from the intestines and not with feces *sensu stricto*; food residues, having undergone a limited amount of chemical transformation, do not contain as many inhibitors as modern and fossil feces do. All of the operations were carried out in a room dedicated to the manipulation of ancient DNA. The room is equipped with UV light and contains a bench microcentrifuge, a Speed-Vac concentrator, and positive-displacement pipettes. Strict cleaning criteria were routinely followed, including frequent treatment with bleach. Negative controls were performed throughout the procedure.

PCR Amplification and Sequencing. To set up a PCR system for mammal DNA, we aligned the 12S ribosomal RNA gene (12S rDNA) sequences of 14 mammals and designed a pair of oligonucleotide primers (Mbos L1269: CATGAAGCACGCACACACCG; Mbos H1346: CCAGTATGCTTACCTTGTTAC) that bind to a fragment of 117 bp in length (calculated on the basis of the *Bos taurus* sequence). For plant DNA analysis, we

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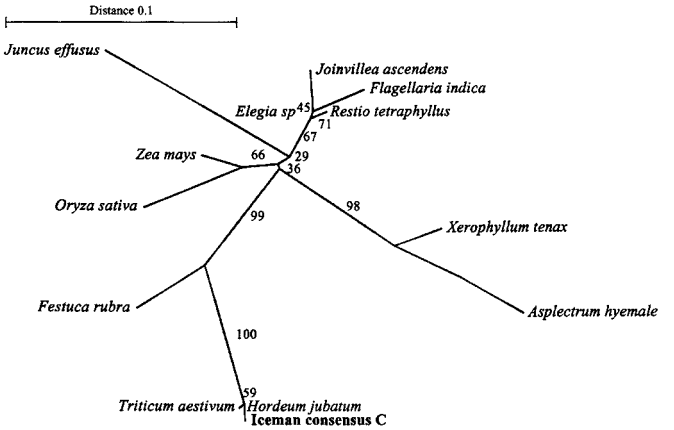

Fig. 1. Bootstrapped neighbor-joining 18S rDNA tree of the Poales order inclusive of the Iceman consensus sequence C. The tree is based on the families Restianaceae (*Restio tetraphyllus* and *Elegia* sp.), Joinvillaceae (*Joinvillea ascendens*), Flagellariaceae (*Flagellaria indica*), Juncaceae (*Juncus effusus*), and Poaceae (*Zea mays*, *Oryza sativa*, *Festuca rubra*, *Triticum aestivum*, and *Hordeum jubatum*) families. The last of these is further subdivided into the Panicoideae (*Z. mays*), Ehrhartoideae (*O. sativa*), Pooideae (*F. rubra*), and Triticeae (*T. aestivum* and *H. jubatum*) tribes. The outgroup is represented by *Xerophyllum tenax* (Liliales) and *Asplectrum hyemale* (Asparagales).

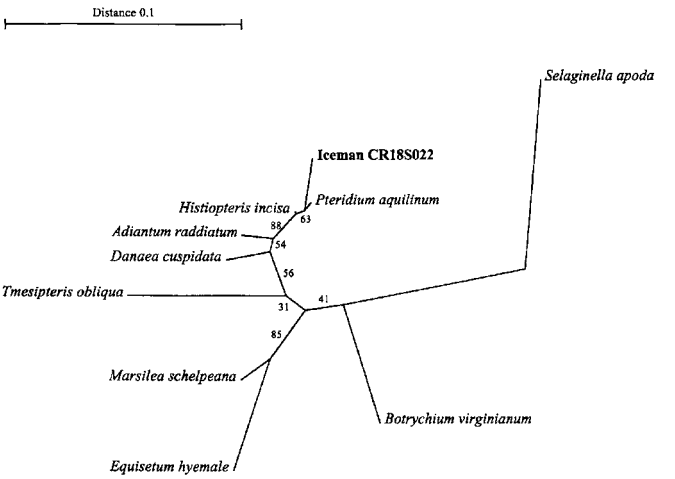

Fig. 3. Bootstrapped neighbor-joining 12S rDNA tree of the Filicopsida class inclusive of the Iceman CR18S022 sequence. The tree is based on the Ophioglossales (*Botrychium virginianum*), Hydropteridales (*Marsilea schelpeana*), Equisetales (*Equisetum hyemale*), Marattiales (*Danaea cuspidata*), Psilotales (*Tmesipteris obliqua*), and Filicales (*Adiantum raddiatum*, *Histiopteris incisa*, and *Pteridium aquilinum*) orders. The outgroup is represented by *Selaginella apoda* (Lycopodophyta).

designed the following pairs of oligonucleotide primers as follows. The first system (Angio 1f, TGCAGTTAAAAAGCTCGTAG; Angio 2r, GCACTCTAATTTCTTCAAAG) was designed on the basis of the alignment of the 18S ribosomal RNA (18S rDNA) sequence of 12 monocotiledonous and dicotiledonous plants. The primers bound to a 159-bp fragment (calculated on the basis of the *Boquila trifoliata* gene sequence). Actually, they were shown capable of binding not only to angiosperm DNA but also to the DNA of gymnosperms, pteridophytes, and fungi. The second system (CalrbcL170f, TAGCGGCGGAATCTTCTACT; Cal rbcL240r, TATGATAGCATCGTCGTTTG), was designed on the basis of the alignment of the chloroplast ribulose bisphosphate carboxylase large subunit (*rbc*L) gene sequence of 12 monocots and dicots. The primers bound to a 90-bp fragment

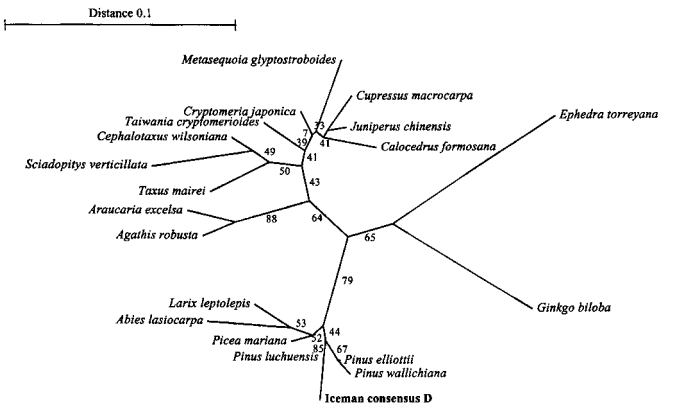

Fig. 2. Bootstrapped neighbor-joining 18S rDNA tree of the Coniferales order inclusive of the Iceman consensus sequence D. The tree is based on the Cupressaceae (*Juniperus chinensis*, *Cupressus macrocarpa*, *Calocedrus formosana*, *Metasequoia glyptostroboides*, *Cryptomeria japonica*, and *Tauwania cryptomerioides*), Taxaceae (*Taxus mairei*), Sciadopityaceae (*Sciadopytis verticillata*), Cephalotaxaceae (*Cephalotaxus wilsoniana*), Araucariaceae (*Araucaria excelsa*, *Agathis robusta*), and Pinaceae (*Larix leptolepis*, *Abies lasiocarpa*, *Picea mariana*, *Pinus luchuensis*, *P. wallichiana*, and *P. elliottii*) families. The outgroup is represented by *Ginkgo biloba* (Ginkoales) and *Ephedra torreyana* (Ephedrales).

(calculated on the basis of the *Triticum aestivum* gene sequence). A third plant system (HrbcL252f, ATCGTTACAAAGGACGATGC; HrbcL320rPCR, AGGTCTAATGGATAAGCTAC) was designed on the basis of the *rbc*L gene of 6 monocots. The primers bound to an 88-bp fragment (calculated on the basis of the *Hordeum vulgare* gene sequence). Primer pairs designed to amplify longer (≥130 bp) portions of the *rbc*L gene proved ineffective. All amplifications were performed in 50 μl of reaction medium containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 enzyme units *Taq* polymerase (Ampli *Taq* Gold, Perkin–Elmer), 200 mM each dNTP, 300 ng of each primer, and 1 μl of DNA preparation (we tested serial dilutions from 1:10 to 1:100). The reaction mixture was pretreated with DNase (2 enzyme units for 30 min at room temperature) to eliminate contaminant DNA. The DNase was subsequently inactivated at 95°C for 15 min. The thermal profile (45 cycles) was set as follows for the primer pairs MbosL1269/H1346, Angio1f/2r, and CalrbcL170f/240r: 94°C for 1 min (denaturation), 50°C for 30 s (annealing), 72°C for 1 min (elongation). The last elongation step lasted 10 min. For the HrbcL252f/320r primer pair, the conditions were the same except that there were 55 cycles.

The PCR systems were directly tested on the ancient DNA preparations, and no positive (i.e., based on modern DNA) control was used. The amplification products were checked by electrophoresis on 2.5% (wt/vol) agarose, then purified by using a High pure PCR product purification kit (Roche Molecular Biochemicals) and directly cloned by using the pGEM-T Easy Vector System (Promega). Recombinant plasmids were isolated by using a Miniprep kit (Applied Biosystems) and insert size and DNA concentration assessed by gel electrophoresis. DNA sequences were obtained by using an ABI-Prism 310 automated DNA sequencer and the BigDye Terminator Cycle Sequencing v 2.0 Ready reaction kit (Applied Biosystems). Cycle sequencing products were purified by Centri-sep spin columns (Princeton Separations, Adelphia, NJ).

Sequence Data Analysis. Results were compared with the reference sequences in GenBank by using the National Center for Biotechnology Information (NCBI) BLAST search (8). Distance matrices were obtained from the aligned sequences and cor-

Table 1. Taxonomic identification of the consensus sequences for the 18S rRNA, rbcL, and 12 rRNA gene clones from the Iceman's intestinal content

Consensus	No. of clones	Type of sequence	Taxonomic identification*								Base similarity
			Kingdom	Phylum	Class	Order	Family	Tribe†	Genus	Species	
A	12	18S rRNA	Fungi	Basidiomycota	—	—	—		—	—	107/107
B	25		Fungi	Basidiomycota	Urediniomycetes	—	—		—	—	115/119
C	4		Viridiplantae	Streptophyta	Liliopsida	Poales	Poaceae	Triticeae	—	—	117/117
D	13		Viridiplantae	Streptophyta	Coniferopsida	Coniferales	Pinaceae	—	Pinus	—	116/118
E	8		Fungi	Ascomycota	—	—	—		—	—	116/117
F	8		Fungi	Basidiomycota	Urediniomycetes	—	—		—	—	118/119
G	5		Viridiplantae	Streptophyta	Eudicotyledons	—	—	—	—	—	112/118
CR18S022	1		Viridiplantae	Pteridophyta	Filicopsida	Filicales	—	—	—	—	116/119
I18S001	1		Fungi	Basidiomycota	Heterobasidiomycetes	—	—		—	—	114/117
H	15	rbcL	Viridiplantae	Streptophyta	Liliopsida	Poales	Poaceae	—	—	—	48/48
I	12		Viridiplantae	Streptophyta	Eudicotyledons	—	—	—	—	—	50/50
J	8		Viridiplantae	Streptophyta	Liliopsida	Poales	—	—	—	—	50/50
K	9	12S rRNA	Metazoa	Chordata	Mammalia	Artiodactyla	Cervidae		*Cervus*	*elaphus*	76/76
L	2		Metazoa	Chordata	Mammalia	Artiodactyla	Bovidae		*Capra*	*ibex*	76/76

*The degree of confidence of the identification was checked by the construction of the corresponding phylogenetic trees as shown in Figs. 2–5. It can be observed that the accuracy of the 18S rDNA identification varies widely among the different kingdoms and classes, the highest being reached by the Coniferopsida (Viridiplantae) and the lowest with the Fungi.

†Used exclusively for the Viridiplantae kingdom.

rected for the superimposed mutations. Phylogenetic trees were constructed by using the TREECON program (9) with a neighbor-joining algorithm (10). The robustness of the inferred topologies was tested by bootstrap resampling of trees (11).

Aspartic Acid Racemization Analysis. The level of aspartic acid (D/L-Asp) racemization in grass samples (about 3 mg each) coming from the Iceman's outer clothing was determined as reported (12).

Criteria of Authenticity. For the Tyrolean Iceman, evidence that the ancient DNA is preserved was repeatedly given in the past by showing that not only the original mitochondrial DNA of the man (13), but also that of his intestinal microflora (14), and that of the grass clothing (15) could be retrieved. In addition, the present work was conducted following the guidelines of Cooper and Poinar (16). More in detail, given the working conditions implemented in our laboratory, the types of controls performed, and the intrinsic nature of the research (focusing on the quest for plant and animal DNA amplicons), this work complies with criteria (*i*) physically isolated work area, (*ii*) control amplifications, (*iii*) appropriate molecular behavior, (*iv*) reproducibility, (*v*) cloning, (*vii*) biochemical preservation, and (*ix*) associated remains. As this study does not consider the analysis of ancient human DNA, the application of the remaining two criteria, independent replication (*vi*) and quantitation (*viii*), is unnecessary.

Results

Aspartic Acid Racemization. Indirect evidence for ancient DNA survival can be obtained by assessing the extent of aspartic acid racemization. This test is based on the observation that aspartic acid racemization and DNA depurination proceed at a very close rate in a given specimen (17). Practically it is assumed that a D/L-Asp higher than 0.08–0.1 (uncorrected for racemization caused by the experimental procedure) indicates that no authentic DNA is left in an archaeological sample, whereas lower values are compatible with its conservation (18). In a previous investigation (14), we determined the D/L-Asp for tissue samples from the Iceman's corpse and found it to be in the range of 0.06–0.08.

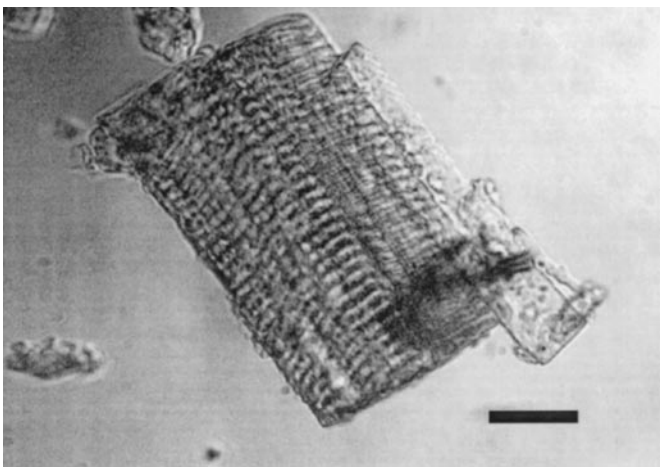

Fig. 4. A muscle fiber from the Iceman's ileum. (Bar = 10 μm.)

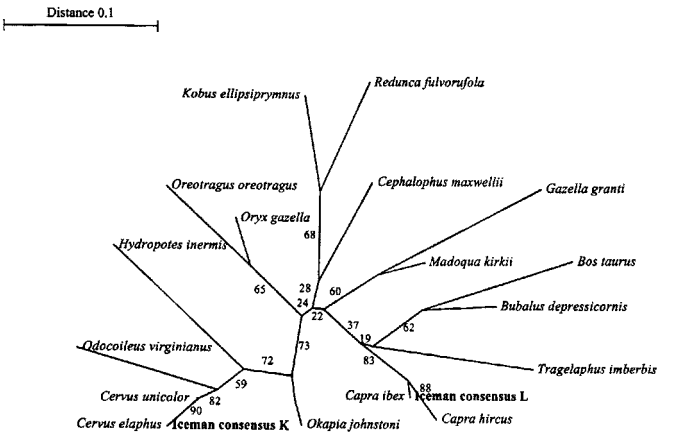

Fig. 5. Bootstrapped neighbor-joining 12S rDNA tree of the Pecora infraorder inclusive of the animal consensus K and L sequences from the Iceman's ileum and colon, respectively. The tree is based on the Bovidae (*C. ibex*, *C. hircus*, *Tragelaphus imberbis*, *Bubalus depressicornis*, *Bos taurus*, *Modoqua kirki*, *Gazella grantii*, *Cephalophus maxwellii*, *Redunca fulvorufola*, *Kobus ellipsiprymnus*, *Oryx gazella*, and *Oreotragus oreotragus*) and the Cervidae (*Hydropotes inermis*, *Odocoileus virginianus*, *Cervus unicolor*, *C. elaphus*) families. The outgroup is represented by *Okapia johnstoni* (Giraffoidea).

In the present study, we analyzed fragments of grass from the outer clothing of the hunter/warrior and found D/L-Asp ratios ranging between 0.06 and 0.07. These figures, in addition to the results of the above-cited studies on the retrieval of ancient human, plant, and bacterial DNAs from the Iceman's body and equipment, make a strong argument in favor of the possibility of recovering authentic DNA from the intestinal content.

Plant DNA. The DNA extracted from the colon and ileum samples were PCR amplified by using the Angio 1f/2r oligonucleotide primer pair. This system is designed to bind to an approximately 159-bp-long fragment of the nuclear 18S ribosomal RNA gene (18S rDNA). Both specimens yielded amplification products of the expected size. Amplification products were then cloned and sequenced until the same groups of related sequences were repeatedly found. Sequences were grouped into clusters, and the consensus sequences were compared with the sequences deposited in GenBank as of March 2002 by using a BLAST search (for sequence details, see Figs. 6 and 7, which are published as supporting information on the PNAS web site, www.pnas.org). The 77 amplicons from the 18S rDNA libraries can be grouped into 9 clusters (A, B, C, D, E, F, G, CR18S022, and I18S001), 5 of which (A, B, E, F, and I18S001) correspond to fungi, and 4 (C, D, G, and CR18S022) correspond to plants.

To better identify the taxa, we inserted each of the four plant sequences into a corresponding phylogenetic tree. The results (referring to the C, D, and CR18S022 sequences) are shown in Figs. 1–3. Sequence C (Fig. 1) is identified at the tribe level (Triticeae), sequence D (Fig. 2) at the genus (*Pinus*) level, whereas sequence CR18S022 (Fig. 3) is at the order (Filicales) one. On the other hand, sequence G (not shown) cannot be identified beyond the class (Eudicotyledons) level. The results of the phylogenetic analyses are summarized in Table 1.

Normally, plant remains consist of so-called macroremains (remains of food) and microremains, the latter being represented by pollen. One way in which pollen is ingested is by adherence to the swallowed food plants. This type of pollen is classified as "economic pollen." On the other hand, the pollen content of the atmosphere is unintentionally incorporated in the food residue by breathing or by drinking of water. This is defined as "background pollen," and reflects the general composition of the vegetation in the area where the person or animal lived. The use of the 18S rDNA system does not allow one to determine whether the plant DNA comes from pollen or from macroremains, as this system is directed to a nuclear gene that is present in both kinds of residues. For this reason, we also tested the DNA extracted from the colon and ileum fecal samples with two PCR systems using primer pairs (rbcL170f/240r; HrbcL252f/320r) designed to bind to a 90-bp-long fragment of the chloroplast *rbc*L gene, because pollen, contrarily to macroremains, does not contain chloroplast DNA. The first (rbcL170f/240r) system yielded an amplification product from the colon DNA preparation, but not from the ileum DNA preparation. The colon amplicons grouped into two clusters (I, J). Phylogenetic analysis of the consensus sequences shows that the first cluster corresponds to unidentified members of the Eudicotyledon class, whereas the second cluster is identified at the order (Poales) level. On the other hand, the second (HrbcL252f/320r) system produced an amplification signal with the ileum DNA preparation, but not with the colon DNA preparation. The ileum clones all grouped in a single cluster (H) of sequences phylogenetically identified at the family (Poaceae) level.

Fungal DNA. As described in the previous paragraph, 5 consensus sequences (A, B, E, F, and I18S001) from the 18S rDNA library correspond to fungi. A phylogenetic analysis allows one to identify three sequences (B, F, and I18S001) at the class level (Table 1); the first two are Urediniomycetes, and the third is Heterobasidiomycetes. The remaining (A, E) sequences are identified at the phylum (Basidiomycota, A; Ascomycota, E) level only.

Table 2. Comparison of the results of DNA and microscopic analyses of plant and animal remains in the Iceman's intestines

Type of remain	Location	DNA analysis*	Microscopy†
Plant macroremains	Colon	Dicotyledons (60%)	Cereals (82%)
		Cereals (40%)	Dicotyledons (18%)
	Ileum	Poaceae (100%)	Not tested
Pollen	Colon	Pinus (73%)	Ostrya (55%)
		Cereals (22%)	Corylus avellana (20%)
		Fern spores (5%)	Pinus (4%)
			Betula (4%)
			Primulaceae (3%)
			Fern spores (2%)
			Caltha (2%)
			Other pollen types (10%)‡
	Ileum	—	Not tested
Animal remains	Colon	*C. ibex*	Muscle fibers
	Ileum	*C. elaphus*	Muscle fibers§

*The relative abundance is based on the number of the corresponding 18S rDNA or rbcL clones as in Table 1.
†Data from the literature (3, 4) unless otherwise specified.
‡Including Alnus, Tilia, Abies, Fagus, Picea, Vaccinium, Salix, Gramineae, Asteraceae, Umbelliferae, Rosaceae, Rubiaceae, Saxifraga, Valerianaceae, Chenopodiaceae, Plantago, Polygonum, Rumex, Urticaceae, Cerealia, and Papilionacea.
§This paper.

Animal DNA. Observation under a light microscope of the food residues from both the ileum and colon of the mummy allows one to easily spot minute fragments of well-preserved muscle fibers (Fig. 4). The presence of muscle fibers in the colon, on the other hand, had been previously reported (3, 4). To identify the species of provenance, we PCR amplified the DNA extracted from the ileum and colon samples by using the MbosL1269/H1346 oligonucleotide primer pair. This system is designed to bind to an approximately 117-bp-long fragment of the (mitochondrial) 12S ribosomal RNA gene (12S rDNA) of a wide range of mammals. Both specimens produced amplification bands of the expected size. Amplification products were then cloned and sequenced. The clones of the colon library (21) were found to group in two clusters, of which the first (19 clones) corresponded to human DNA (presumably originating from the mummy itself and/or from contaminations), whereas the second (consensus L) was closest to domestic goat (*Capra hircus*) with 3 substitutions. For this reason, after all of the ancient DNA analyses had been completed, we sequenced a corresponding portion of the 12S rRNA gene of ibex (*Capra ibex*) and found that it perfectly matched the Iceman's sequence. The screening of the ileum library (17 amplicons), on the other hand, showed that 8 clones were of human origin, and the other 9 clones (consensus K) corresponded to the database sequence (GenBank accession no. AF091707.1) of red deer (*Cervus elaphus*) with two substitutions. However, when we checked a specimen of modern red deer from central Europe, we found that its sequence was identical to the ancient one. On the other hand, a careful comparison of the AF091707.1 sequence with other Cervoidea sequences showed that the two substitutions were likely to be sequencing errors. Fig. 5 shows the colon and the ileum animal consensus sequences within a phylogeny of the Pecora infraorder (see also Table 1).

Discussion

Plant Microremains. We can now compare (Table 2) the results of our DNA analysis with those of previous ones performed by using light and electron microscopy (3, 4). The first show that the

plant 18S rDNA spectrum of the colon is dominated (73%) by the DNA of conifers, followed (22%) by that of cereals (Triticeae) and (5%) of ferns (Filicales). The most likely explanation is that pinus and fern sequences reflect the presence of background pollen and spores in the intestines. This conclusion is supported by the lack of pinus and fern sequences in the rbcL libraries. One may wonder how pollen sequences are obtained, as the pollen coat offers a strong obstacle to DNA extraction. We can only observe that conifer 18S rDNA sequences have also been reported in a previous study on fossil ice cores from northern Greenland (21). The most plausible interpretation of this result is that the DNA comes from ice-trapped pollen. It seems therefore that a prolonged period at low temperature, and possibly the effect of the pressure, may loosen the resistance of the pollen coat, thus making it possible for DNA to be extracted. Pollen of conifers and spores of ferns, on the other hand, were also detected in the course of the examination of the colon content conducted by using microscopy, though in this type of analysis the prevailing types of pollen were those of hop hornbeam (*Ostrya carpinifolia*) and hazel (*Corylus avellana*), which accounted for 55% and 20% of the pollen counts, respectively (Table 2).

The vegetation in the Alps correlates closely with climatic variations. After the end of the last Glacial period, the altitudinal zonation of the forests became progressively established: (*i*) mixed deciduous forest (oak, elm, lime, ash, maple, and hornbeam) in the valleys; (*ii*) mixed deciduous and coniferous forest (spruce, fir, beech, hazel, birch, and other deciduous species) at intermediate altitudes; and (*iii*) subalpine coniferous forest (Arolla pine, dwarf mountain pine, Scots pine, larch, and spruce) up to the tree line (24). With regard to the ferns, these plants grow in moist habitats of the Alps in a wide range of low and intermediate altitudes. In the Neolithic period, ''slash and burn'' was the method adopted to clear patches for cereal growing. On the other hand, the herb-rich alpine meadows and grasslands were ideal pastures for farm stock. If we now consider the arboreal 18S rDNA spectrum of the colon (pines) and the pollen spectrum, as determined by microscopy, we can observe that both fit with the distribution of the above-described vegetation on the Neolithic Alps, though they apparently correspond to different altitudinal zones of vegetation. Among the causes of discrepancy, one could consider, for example, variation from one sampling to another because of differences in the content composition inside the colon.

Plant Macroremains. The presence of the macroremains of Poaceae, possibly cereals, in the ileum is suggested by the *rbc*L DNA analysis (cluster H). The same type of analysis indicated the presence of Poales (cluster J) and Eudicotyledons (cluster I) macroremains in the colon. As the 18S rDNA analysis of the colon content indicated the presence of Triticeae, we may presume that the Poales fraction of the colon macroremains is composed by cereals. If we compare the result of the molecular and of the microscopic analysis of the colon (Table 2), it can be observed that although both show the presence of cereals and dicotiledons, the relative proportions of the two types of remains vary considerably from one analysis to another. In this case, the different results could, possibly, be explained by the fact that cereal residues possibly keep morphological details better than dicots, thus leading to an underestimation of the latter when the examination was performed by microscopy.

Fungi. Identification of fungi by use of the 18S rDNA sequence is less accurate than that of plants. However, the fact that both the Urediniomycetes and the Heterobasidiomycetes classes include psicrophilic genera such as Leucosporidium and Mrakia and that, in addition, the consensus sequence F is very close to a fungal sequence (T44NS-7) previously obtained in a study on the DNA of the man's grass clothing (20), suggest that the fungi present in the intestines are most probably representatives of the cold-adapted microflora that developed after the corpse and the equipment became entrapped in the ice (22).

Animal Macroremains. In the recent past, the reconstruction of the diet of the prehistoric hunter/warrior was the object of some controversy. Stable carbon and nitrogen isotope analysis performed on the Iceman's hair led Macko and colleagues (19) to conclude that the man was a vegetarian, or even a vegan. This conclusion was rejected by Dickson and colleagues (4) on the basis of two kinds of evidence: (*i*) paleobotanical analyses showing the presence of unidentified muscle fibers in the food residue from the Iceman's colon together with a prevailing proportion of plant fragments (mainly cereals of the wheat and wheat–rye type); and (*ii*) reinterpretation of the Macko and colleagues stable isotope analysis showing that the data were indicative of an omnivorous rather than a vegetarian diet.

Of the utmost interest, therefore, are the results of the DNA analysis of the animal component of the intestinal content, although they are obviously indicative only of the composition of the last meals and not of the diet. In the course of the first archaeological exploration of the Iceman site, two small bone splinters were discovered at the base of the rock shelf, in addition to the other remains. Anatomical and zoological investigations performed by von den Driesch and Peters (23) led them to conclude that the pieces were lateral apophyses of a fourth and a fifth cervical vertebra of a male Alpine ibex (*C. ibex*). This discovery gave rise to the hypothesis that this meat had represented the Iceman's ultimate food reserve. Such a hypothesis has resisted unchallenged for many years (1–4). We are now able to demonstrate that although the use of ibex meat is confirmed by the result of the analyses of the colon content, the last meal of the Tyrolean Iceman was actually composed of red deer meat. The use of red deer meat as a food supply is perfectly consistent with the paleozoological identification of the materials used by the iceman to manufacture his equipment: domestic goat and red deer in the leggings and the punch of a retoucher (an implement used to sharpen the edge of stone tools), a long curved spike, and a bundle of four points were all made from red deer antler (1, 2). According to some authors (24, 25), the deforestation in European Mesolithic was mainly aimed at favoring the growth of red deer herds, which were maintained in a state of semidomestication by means of selective hunting. The strict connection between Neolithic man and red deer is witnessed by the wealth of artistic representation of this animal among the archaeological finds in the central and eastern Alps; the polished and carved stones of the Val Camonica, in particular, evidence how the red deer was central to the interests of the prehistoric populations along the Alpine arc (26).

Conclusions

According to the present DNA analysis, the last journey of the hunter/warrior was made through a subalpine coniferous forest, where he possibly had a first meal, composed of cereals, other plant food, and ibex meat, and ended with his death in a rocky basin at over 3,200 m above sea level, not before his having had a further meal based on red deer meat and, possibly, cereals.

It may be of interest to compare the last meals of Ötzi with the ''last meals'' of other prehistoric individuals. All of the more so because it has been recently proposed that the Iceman might have been the victim of a ritual sacrifice (27). The best source on this matter is perhaps given by the studies on the so-called ''bog bodies'' such as the Tollund, Grauballe, Borremose, Huldremose, and Lindow II and III individuals (28). Some of them (Tollund, Grauballe and Lindow II, for example) clearly died of a violent death. Analyses performed through the years have shown that there is great variation in the plants eaten, and this

has given rise to the view that some of the last meals may have had ritual significance, though it is still matter of speculation whether the above-mentioned individuals were actually victims of sacrifices or, rather, of executions. Tollund Man, for instance, had eaten many species, including barley, willow-herb, linseed, and gold of pleasure. Grauballe Man and Lindow II had wheat and barley as major cereals in their intestines, together with some weed seeds, whereas a Borre Fen individual had no cereal component, just wild species. On the other hand, corn spurrey *Spergula arvensis* is present in the Huldremose, Grauballe, and Borre Fen individuals.

We can observe that, regarding the presence of cereals, the last meals of the Iceman seem close to those of Grauballe and Lindow II. On the other hand, virtually nothing can be said about the animal component in the intestinal content of the bog bodies, because of the poor state of histological preservation of the remains (in the case of Grauballe Man, the consumption of meat has been inferred from the finding of ova of the red squirrel parasite *Eimeria mira*) and of the impossibility of performing molecular analyses because of complete DNA degradation (29).

We can further attempt to put the last meal and the death of Ötzi on a temporal scale starting from the observation that his stomach was empty. Forensic literature reports that the stomach starts emptying within 10 min from the first food ingestion. The "head" of a solid meal proceeds at a speed of $\approx$2 m/h, thus reaching in about 3 h the ileo-caecal valve, where it can stop for a further hour. It is known, however, that the speed of stomach emptying can vary widely according to meal composition (whether, for example, the meal is richer in carbohydrates rather than in meat or lipids). In addition, this parameter can vary according to the state of health of the individual, being substantially slower in gastropathic subjects and, what is even more important, in case of protracted emotional stress (30). The latter condition, in particular, seems to apply to the Iceman, because, as reported in the Introduction, he had been hit by an arrow that did not kill him immediately (31).

We can finally observe that although the composition of his last meals offers no obvious contribution to the hypothesis on whether the Iceman was or was not the victim of a ritual sacrifice, the finding of ibex and deer meat certainly strengthens the one that, among other possible social roles, he covered that of hunter.

Almost 30 years ago, Nanna Noe-Nygaard (32) showed that Mesolithic deer and wild boar hunters used to aim their arrows and spears at the left shoulder blade as this gave them the best chance of killing the prey at the first shot. As the arrow that struck Ötzi actually pierced his left shoulder blade, it seems to us much more reasonable to assume that, rather than of a ritual sacrifice, he had been the victim of some rivalry among big game hunters.

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