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Isolation of nucleic acids and cultures from fossil ice and permafrost

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Owing to their constant low temperatures, glacial ice and permafrost might contain the oldest nucleic acids and microbial cells on Earth, which could prove key to reconstructing past ecosystems and for the planning of missions to other planets. However, recent claims concerning viable cells and microbial nucleic acids obtained from ice- and permafrost cores from hundreds of thousands to millions of years old are not properly authenticated and the findings could be the result of contamination. Here, we discuss the processes that restrict the long-term survival of DNA and/or RNA molecules in ice and permafrost, and highlight sources of contamination that could result in false claims. Additionally, we present a set of precautions, controls and criteria to help ensure that future cultures and sequences are authentic.

In recent years, both glacial ice and permafrost (permanently frozen soils) have been the focus of much attention. Owing to their constant low temperatures, they are believed to be ideal for the long-term preservation of microorganisms and biomolecules. Several authors claim to have isolated ancient viable microbes and DNA and RNA molecules from ice-cores up to 100 thousand years (kyr) old and from permafrost-cores up to 2–3 million years (MY) old [1–14]. If true, these discoveries could fundamentally alter our views about microbial physiology, ecology and evolution (Box 1). Small amounts of permafrost (2 g of soil) have also been shown to contain DNA sequences from various mammals, including woolly mammoth, horse and steppe bison up to 30-kyr old and plant DNA as old as 300–400 kyr [15]. This discovery makes permafrost-preserved DNA potentially important in the study of past ecosystems (Figure 1). However, both the culturing of ancient microorganisms and the amplification of ancient DNA and RNA molecules from ice and permafrost are beset with problems.

The genomes of dead and dormant organisms degrade with time. Theoretical and empirical calculations show that short DNA fragments (100–500 bp) do not survive in the geosphere for more than 10^4 years in temperate environments and 10^5 years in colder ones. This is predominantly due to the spontaneous hydrolytic and oxidative damage that accrues [16,17] (Box 2). RNA has an even shorter half-life [16] and an upper limit of 1 MY has recently been suggested for all amplifiable nucleic acids [18].

The DNA of resting cells, such as bacterial endospores, might survive longer than theory predicts, probably because of special adaptations such as DNA-binding α/β-type small acid soluble proteins (SASP), which reduce the rate of DNA damage to the genome [19]. Nevertheless, because endospores have no active DNA repair, DNA damage accumulates within their genome as a function of the environment, eventually becoming lethal [20]. Likewise, continual metabolic activity and DNA repair persisting within trapped ancient microbes might enable cell and DNA survival beyond what theory predicts [21]. Although there are some indications of subsurface metabolic activity in permafrost settings [22], direct evidence for DNA repair within these cells is lacking. Thus, from a theoretical standpoint, the survival of viable cells and nucleic acids for hundreds of thousands to millions of years in ice and permafrost must be viewed cautiously.

A further problem is the risk of contamination. The use of nonspecific media for the culturing of microorganisms and the extreme sensitivity and ability of PCR to amplify trace quantities of contaminant DNA and RNA, cast serious doubts on the claims of nucleic acid isolation and cell survival in ancient ice and permafrost to date. There is

Box 1. The big picture

- DNA and RNA molecules are weak and degrade depending upon the level of water, oxygen and most importantly the temperature of the local environment.
- Permafrost and glacial ice contain the lowest temperature settings of any geological environment on earth.

DNA and organic molecules survive longer in colder environments as the rates of decay are slowed down an order of magnitude for every 10 °C drop in temperature.

- DNA and RNA and microorganisms should in theory survive longest in ice and permafrost.
- In theory, one should be able to extract DNA and RNA from these media up to several hundred thousand years and even a million years in age.
- The possibility of obtaining culturable (viable) cells from ice and permafrost has yet to be reproducibly shown.

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Box 2. DNA and RNA damage

DNA and RNA molecules are particularly prone to spontaneous hydrolytic damage. Cleavage of the phosphodiester bond in the sugar backbone by hydrolytic attack results in single-strand breaks. In a fully hydrated system, phosphodiester cleavage in DNA occurs about once every 2.5 h at 37 °C [41]. Single-strand nicks can also be generated by hydrolytic cleavage of the glycosidic bonds connecting the bases to the sugars, causing base loss (depurination and depyrimidation) and subsequent strand cleavage by β elimination. In hydrated DNA, both depurination and β-elimination occur once every 10 h at 37 °C. Depyrimidation is estimated to occur at a rate of 5% of that for depurination [16]. The lack of the 2′ OH group in DNA compared with RNA increases the strength of the phosphodiester bonds of the sugar backbone, but weakens the glycosidic bond that joins the bases to the sugars. Thus, RNA has a slower rate of depurination than does DNA, yet direct cleavage of its phosphodiester bonds will be more rapid [38]. Therefore, RNA molecules are not expected to survive as long in the geosphere, compared with DNA. Single-stranded nicks are largely responsible for the reduction in the number of amplifiable template molecules in fossil remains, the short amplification products (usually 100–500 bp) and lesions that block the action of DNA polymerases (Table I).

The miscoding bases hypoxanthine, uracil, thymine and xanthine can be generated by the hydrolytic deamination (hydrolytic cleavage of their amino groups) of adenine, cytosine, 5-methylcytosine and guanine, respectively. Cytosine is particularly prone to this reaction [41]. Miscoding lesions do not block DNA polymerases but do cause misincorporation of erroneous bases during PCR (Table I).

Free radicals, such as peroxide ($-O_2$) and hydroxy (-OH), as well as hydrogen peroxide (H_2O_2), cause oxidative damage to DNA and RNA. These radicals are likely to play an important role in limiting the half-life of DNA and RNA [16,41]. Many oxidative lesions block extension of polymerases during PCR, preventing amplification and eventually causing chimera sequences via 'jumping PCR' (i.e. extended primers acts as primers in subsequent PCR cycles binding nonspecific to template DNA [42]) (Table I).

DNA is also prone to condensation-type reactions via the exocyclic amine groups, which can react with the carbonyl groups of reducing sugars (Maillard products) potentially causing DNA–protein crosslinks [43]. In addition, DNA can be interstrand crosslinked via bifunctional alkylating agents such as phosphoramide mustard, cisplatin, melphalan and others, forming a covalent crosslink between the DNA strands [14,44]. Crosslinks are likely to prevent DNA amplification (Table I).

Table I. Damage in ancient DNA and the effects on PCR amplification

Processes	Modification	Effects	Refs
Hydrolysis	Strand break	Few template molecules → Contamination	[16,24,42]
		Short fragment length → Short PCR products	[16,24,42]
Hydrolysis and oxidation	Base modifications	Blocking lesions → 'Jumping PCR'	[8,38,42]
		Miscoding lesions → Base misincorporations	[16,38,45]
Alkylation and Maillard reaction	Crosslinks	DNA-DNA cross links → Nonamplification	[14,37,44]
		DNA–protein cross links → Nonamplification	[43]

increasing evidence that microbes and microbial DNA are ubiquitous in all environments and laboratory settings, making the risk of contamination with contemporary microbial cells and microbial DNA and/or RNA extremely high. As only ~1–5% of the potential modern microbial diversity, (i.e. extant microbial diversity) are estimated to be currently known [23], finding an unpublished microbial sequence or culture does not imply its authenticity. This makes the authentication of any ancient bacterial cell or DNA sequence a challenge. In comparison, plant and animal ancient DNA (excluding human) studies are less prone to contamination. Nevertheless, previously produced PCR products provide a serious contamination threat [24]. The high risk of contamination, especially in the case of microbial work, is likely to be the main reason for the conflicting results within this area (Box 3) and calls for standardized procedures within the field.

DNA and RNA degradation

Processes

DNA and RNA molecules are relatively unstable in comparison to other cellular materials, such as lignin and cutin. In metabolically active tissues, damage to the genome is rapidly and efficiently repaired via a host of repair pathways [16]. However, in inactive (dead or dormant) cells, damage to the DNA and RNA molecules

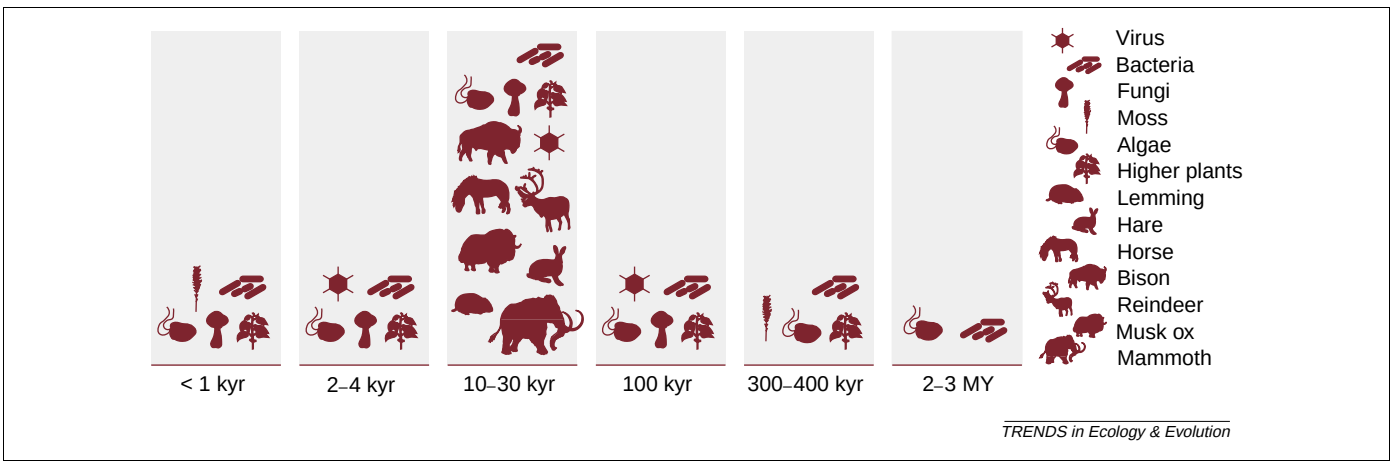

Figure 1. DNA and/or RNA sequences and viable cultures reported from glacial ice and permafrost of various ages [1–15]. This does not include the many DNA sequences from bone and soft tissue remains from permafrost settings. Abbreviations: Kyr thousand years BP; MY millions of years.

Box 3. Conflicting results

In spite of preliminary success in the retrieval of DNA and/or RNA sequences and viable microbes from polar ice and permafrost, the results published to date are conflicting. For example, neither viable cells nor amplification products have been obtained from the GISP2 or the GRIP ice-cores, respectively, using several liters of ice [9,13], in spite of previous reports to the contrary from just a few milliliters of ice melt [1,6]. Others claim the revival of viable microbial cells and amplification of viral RNA (350 bp) from ice-core samples up to 100 thousand year (kyr) old, from the Vostok drill site in Antarctica [2] or the GISP2 site in Greenland [5,6]. However, amplification products as small as 500 bp could not be obtained from core samples 2–4 kyr old drilled at the low latitude Hans Tausen ice cap [8] containing a relatively higher microbial cell count (Table 1, main text). Furthermore, it has been claimed that endogenous bacterial DNA (~1.4 kb [7] and ~900 bp [11]) has been found in ice-core samples derived from the refrozen water of Lake Vostok, an ancient lake beneath the East Antarctic Ice Sheet. However, one of the organisms (*Afipia*) identified [7] is a common laboratory contaminant [32]. It is a matter of concern that the only sequence identified to the same genera by the two research laboratories *Aquabacterium*) [7,11] was additionally recovered from the PCR controls [7]. Furthermore, later attempts to reproduce the initial sequence results from the Lake Vostok ice [7] have been unsuccessful [46]. Likewise, recent attempts to obtain microbial DNA sequences from 1.5–2 million-year-old permafrost samples from Kolyma Lowland and Laptev Lowland have failed [14] despite previous claims of viable cultures in such samples [3,4]. These conflicting results could be attributed to differences in methodological efficiency, but are more likely to be due to contamination: this underlies the need to establish clear standardized procedures.

accrues. Aggressive although incomplete degradation via endogenous and exogenous nucleases, as well as spontaneous hydrolysis, oxidation and alkylation, also limits the half-life of all biomolecules. These spontaneous processes are, among other things, dependent upon the availability of free water, oxygen-free radicals, pH, heavy metal ion chelation and the availability of alkylating agents [16,18,24] (Box 2). Colder environments are therefore better environments for the long-term storage of nucleic acids, given that rates of reaction generally drop an order of magnitude for every 10-degree drop in temperature [25].

Glacial ice

High-altitude polar ice caps with temperatures as low as −30 °C to −50 °C and no surface melting can be considered dry environments. Nevertheless, they do contain acidic liquid veins, which run along the triple boundaries of the ice crystals [26,27] potentially exposing the DNA and/or RNA molecules of dead and dormant cells to hydrolysis. Furthermore, low-altitude ice caps and some bedrock ice contain up to 1% free water and DNA will therefore be subject to hydrolytic damage. Even tightly hydrogen-bonded water molecules found in the major grooves of DNA, which support the molecules structure does not freeze. Whether these molecules are able to damage the DNA molecule is unknown.

There is little if any diffusion of oxygen through glacial ice below the first 70–80 m, when the snow becomes solid. However, DNA and RNA molecules are exposed to oxidation before this occurs. As the solidification can take several hundred to several thousand years, depending on the rate of snow accumulation, nucleic acids will initially be subjected to oxidative damage.

Permafrost

In permafrost, ice comprises 92–97% of the total water volume, whereas the remaining 3–8% is in an unfrozen state, depending on the temperature and sediment texture [12]. Therefore, nucleic acids in permafrost will be exposed to spontaneous hydrolytic damage. The rate of hydrolysis is likely to occur at a higher rate in permafrost than in high-altitude polar ice simply because of the higher temperatures (−9 to −12 °C in northeast Siberian permafrost and −22 °C in Antarctic permafrost, compared with −20 °C to −50 °C in high altitude polar ice caps).

The high methane values (up to 40 ml kg^{-1}) and redox potentials of +40 to −250 mV in northeast Siberian permafrost suggest largely anaerobic conditions [28] and, therefore, oxidative damage to the DNA and/or RNA molecules might be minor. This is in contrast to Antarctic permafrost, which contains redox potentials of +260 to +480 mV, suggesting largely aerobic conditions [28]. In addition, Antarctic permafrost sustains slightly alkaline pH (Miers Valley, Antarctica, pH = 7.95–8.45; Taylor Valley, Antarctica, pH = 8.95–9.5) [12] as opposed to neutral pH in northeast Siberian permafrost. These differences are likely to make nucleic acids in Antarctic permafrost more prone to oxidative and alkylation damage.

Temperature dependence

The effect of temperature on spontaneous chemical decay is described by the Arrhenius equation: $k = Ae - Ea/RT$, where k is the rate constant, A is the pre-exponential factor that depends on the reaction, Ea is the activation energy, R is the gas constant (8.31 KJ mol^{-1} at 1 atm) and T is the temperature (Kelvin). Accordingly, any decrease in temperature induces an exponential decrease in the reaction rate [29]. Rough calculations on the influence of depurination (a hydrolytic process largely responsible for DNA fragmentation; Box 2) on DNA survival suggest that a

Table 1. Cell counts (dead and viable) and DNA concentrations in ancient ice and permafrost[a]

Sample	No. of cells	Refs	Ng DNA	Refs
Hans Tausen (ice; Greenland) 82,5°N, 37,5°W, 1270 masl. 2-4 kyr	10^3 l^{-1}	[40]	ND[a]	
Kangerlussuaq (ice; Greenland) 67,09°N, 50,02°W 8-70 kyr	ND		0.7–3 l^{-1}	[13]
GRIP (ice; Greenland) 72,30°N, 37,30 °W, 3232 masl. 800 yr	Undetectable	[40]	Undetectable	[13]
Kolyma and Laptev Sea Lowland (permafrost; Siberia) 69–72°N, 140–156°E 10-600 kyr	10^7 g^{-1}	[14,15]	12–160 g^{-1}	[14]
Beacon Valley (permafrost; Antarctica) 77°50S, 160°36E 8.1 MY	$10^7 g^{-1}$	[14]	Undetectable	[14]

[a]Abbreviations: kyr, thousands of years; MY, millions of years; ND, no data.

bacterial genome of 3.0×10^6 bp (alternating purines and pyrimidines in a ratio of 1:1, and assuming constant activation energy) will be broken into fragments of *ca.* ~100 bp in length within 500 years at 15 °C, 81 000 years at −10 °C and 1.7 million years at −20 °C (Figure 2).

Additional factors influencing the Arrhenius equation are pH and heavy metal ion chelation (via the activation energy) and pressure (by changing the gas constant). The presence of higher concentrations of heavy metals, soil humic acids and microbial cells in permafrost than in glacial ice makes DNA in permafrost more prone to microbial degradation, crosslinking and condensation type reactions. However, in spite of the increased soil components of permafrost increasing DNA degradation, permafrost has negligible pressure compared with deep glacial ice, which can contain pressures up to 300 bar. This is likely to increase the rates of spontaneous decay, reducing the half-life of any DNA or RNA molecule. Nevertheless, the constant low temperatures of high-latitude polar ice caps and permafrost are likely to make them among the best environments on Earth for the long-term storage of microbial cells and nucleic acids.

Contamination

Conflicting results

Several papers have reported the successful retrieval of ancient DNA and/or RNA sequences and viable cells from glacial ice and permafrost [1–15]. These papers reveal highly conflicting results (Box 3). Although this could be attributed to differences in methodological efficiency, they are more likely to be the result of contamination.

The risk of contamination is high for the amplification of nucleic acid by PCR and for the culturing of cells from unspecific media. Given the low number of viable cells and nucleic acids present in glacial ice and permafrost (Table 1), contamination of samples with modern exogenous cells and DNA molecules is a serious concern. There are two priority issues that must be addressed to improve the likelihood of successful, contaminant free extraction of cells and DNA and/or RNA from ice and permafrost; contamination derived from (i) the drilling and coring procedure; and (ii) laboratory reagents and techniques.

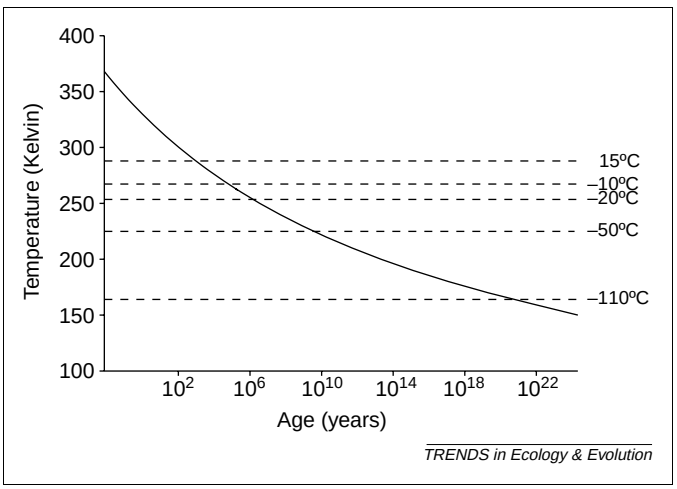

Figure 2. Long-term survival of 100 bp of DNA as a function of temperature. The calculations are based upon a genome size of 3.0×10^6 bp, the Arrhenius equation and depurination kinetics of Lindahl and Nyberg [39] (i.e. a depurination rate of 4×10^{-9} sites sec^{-1} at 70 °C, pH 7.4, and a constant activation energy of 31 kcal mol^{-1}). We have simplified calculations assuming damage is distributed equally over the genome at all purine sites.

Drilling and handling

The most significant source of contamination probably derives from sampling. Permafrost cores are preferentially drilled for the isolation of microorganisms. Special rotation-column coring methods that lack drilling fluids have been developed to minimize contamination from externally derived cells, but not from externally derived DNA and RNA molecules [14,15]. Ice-cores, however, are predominantly drilled for isotopic studies, which provide information about past climate, and no special procedures are used to minimize biological contamination during the coring process.

Different ice-core drilling methods vary with respect to the level of contamination. Mechanical drilling lacking drilling fluid in the borehole results in numerous small cracks, making it difficult to clean the ice-core samples post coring, thus increasing the risk of contamination. Drilling performed with fluid such as Exxol D60 (lamb oil) and HCFC (Freon 141B) often results in good-quality ice-cores with few noticeable cracks. Although the drilling fluid used is likely to be contaminated itself (although this has not yet been systematically tested), it only penetrates the ice where there are cracks [30]. To test the efficacy of all drilling methods, and the extent of contaminant penetration into the cores (small cracks are invisible), all drilling equipment and fluids should be systematically spiked with recognizable microorganisms, such as *Serratia marcescens*, as has been done with permafrost coring [3,14,15]. Finally, during the logging, cutting and storing of the cores in the field, sterile gloves, caps and facemasks should be worn to minimize human-derived microbial contamination (Table 2).

Sample contamination

In the laboratory, various procedures have been proposed to minimize sample contamination [2,5,7–9,14,15]; however, to date, none have systematically compared the relative efficiencies of these methods. Removing 1–3 cm of the surface with a pre-sterilized microtome knife (treated with 5% sodium hypochlorite) appears to be highly efficient in minimizing sample contamination [8,14,15]. The removal of the outer surface of ice cores should be done in a laminar flow hood in a room maintained at −20 °C, to avoid the formation of water film on the surface. The small size of the permafrost samples needed for biological studies (a few grams) makes it possible to handle short core samples (e.g. ~10 × 10 cm) in a positive airflow hood or glove box in a clean room whilst removing the surface of the core (Table 2).

Laboratory contamination

Microorganisms are ubiquitous in all environments and settings. Contamination with bacterial DNA is evident even in laboratories dealing with whole-genomic DNA and contemporary low biomass samples [31,32]. It is therefore prudent to assume that most laboratory reagents and tools are contaminated with microbial cells and microbial

Table 2. Precautions, controls and criteria for ice and permafrost genetics and culturing

Procedure	Precaution	Controls	Criteria for all procedures
Drilling	Fluid-based drill (ice)	Recognizable contaminant added[a]	Empty controls
	Mechanical drill (permafrost)		IR[b]
	Sterile gloves, face mask and cap		Independent reproducibility
Inspection for cracks (ice)[c]	Freeze laboratory (ice)	Air	Cloning and/or sequencing
Sub-sampling	Dedicated clean laboratory (permafrost)[d]		Age-dependent patterns[e]
Removal of surface	Positive flow bench or glow box		Quantification[g]
	Body suit, sterile gloves, face mask, cap		Preservation[g]
	Sterilized tools[f]		
Melting (ice)	Dedicated clean laboratories[d]	Air	
Concentration (ice)	Positive flow bench or glow box	Filter (ice)	
DNA and/or RNA extractions	Body suit, sterile glows, face mask and cap	Extraction (ratio 1:5)	
PCR set up	Cleaned reagents, tools and tubes[f]	PCR (ratio 1:1)	
Culturing		Multiple media	

[a]Easily recognizable cultures and/or DNA that can be amplified by PCR to examine the effects of penetration [14,15].
[b]Inverse relationship between amplification strength and fragment length [8,14,18,24,34].
[c]Use light table [8,13].
[d]Physically isolated from other laboratories, fully equipped, separate ventilation system, nightly UV irradiation. Do not use same laboratories for ancient DNA and/or RNA work and culturing of ancient microbes [8,14,15,24].
[e]Age-dependent patterns in, for example, diversity and DNA and/or RNA damage if comparing samples of different ages preserved in similar conditions [14].
[f]Treated with 5% sodium hypochlorite, 2.5M HCL, UV radiation or baking [8,14,15,24].
[g]DNA and/or RNA quantification [14,18,34] and indirect and/or direct evidence of DNA and/or RNA preservation by, for example, amino acid racemization [17], enzymatic assays [37] or mass and/or gas spectrometry [38] should be added to the list of criteria for surprising results that largely contradict theoretical expectations for DNA/RNA and cell survival.

nucleic acids. In addition, previously produced PCR products constitute a significant source of contamination.

Buying laboratory reagents and equipment labeled 'sterile' does not guarantee that they are free of either cells or nucleic acids [33]. Autoclaving kills microbes but cleaves DNA into short, amplifiable, fragments ($\leq$100 bp). Ethanol is a better bacteriostatic agent than it is a sterilant and does not destroy DNA or RNA. Therefore, to reduce the risk of contamination efficiently, it might be helpful to treat all reagents with ultra-filtration (30–50 K MW cutoff), tubes and water with UV-irradiation (75 W, 42 H), plastic and glassware via baking (>180 °C, 12 h) and/or 5% sodium hypochlorite or 2.5M HCl for 48 h [8,14,15,24] (Table 2).

Clean laboratories

Handling of all cores, culturing experiments, DNA extraction and the PCR setup must be carried out in positive air hoods or glove boxes in fully equipped laboratories dedicated to low template number samples ('clean' laboratories). These laboratories should be physically separated from other molecular or microbial laboratories, should have separate ventilation systems and be under nightly UV-irradiation. Equipment and surfaces should be cleaned regularly with bleach. Personnel should also wear full body suits, sterile gloves, caps and facemasks [8,14,15,24] (Table 2).

Controls

Controls should be conducted for every experimental step: an air control to monitor possible contamination from the air circulating within the hood or glove box; a filter control for ice-core studies for the concentration of melt water; culture controls using petri dishes containing only media, DNA extraction controls using DNA extractions lacking samples (performed in ratio of 1:5) and PCR controls (performed in a ratio of 1:1) [8,14,15,24] (Table 2). Blank controls can appear clean after PCR, whereas low-level contaminants in sample extracts can still be amplified owing to 'product carryover' or carrier effect [24]. Likewise, empty blank controls are not a test for sample contamination. Therefore, some specific additional criteria are needed to authenticate ancient DNA and/or RNA and cultures from ice and permafrost.

Authenticating results

Criteria of authenticity

Nucleic acid and microbial isolation from ice and permafrost should be conducted following the set of criteria given below and in Table 2. The criteria appear in order of importance. Independent reproducibility of sequence and/or culturing results as well as the cloning and sequencing of amplification products are essential and should be performed as often as possible. These criteria provide essential direct or indirect evidence to support the authentication of the sequence or culture in question.

Reproducibility

Results should be replicated independently by another laboratory using the same core sample or, ideally, a different sample of the same age drilled in close proximity. The independent replication might be complicated by an uneven distribution of cells in the sample(s); therefore, it is wise to pool several independent DNA extracts before PCR amplification and to pool several PCR reactions before cloning to increase homogenization and, hence, replication. For studies showing high sequence diversity, statistical methods, such as a bootstrap test, can be used to test the reproducibility of results [14,15,18,24,34]. Many DNA claims have failed to meet this essential criterion of authenticity [18,34,35,36]. The need for independent replication of results in ice and permafrost studies is made evident by the considerable variation in results obtained by different groups (Box 3).

Cloning and sequencing

Amplification products should be cloned and many clones sequenced. This enables the determination of damaged-

Box 4. The hunt for extraterrestrial life forms

The search for life on other planets, especially Mars, is becoming a central focus of both the National Aeronautics and Space Administration (NASA) and European Space Agency (ESA). A task force is planned to explore the Martian surface and even to bring back Martian samples to Earth for comprehensive analyses [47].

Mars and Europa, a moon of Jupiter, are the main candidates in the hunt for life beyond Earth (Figure I) because of the assumption that there was free water in the past on Mars and that it is still present on Europa. Ice plays a central role in this search: Mars has ice caps at each pole that are believed to be ~100 million years old [48] and have a ground floor of permafrost. Europa is believed to have an ocean covered by a surface of ice several kilometres thick.

There are two routes by which simple biomolecules, such as amino acids, could be shared between Earth and Mars: (i) by convergent evolution; and (ii) by Panspermia. The first idea is supported by evidence suggesting that Earth and Mars were ecologically similar 3.5–3.9 billion years ago (when life evolved on Earth), both had a thick atmosphere of CO_2, volcanic activity and surface water [49] and, as shown by 'The Miller–Urey primordial soup experiment', are likely to have contained simple amino acids and purine bases [50]. The Panspermia hypothesis, that life might have been exchanged between planets, is supported by studies showing that bacterial endospores can survive in space for several years [19] and by the suggestion of a significant transport of material from Mars to Earth over several millennia [51].

The Martian polar ice caps are believed to sustain temperatures of between −50 °C and −110 °C [52], and are thus likely to be a suitable place to look for simple chemical molecules, such as amino acids. The same is true of the Martian permafrost. Rough calculations using the Arrhenius equation suggest that 100 bp of DNA can theoretically survive 3.4×10^{9}–3.10^{21} years at −50 °C and −110 °C, respectively (Figure 2, main text). Although the calculation is highly simplified, it does suggest that any nucleic acids on Mars would be preserved for periods of time significantly longer than can expected on Earth.

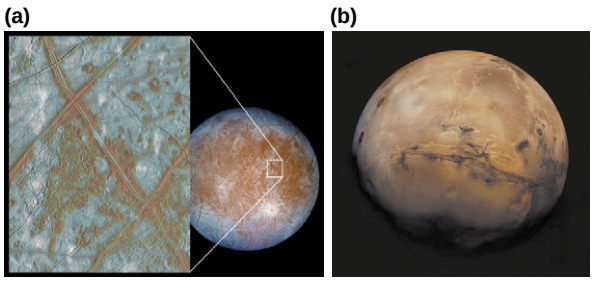

Figure I. Main candidates in the search for life beyond Earth. **(a)** Europa, one of the moons of Jupiter, with a thick shell of ice. **(b)** Planet Mars. Reproduced from http://photojournal.jpl.nasa.gov/.

induced errors, level of sequence diversity, potential nuclear insertions and readily identifiable contaminants [8,14,15,18,24,34].

Age related patterns and associated remains

The discovery of previously unknown cultures or novel microbial nucleotide sequences should not be used as a criterion for their authenticity. However, clear age-related patterns in DNA and/or RNA damage from samples preserved under the same environmental conditions might provide evidence of authenticity [14]. The retrieval of additional DNA sequences from non-exogenous sources, such as extinct animals and plants might provide additional indirect evidence for DNA survival [15].

Appropriate molecular behaviour

There should be an inverse relationship between PCR amplification efficiency and fragment length (i.e. the concentration of short templates should be relatively higher compared with that of longer ones because of sequence fragmentation). However, this pattern can only be expected if the sensitivity of all primer pairs being used has the same amplification efficiency [8,14,18,24,34]. This should hold even in studies of ancient viable microbial cells, because each viable cell should be accompanied by larger numbers of dead cells of the same type.

Quantification and nucleic acid survival

Quantifying the number of starting template molecules using quantitative or 'real time' PCR, can provide information about possible contamination. It is difficult to reproduce results when PCR begins with <1000 template molecules [18,34]. Assessing the total amount, composition and relative extent of change in amino acids can provide indirect evidence of DNA survival [17]. Direct evidence of the state of the DNA can be addressed by enzymatic assays [37] or by gas chromatography/mass spectrometry [38].

Conclusion and prospects

The culturing of ancient viable microorganisms and the recovery DNA and RNA sequences from glacial ice and permafrost holds tremendous promise. At the lowest temperatures of any geological setting, glacial ice and permafrost are likely to contain the oldest endogenous nucleic acids on Earth. Progress in this field will be of great importance not only in microbial ecology and evolutionary biology, but also in the search for extraterrestrial 'life' (e.g. amino acids and simple ribonucleotides) on Mars and Europa (Box 4). However, before this can become a fully fledged area of biological research, biochemical work targeting the survival of nucleic acids and microorganisms in these environments must be carried out. Strict adherence to the above-mentioned protocols, controls and criteria (Table 2), is essential for establishing reputable data.

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