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Molecular ecology of a Neolithic meadow: the DNA of the grass remains from the archaeological site of the Tyrolean Iceman

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Abstract. The paper reports on the molecular analysis of samples of approximately 5,300-year-old grass found at the alpine archaeological site where the so-called Tyrolean Iceman was discovered. The grass comes from a 'cloak' made of long grass blades and/or the stuffing of the 'snow footwear' worn by the Iceman. The results show that while the largest fraction of the DNA extractable from the grass is of 'foreign' origin, a much smaller part belongs to the original genetic material of the grass itself, and can be used as a valuable taxonomic clue to the plant species utilized by neolithic men to manufacture their equipment. On the other hand, the 'foreign' DNA, or at least a portion of it, comes from microorganisms – mainly filamentous fungi and unicellular algae – which seem to have been associated with the grass since the time the grass was harvested.
Key words. Frozen plant remains; grasses; plant pathogenic fungi; Tyrolean Iceman; ancient DNA; Neolithic.

DNA in archaeological plant specimens

Molecular genetic techniques are providing an increasing wealth of useful information on the relationship between crop species and their wild relatives, and the extent of genetic diversity in such species. The application of gene technologies to extinct organisms can be considered to be the ultimate extension of studies of the world's genetic resources; DNA analysis of ancient specimens can provide insights into past genetic diversity and, implicitly, an estimate of the loss of genetic diversity through the centuries.

Fundamental to all this is knowledge about whether and to what extent, the original heridity material of a land plant is able to survive through long time spans. A paper by Neil Hallam[14] reports what can be taken as a first indication that nuclear cell components can survive in an archaeological plant specimen. This scholar examined thin sections of emmer wheat (*Triticum dicoccum*) grains from Thebes and from the silos of Fayum, Egypt, by electron microscopy. The grains from Thebes had been dated 5000–4000 years before present (BP) on archaeological evidence, while those from Fayum had been radiocarbon dated 6441 ± 180 ^{14}C-years. The micrographs published by Hallam clearly show that the fine structure of the seeds has been preserved to an amazing extent, with nuclei, nucleoli and chromatin still perfectly distinguishable. A subsequent biochemical analysis of the same material performed by Daphne Osborne[22] showed, in fact, faint traces of severely degraded DNA and/or RNA. Very short nucleic acid fragments were isolated by Rollo[25] in the early 1980s from 3300-year-old seeds of cress (*Lepidium sativum* L.) from the tomb of 'architect' Kha, a high-ranking officer of the Thebes necropolis. When characterized by enzymatic digestion, polyacrylamide gel electrophoresis and molecular hybridization with cloned plant genes, the nucleic acid fragments were shown to be ribonuclease-sensitive, low-molecular weight (<100 bases in length) and still capable of binding to modern plant nuclear genes for ribosomal rRNA (rDNA). In the same years, using a DNA extraction procedure based on the use of cetyltrimethylammonium bromide (CTAB) to separate nucleic acids from polysaccharides, Rogers and Bendich[24] succeeded in isolating high molecular weight (up to 30 kilobase pairs, kbp) DNA from a large collection of old and ancient plant materials, including mummified plant seeds from packrat middens dating back to 500–45,000 years ago.

Unfortunately, in these experiments no specific hybridization test was applied to determine whether the DNA was endogenous to the seeds or due to microbial contaminants. On the other hand, the microbial origin of the high molecular weight DNA extracted from approx 2,300-year-old seeds from a wet site of southern Italy was demonstrated by Rollo et al.[27] by molecular cloning of the 'ancient' DNA, followed by labelling of the clones and hybridization with reference DNA. Evidence of the presence of endogenous DNA in maize kernels, radiocarbon-dated 980 ± 95 ^{14}C-years from a Huari archaeological site (Peruvian coast), was provided by Rollo et al.[28] using the polymerase chain reaction (PCR). In this case, short nuclear and mitochondrial DNA tracts could be replicated in vitro and the copies characterized by restriction endonuclease digestion. Helentjaris[16], on the other hand, reported on the extraction of high molecular weight DNA – with only moderate degradation, compared to modern samples – from five cob samples from an Anasazi Indian site (12th–13th century AD). More recently Rollo et al. were able to recover DNA fragments 90 bp long – corresponding to

the termini of the so-called *Mu*-elements, a class of transposable elements diffused in the genus *Zea* – from 1000- to 2000-year-old maize samples from Peru[29]. The *Mu*-element tracts were subsequently characterized by nucleotide sequencing and single strand conformation polymorphism (SSCP) analysis[1].

The findings of the so-called Tyrolean Iceman and his neolithic equipment has provided scientists with a unique opportunity of studying the long-term survival of DNA in particularly well-preserved human and plant remains[26]. The description of the results of the analyses performed on some samples of grass from the Iceman's equipment is the subject of the present report.

The Tyrolean Iceman

A mummified human body, later recognized as that of a Neolithic herdsman/hunter, was spotted on 19th September 1991 by two German tourists at 3270 m above sea level in Alta Val Senales, Italy, very close (92.6 m) to the Austrian/Italian border. In the days following the finding and the subsequent recovery of the body from the ice, several pieces of equipment such as a bow made of yew, a quiver with arrows, a copper axe, a stone knife and remnants of garments were found in close proximity to the body. It was soon realized that the particular nature of the site – a shallow rocky basin filled up with ice and snow – had made possible the preservation of many, otherwise highly perishable, materials such as human hairs, leather, hide, and even hay, tree leaves and fungal fruiting bodies. In particular, the remains of Neolithic 'snow footwear' made of leather and hide and employing hay as an insulating material were still dangling from the legs of the mummy at the moment of its recovery from the ice, during the first survey of the site (September 1991), and a sort of cloak ('Grasmatte') made of woven tufts of grass was later found nearby[9,20]. A second archaeological expedition, carried out during August 1992, led to the recovery, among other plant remains, of additional grass fragments which most probably came from the very long blades making up the 'cloak'. While the artefacts found during the first survey of the site were brought to the Roman Germanic Museum in Mainz, Germany, where most of them underwent conservation treatment, those found in 1992 were put in a refrigerated container soon after their recovery from the ice, and then transferred to Bolzano, Italy, where they were simply kept in a refrigerator at -2 to $+5$ °C. The relative abundance of the grass fragments recovered, and the proper conditions employed for their conservation, made it feasible to use part of them for DNA analyses. As the team officially appointed to study the DNA of the plant remains of the Iceman's site, we could obtain several samples of grass just a week after the end of the 2nd archaeological expedition, and use them for molecular analyses.

The human body and the grass artifacts recovered in the course of the first (1991) excavation of the site were radiocarbon dated using accelerator mass spectrometry (AMS) in laboratories at Gif-sur Yvette, Uppsala, Geneva and Oxford[2,37], thus their age is well known. As a precaution, however, we sent a sample of the grass found in 1992 to Krueger Enterprises Inc., Geochron Laboratories Division, Massachusetts, for AMS dating. The result was that the grass remains are $4{,}555 \pm 48$ ^{14}C-years old, which means that they date back to 3362–3136 BC (one sigma) or 3492–3049 BC (two sigma). These figures fit very well with the age of the body for which the uncalibrated ages of $4{,}523 \pm 27$ ^{14}C-years (skin) and $4{,}576 \pm 27$ ^{14}C-years (bone), were determined[2].

A quest for Neolithic grass DNA

The grass family is the most important one among the flowering plants, including the cereal grains as well as many forage crops; the members of this family dominate the prairies, steppes and savannas over large regions of the Earth. The grass family is also among the largest families of flowering plants, with an estimated 500 genera and 8,000 species. Fossil evidence suggests that the grass family is relatively young (50 to 65 million years old) and the grasslands were present on several continents by the end of the Eocene[40,42]. Because of the economic importance of many grass species, grass systematics has been the focus of considerable study. However, highly reduced floral structures and an apparently high degree of convergent evolution have made both subfamilial classification and phylogenetic reconstruction a difficult task within this family. This fact has promoted scholars to exploit diverse sources of systematic evidence, including biogeography, morphology, anatomy, physiology, cytology, biochemistry and, more recently, molecular biology[10]. Although desiccated grass (wheat and barley) leaf blades and haulms have also been recovered from Neolithic archaeological sites in Egypt, the state of preservation of the grass from the Iceman's site is unrivalled.

For our analysis, we utilized fragments of grass (each about 4 cm in length, equivalent to approximately 16 mg dry weight) from three samples (T27, T44 and T182) from the Iceman's site. The fragments were put in mortars and following the addition of an SDS/EDTA/phenol-based grinding medium, to inhibit nuclease activity and facilitate the lysis of the tissues, homogenized with pestles. The grass homogenate was sequentially submitted to extraction using phenol, chloroform and ether, to eliminate proteins and lipids and leave nucleic acids (DNA and RNA). The nucleic acid fractions were eventually precipitated from the suspension in the cold using ethanol, and the sediment was desiccated and

resuspended in a sterile medium for further analyses[31]. In the following steps, the presence, concentration and state of preservation of the nucleic acids were determined by fractionating the preparation using gel electrophoresis followed by treatment of the gel with ethidium bromide.

The result shows that the T182, T44 and T27 preparations from grass from the Iceman's site contain appreciable amounts of DNA. Electrophoresis separated the DNA into high, middle, and low molecular mass fractions; the first was composed of fragments about 10,000–20,000 bp in length. The amount of extractable DNA differed in the three samples, ranging from about 160 ng (sample T182) to 3μg (sample T27). By treating the gel with pancreatic ribonuclease, little if any change in the fluorescent pattern of the nucleic acids could be observed, indicating that neither RNA nor modified DNA was present in a significant amount.

The observed electrophoretic patterns are common to most DNA preparations from plants, humans, animals, fungi and bacteria; therefore, on the sole basis of this test, nothing can be said about the possible origin of the DNA extracted from the grass.

The second question we tried to answer concerned the origin of the DNA extracted from the grass remains. If one is dealing with an ancient specimen, the simplest answer to this question, i.e. that the DNA isolated from a grass blade is grass DNA, is not necessarily the right one. Several reports[27,36] show that bacterial and fungal contamination can be a rather common occurrence in ancient plant, animal or human remains. To obtain an insight into the source of the DNA extracted from grass from the Iceman's site, DNA samples from the three preparations were fractionated on a gel. DNAs isolated from modern grass samples (fresh and desiccated specimens collected in the field) were separately loaded on the same gel. Following the electrophoretic run, the DNAs were transferred from the gel to a nylon membrane and tested for their capacity to bind to a radioactive nuclear DNA probe (18 and 25S rDNA) from flax[12,39]. The rationale of the test was that the flax probe, under the conditions employed, would bind to any plant DNA, irrespective of the plant species, but would not bind to any bacterial DNA. Subsequently, the negative films of the UV pictures of the gel and the autoradiographic films were scanned by a densitometer (fig. 1), with the exception of those from sample T182, which gave too faint a trace on the two films to be analyzed in this way. Finally, the area under the corresponding densitometric profiles was determined and the ratio, radioactivity bound/amount of DNA on the gel, calculated for the different specimens. We found that the ratio was higher (3.29) for the fresh specimen, lower for the (modern) desiccated one (0.57) and reached the lowest value in the cases of the two ancient ones, T27 and T44 (0.31 and 0.35, respectively). By taking the ratio for the fresh specimen as 100% we estimated that in the desiccated sample the percentage of grass DNA was only 17%, a figure which fell to 9% (T27) and 10% (T44) in the two ancient specimens.

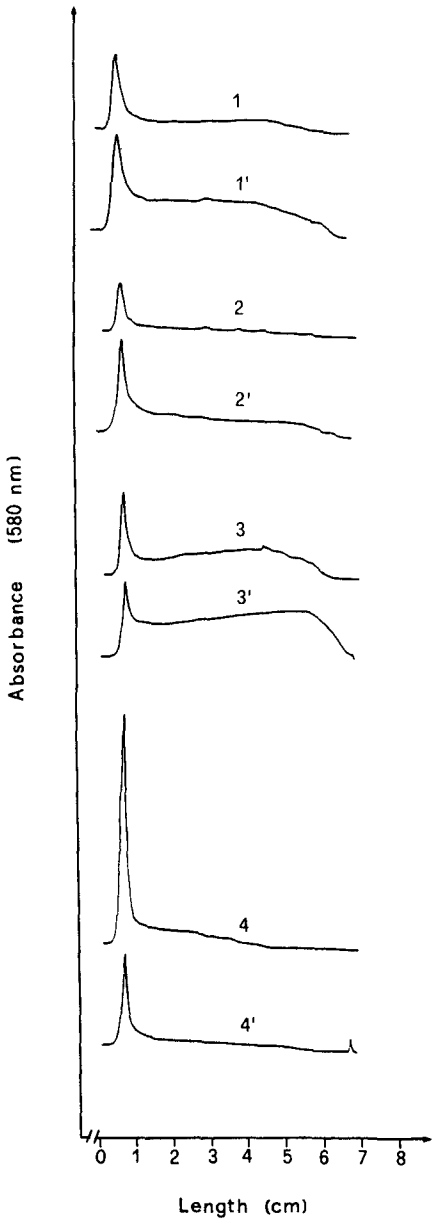

Figure 1. Analysis by gel electrophoresis and molecular hybridization, using a plant (flax) rDNA probe, of the DNA isolated from two Neolithic (T44, T27) and two modern grass samples. Densitometric traces of the negative film of the UV picture of the gel (1', 2', 3', 4') and of the autoradiographic film (1, 2, 3, 4). Traces: 1, 1', sample T44; 2, 2', sample T27; 3, 3', modern desiccated grass; 4, 4', fresh grass.

The result of the hybridization experiment thus seemed to indicate that though certainly mixed with non-plant DNA, some of the original genetic material of the grass was left. One has, however, to be very careful in drawing even this apparently cautious conclusion as, unfortunately, while it is true that a plant nuclear rDNA

probe discriminates very efficiently between plant and bacterial DNA, the same is not equally true if the probe is employed to discriminate between plant, algal and fungal DNA. This means that the hybridization signals produced by the probe could be due to fungal and/or algal DNA as well. Therefore, the only legitimate conclusion we are entitled to draw from the results of the molecular hybridization experiment is that at least an appreciable fraction of the DNA extracted from samples T44 and T27 does not come from a bacterial contaminant.

The Cox *II intron sequence as a marker for plant DNA*

In a following step, attempts to isolate DNA tracts which could be recognized as bona fide remnants of the original genetic material of the grass were made using the polymerase chain reaction (PCR).

Among the many DNA tracts, genes or non-coding sequences making up the genome of a plant, the intervening sequence (intron) of subunit II of the mitochondrial gene for cytochrome *c* oxidase (*Cox* II) is particularly suited as a PCR target in experiments aimed at verifying whether some of the original genetic material is left in an ancient plant sample. This is because the DNAs of bacteria and fungi do not contain the *Cox* II intron, whereas it marks the DNA of all land plants[6]. In addition, as the intron is located on the DNA of a cell organelle and as each plant cell contains many copies of it, the chances that at least a few copies of the intron are left in the sample are higher than for many nuclear sequences which are normally present in only two copies per cell.

A PCR system designed to amplify a 420-base-long tract encompassing a short portion of the *Cox* II gene and about one half of the intervening sequence, was tested using the three DNA preparations (T27, T44 and T182) from the grass from the Iceman's site. Preliminary tests, however, showed that without further treatment the DNA preparations were not amplifiable using PCR. This problem is common to many old and ancient DNA preparations, and is due to inhibitors which copurify with the DNA[27]. We therefore submitted the DNA to a further purification step and then used it as a substrate for a PCR reaction. After the application of 30 PCR cycles, aliquots of the PCR mixture were fractionated by agarose-gel electrophoresis and the gel was stained with ethidium bromide and photographed under UV light. No PCR product was found with the DNA from sample T182 as a substrate, but the other two samples gave rise to fluorescent bands of the expected length (420 bp). In a subsequent step, the DNA fragments were re-amplified using PCR (20 cycles), cloned in a plasmid vector[4], and the nucleotide sequences of the PCR clones were then aligned with the corresponding tracts of the DNA of cultivated grasses such as wheat, rice and maize. The strong homology (98–99%) between the PCR clones and the reference sequences indicated that some plant DNA was indeed left in the ancient specimens.

The rbc*L gene sequence as a clue to grass taxomony*

In recent years there has been a growing interest in the use of the gene for the large subunit of the ribulose-1,5-biphosphate carboxylase (*rbc*L) as a clue to systematics and as a tool for reconstructing plant phylogeny[7,17]. As a chloroplast encoded gene, *rbc*L is present in many identical copies in each leaf cell. Actually its degree of reiteration is normally very high, much greater than that of a mitochondrial gene such as *Cox* II. As a consequence, the chances of finding copies of this gene in a DNA preparation from an ancient plant specimen are also correspondingly higher.

The *rbc*L gene has been used by E. Golenberg and P. Soltis[13,38] as a marker for plant DNA, to show that some of the original genetic material of 17–20 million year-old fossil leaves was left. An interesting property of *rbc*L is that, unlike the *Cox* II intervening sequence whose nucleotide sequence remains virtually unchanged in the different plant taxa, the sequence of the former varies from genus to genus, or in some cases even from species to species. Therefore, in addition to a confirmation that some of the original genetic material of the grass has survived, the search for and analysis of the nucleotide sequence of this gene can offer a clue to the taxonomy of the plant specimen. A drawback of this approach is that since the *rbc*L gene is common to many photosynthetic organisms – land plants, algae, and cyanobacteria all possess it – the chances of catching unwanted DNA sequences from ancient and modern environmental contaminants are high. At least in principle, however, unwanted *rbc*L gene copies produced by the amplification process can be identified by cloning the amplified DNA and sequencing individual clones.

As PCR primers, we employed two oligonucleotides designed to bind to the ends of a 529 bp segment of the maize gene. The nucleotide sequences of the binding sites of the primers are rather conserved in the different groups of grasses. With this PCR system (30 cycles), the DNAs extracted from the T27 and T44 samples produced bands of the expected length, but nothing was obtained from sample T182. Following further amplification (20 cycles), the DNA fragments were cloned in a plasmid vector and the nucleotide sequences of several individual clones was determined. The sequences were then compared with the corresponding tracts of the DNA of grasses, other land plants, algae and cyanobacteria, obtained from DNA data banks. Two clones (T2720 and T2717) were found to be identical and to show a very strong homology with all the grass *rbc*L

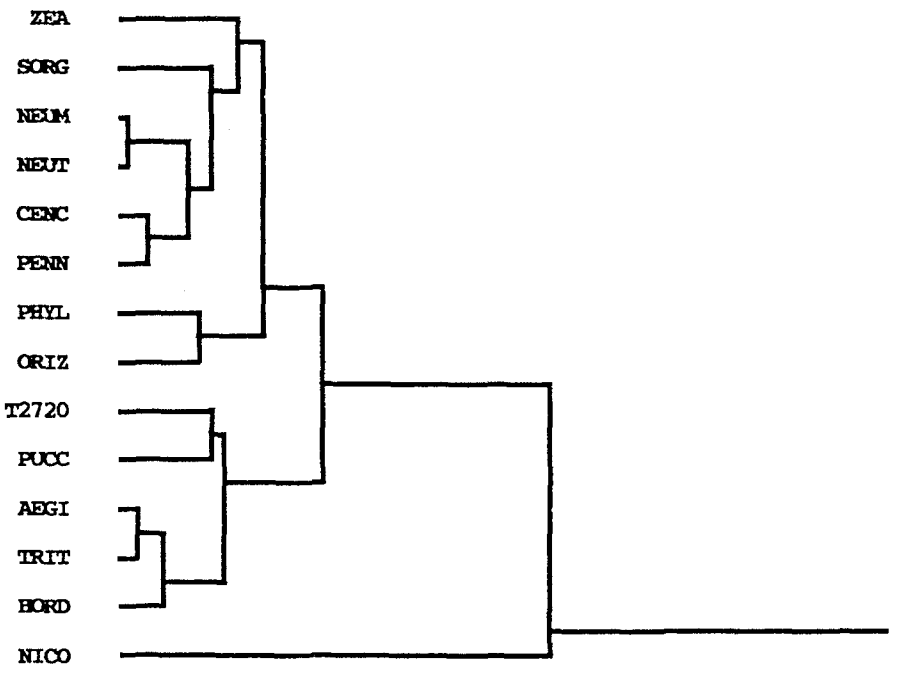

Figure 2. Phenetic tree of grasses including the grass from the Iceman's site. The tree is based on the comparison of a 483 bp long fragment of the *rbc*L gene. AEGI, *Aegilops crassa*; CENC, *Cenchrus setigerus*; HORD, *Hordeum vulgare*; NEUM, *Neurachne munroi*; NEUT, *N. tenuifolia*; NICO, *Nicotiana tabacum* (outgroup species); ORIZ, *Oriza sativa*; PENN, *Pennisetum glaucum*; PHYL, *Phyllostachys sp.*; PUCC, *Puccinellia distans*; SORG, *Sorghum sp.*; TRIT, *Triticum aestivum*; ZEA, *Zea mays*; T2720, Iceman's grass.

sequences tested, while the other clones showed weak homology or no homology at all. A tree based on grass *rbc*L sequences, which includes the sequences of these clones, is shown in figure 2. According to this tree the grass from the Iceman's site falls within a branch of the Gramineae family corresponding to the subfamily Pooideae which includes cereals such as barley and wheat.

Remnants of a 'Neolithic' microbial world?

Further sequence analyses of the *rbc*L clones from samples T44 and T27 gave a rather complicated picture for the DNA from the grass from the Iceman's site. As a matter of fact, though sequence comparison shows that two clones (T2720 and T2717) certainly come from the genetic material of the grass itself, this is not the case with the other clones. This fact is best exemplified by the analysis of clone T4413. The nucleotide sequence of this clone shows only low (77%) homology with that of clone T2720 and, in general, with grass DNA. It shows a slightly higher homology with the *rbc*L gene of a freshwater unicellular alga such as *Chlamydomonas reinhardii* (80%). Other clones show homology with the DNA of dicotyledonous plants (91–96%), a result which is puzzling. If the possibility of laboratory contamination by dicotyledonous plant DNA is excluded, one has to look elsewhere to find an explanation for this curious finding. Perhaps it could lie in the fact that, in order to avoid the risk of washing away small DNA molecules, the grass specimens were not cleaned. Thus, some ancient plant debris from the archaeological site may have remained stuck to the grass and undergone the DNA extraction procedure. This is obviously just a tentative explanation and further research work is required to clarify this point. The presumed origins of 26 clones from samples T27 and T44 are shown in figure 3. In fact, *rbc*L sequences of exogenous origin seem to make up most of the sequences and this situation may be common to all the grass remains from the site. In addition, it has to be considered that the PCR system

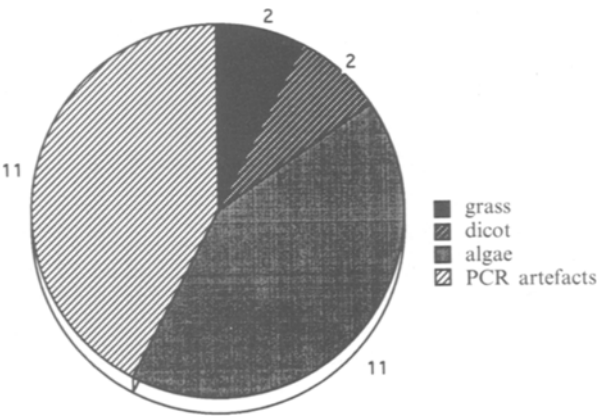

Figure 3. Presumed origin of 26 PCR clones from DNA from the ancient grass sample. The DNA was amplified using *rbc*L primers.

employed can only detect DNA from photosynthetic organisms, which means that if the DNA of non-photosynthetic bacteria, fungi and protozoans were present in the preparation it would not have been detected. Thus the proportion of 'foreign' DNA molecules in the grass from the Iceman's site is, possibly, even higher than it appears from the pie chart.

A preliminary survey of three fragments of the ancient grass using a scanning electron microscope (SEM), aimed at verifying whether anything resembling a filamentous fungus or a unicellular alga was associated with the grass, showed that fungal hyphae actually were present in one sample out of three. The distribution and length of the hyphae suggested that they could belong to a plant parasite which was already present on the grass when it was harvested 5,300 years ago, rather than to a modern saprophyte which colonized the archaeological specimens after their recovery from the glacier. This exciting possibility prompted us to investigate the microbial flora associated with the ancient grass more thoroughly. With this in view, further and more systematic SEM observations were performed; in addition, the range of the DNA analyses, which was initially restricted to plant and algal nucleotide sequences, was widened to include those of fungi.

The SEM survey revealed that several types of biological structures are associated with the grass fragments: fungal hyphae, chrysophycean stomatocysts[35], diatom frustules and, possibly, bacterial cells. The relative abundance of the different cell structures is shown in figure 4. In particular, if one considers that fungal hyphae are found in almost two-thirds of the fragments examined and that the total mass of the fragments considered corresponds roughly to that of a single piece of grass blade of 4 cm in length, one can appreciate how massive the presence of filamentous fungi in the Iceman's grass artefacts is.

In a subsequent phase of the research, a series of attempts to retrieve fungal DNA from the grass was performed. To identify DNA fragments of fungal origins, we again took advantge of the PCR technique, as it is particularly suited for retrieving specific DNA fragments from complex mixtures of DNA. As a PCR target, we chose the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) gene repeat. This is a DNA tract 250–600 bases in length[15], separating the so-called small subunit rRNA (SSUrRNA) and large subunit rRNA (LSUrRNA) genes. The ITS region includes the gene for the 5.8S rRNA, which is located approximately in the middle of the ITS region. The sub-regions either side of it are called ITS1 and ITS2. Comparative studies of the nucleotide sequences of the rRNA genes provide a means for analyzing phylogenetic relationships over a wide range of taxonomic levels. Nuclear rRNA gene sequences evolve relatively slowly and are useful for studying distantly related organisms, whereas the mitochondrial rRNA genes evolve more rapidly and can be useful at the ordinal or family level. The ITS1 and ITS2 regions of the nuclear rRNA genes evolve fastest and may vary among species within a genus, or among populations[21,44]. Studies performed on herbarium specimens have shown that specific tracts of fungal DNA can be retrieved, using PCR, from desiccated fungal tissues as old as 50 years[3].

To amplify the entire ITS region, we took advantage of two specific oligonucleotide primers ITS1 and ITS4, designed by White et al.[44]. The DNA tract encompassed by these two primers is actually slightly larger, and includes small portions of the SSUrRNA and LSUrRNA genes. All the samples tested produced amplificationbands whose size, as determined by comparison with molecular standards, falls within the size range expected for fungal ITS regions. The fact that a single DNA preparation gave rise to two or even three amplification bands was taken as a preliminary indication that several genetic variants of a fungus, or several species of fungi, were associated with the grass. To obtain further details on this point, the DNAs were cloned in a plasmid vector and the nucleotide sequence of several individual clones was determined. The DNA amplified did indeed correspond to complete ITS regions. Subsequently, the 5.8S gene moieties were compared with the corresponding sequences from a DNA data bank (EMBL Data Library). It was shown that some of the 5.8S gene sequences (T18217 group) are relatively close to the corresponding sequences of the fungi *Fusarium sambucinum* (92.3% homology), *Sclerotinia sclerotiorum* (93.6% homology), a plurivorous plant pathogen, and the yeast *Saccharomyces cerevisiae* (92.3% homology). Another group (T18218) of clones, on the other hand, is of uncertain origin: it seems to come from an organism which is only distantly related with a filamentous fungus (76.4% homology with the DNA of *Puccinia graminis*).

Other clones (T2709 group) analyzed display virtually identical 5.8 S genes and ITS1 spacer regions. When

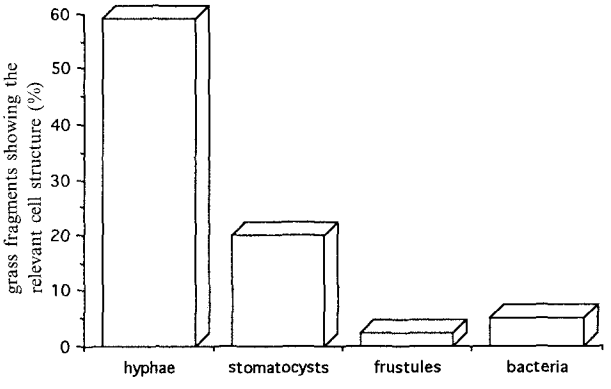

Figure 4. Relative abundance of the different components of the microbial flora of the Iceman's grass as revealed by the SEM.

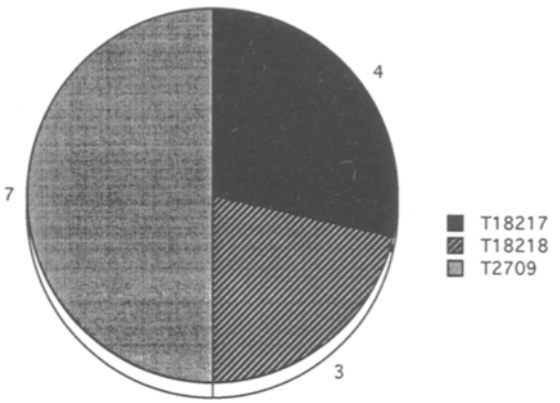

Figure 5. Relative abundance of the different types of fungal 5.8S gene sequences in the DNA extracted from the Iceman's grass, as determined by sequence analyis of 14 PCR clones from samples T27 and T182.

compared with the corresponding sequences from the data-banks, they show a close relationship with those of well-known phytopathogenic/saprophytic fungi such as *Fusarium sambucinum* (97.4% homology), *Sclerotinia sclerotiorum* (97.4% homology), *Alternaria alternata* (96.8% homology) and *Epichloe typhina* (96.2% homology). The last-named is a parasite infecting over 50 species of grasses (fig. 5).

On Tyrolean grasses and Siberian mammoths

Previous reports on DNA isolation from archaeological and palaeontological plant specimens refer to dessicated ('mummified') seeds kept in a warm environment, or to fossil and sub-fossil leaves and seeds only. Furthermore, they do not present an univocal picture of the state of preservation of the DNA. Cress seeds from the Egyptian necropolis of Thebes (approximately 3,300 years BP) were shown to contain only degraded nucleic acids[25], while approximately 45,000-year-old seeds from fossil packrat middens were reported to contain well-preserved molecules[24]. Equally, only very short DNA fragments could be isolated by Rollo et al. from pre-Columbian maize kernels radiocarbon dated 980 ± 95 ^{14}C-years BP[28,29,43], while the presence of virtually intact DNA molecules in maize samples of a similar age was reported by Helentjaris[16]. Fossilized levels of *Magnolia latahensis* and *Taxodium* 17–20 million years old, from the Miocenic shales of Clarkia, Northern Idaho, were shown to contain gene-size DNA[13,38], but independent analyses on the same material suggested that the DNA in the fossil leaves may have come from bacterial contaminations[36].

Evidence for the preservation of DNA in frozen remains comes from the studies carried out on Siberian mammoths and human bodies from the Arctic. As far as the former are concerned, the analyses performed by Johnson, Olson and Goodman[19] at the start of the past decade are particularly significant. These authors examined the DNA extracted from three mammoth specimens, radiocarbon-dated 40,000 (Magadan specimen) 53,000 (Khatanga specimen) and 10,000 years BP (Yuribei specimen) and submitted the DNA to tests such as thermal denaturation analysis and molecular hybridization to the DNA from the modern Asian elephant (*Elephas maximus*). They found that only a very small (2–5%) fraction of the DNA extracted from the mammoths was capable of binding to that of the Asian elephant, thus showing it to belong to the mammoth itself; the remaining 95–98% was found to originate from bacteria. As PCR was not yet available at that time, no attempt was made by the researchers to isolate mammoth genes. Another report[18] showed that the proportion of elephant-like DNA molecules was possibly only 1 in 10,000 of the total DNA. Short (350 bp in length) mitochondrial DNA fragments whose sequence closely matched the corresponding ones from an Asian elephant were eventually obtained by Russell Higuchi by the application of PCR[5,23]. No satisfying conclusion was reached by the above-cited authors about the origin of the contaminating bacteria, i.e. whether they came from the time of the mammoth's death or were introduced after excavation.

As was shown above, in the DNA preparations from the Iceman's grass equipment only a small fraction of the molecules seems to originate from the grass itself; the remaining fraction is made up of 'foreign' molecules mainly coming from unicellular algae and filamentous fungi. Concerning the original grass DNA, it will certainly be possible in the future to refine the data on the taxonomy of the ancient grass down to the genus or even to the species level by comparing the T2720 sequence with a larger number of reference sequences as they become available in the data banks. With this in view, it is of the utmost importance to determine whether or not the ancient double helices recovered from the grass have been affected by post-mortem modifications such as cytosine deamination or loss or oxidation of bases. This is important because, during PCR amplification, these lesions can give rise to misincorporation of nucleotides which are eventually read as genuine base substitutions, thus altering phenetic and phylogenetic conclusions reached on the basis of nucleotide sequences[8]. The test, initially performed on gel fractionated DNA and based on ribonuclease sensitivity, which in principle could discriminate between intact and modified DNA molecules, is not conclusive because it was performed on bulk DNA. Under these circumstances, the only approach one can use to assess the extent of post-mortem lesions is that of comparing the nucleotide sequence of different PCR clones[30]. The nucleotide sequences of two clones of ancient grass *rbc*L, T2717 and T2720, are identical, which suggests that this DNA has only undergone very limited damage. It may also be of interest in this context to know the maximum

size of the fragments which can be amplified using PCR. The results reported in this paper show that fragments of the *rbc*L gene almost as long as 530 bp can be obtained. On the other hand, attempts to amplify longer (700, 1200 and 1400 bp) portions of the same gene were unsuccessful. Although we do not consider the latter results as conclusive, this fact and the scarcity of original grass DNA in the samples both suggest that some degradation process has taken place.

The results of the analysis of the fungal genes, should be treated with some caution, as we cannot exclude the possibility that at least some of the fungal/yeast DNA sequences isolated from the grass DNA come from modern fungal/yeast contaminants at the site. We can only list a few pieces of microbiological and molecular evidence which seem to speak against this possibility:

1) Following plating of 16 grass fragments on two different culture media for fungi (potato-dextrose-agar and Sabouraud) and incubation of the plates for 10 days at 22 °C, fungal colonies grew on two samples only. This result suggests a rather low level of contamination of the grass samples by viable fungi. It is also possible that the viable fungi come from the plastic envelopes in which the samples had been kept since their recovery from the glacier, and which were not sterile. On the other hand, no growth of yeast colonies was observed on the plates and no yeast cell was detected by the SEM.

2) The nuclear DNA content of a filamentous fungus or a yeast is approximatley 0.05 pg. On the other hand, the amount of DNA extractable from the grass ranges, for fragments of 4 cm in length, from 160 ng (sample T182) to 3µg(sample T27). This relatively large mass of DNA could not have come from the few viable fungi which gave rise to the colonies in the plating test; rather, it seems reasonable to assume that the DNA came from the very abundant hyphae revealed by the SEM survey. The superior resistance of the chitine containing fungal cell wall, compared to the weaker plant cell wall, might have favoured a selective survival of the fungal DNA.

3) The intensity of the amplification bands after PCR correlates well with the amount of the DNA in the different samples. If the amplification bands were due to the DNA of a few contaminating fungal or yeast cells, we would not expect to observe any proportionality between the amount of DNA extracted from a sample and the intensity of the corresponding amplification bands.

4) Sequence comparisons have shown that the fungal 5.8S gene sequences isolated from the T27 grass sample are closely related (96–97% homology) with the corresponding sequences of the DNA filamentous fungi which are typical plant pathogens/saprophytes, such as *Fusarium*, *Sclerotinia* and *Alternaria*. As soon as more precise data are available on the taxonomy of the grass from the Iceman's site, it will be possible to identify and isolate the DNA of present-day specimens of the fungal parasites of the grass, and use this DNAs as standards for sequence comparisons. In this way, the attractive conclusion that the fungal DNA sequences isolated from the Iceman's grass belong to plant parasites of 5,300 years ago will, perhaps, be confirmed.

Note added in proof: While this paper was under proof, K. Haselwandter and M. R. Ebner reported that even the culturable fungi and bacteria might be representatives of the original microflora of the grass. These microorganisms are believed to have survived for 5,300 years. (FEMS Microbiol. Letts *116* (1994) 189–194.)

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