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Nucleic acids in mummified plant seeds: biochemistry and molecular genetics of pre-Columbian maize

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Summary

Nucleic acids fractions were isolated from pre-Columbian maize seeds and characterized using different approaches such as polyacrylamide gel electrophoresis, anti-DNA antibody binding, HPLC fractionation, molecular hybridization with cloned genes, and DNA amplification by the polymerase chain reaction. The nucleic acids were found to be very depolymerized ($\leqslant 140$ base pairs in length) and composed mainly of ribosomal RNA. Despite the very low amount and degree of polymerization of seed DNA, specific maize nuclear Mu1, Mu4, Mu8 and, possibly, Mu5 element components could be detected, thanks to the use of amplification systems as short as 90 bp. The results suggest that evaluation of the relative proportions of Mu-type element components and, possibly, other maize genomic components in single mummified kernels, may offer a new key to the study of ancient maize populations.

1. Introduction

In recent years there have been several reports describing the isolation and characterization of DNA from ancient human, animal and plant remains (for reviews see: Paabo, 1986; Paabo *et al.* 1989; Rogan & Salvo, 1990). The long-term aim of these studies is to provide primary evidence of the genetic structure of ancient human populations, of the taxonomy of extinct animal species and of the events that have led to the domestication of the major crop plants.

The very large collections of plant seeds, deriving from a number of archaeological excavations in the Old and New World during the last two centuries, provide an almost continuous record of the stages through which the domestication of some major crop plants, such as wheat, barley and maize took place (Renfrew, 1969; Zohary & Hopf, 1988). It is well known, however, that the traditional archaeological approach is often unable to give satisfactory answers to the many questions posed by the debate on plant domestication – the 100-year controversy (Iltis, 1983) surrounding origin and domestication of Indian corn or maize (*Zea mays* L. spp. *mays*) is an eloquent example of these difficulties.

To assess the potential of the direct analysis of ancient seed DNA as a possible key to plant domestication studies, we have subjected to detailed biochemical and molecular genetics analysis the nucleic acids fractions isolated from a sample of pre-Columbian (Huari) maize. The study has been in part extended also to other seed remains of different age and origin. Our results demonstrate that, through application of a proper strategy, which takes into account both state of preservation of seed DNA and mode of plant evolution, significant genetic information can be obtained.

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2. Materials and methods

(i) *Samples*

A maize (*Zea mays* L. spp *mays*) sample was provided by Centro Studi Ricerche Ligabue (Venice). The sample comes from a Huari tomb (Peruvian Coast) and is composed of nine excellently preserved maize ears kept in a canvas bag decorated with feathers. Kernels were radiocarbon-dated to 980 ± 95 years BP (reference year: 1950).

Cress seeds (*Lepidium sativum* L.) taken from the tomb of Architect Kha and his wife Merit, West Thebes Necropolis (Mattirolo, 1926) were provided by Soprintendenza alle Antichità Egizie, Turin. The site has been historically dated 3410–3365 years BP.

(ii) *Nucleic acids extraction*

Cress seeds (about 0·5 g) were finely ground in a mortar with a pestle and the powder mixed with 1 ml

of a phenol-based mixture composed of 50 mM-Na_2EDTA, 50 mM Tris-HCl pH 8·0, 1% (w/v) SDS and 6% (v/v) water-saturated phenol. The homogenate was extracted with phenol, phenol/chloroform/isoamilic alcohol (24:24:1), chloroform, ether and eventually ethanol precipitated.

Single maize kernels were put in a 10 ml test tube on a laboratory wheel and washed externally for 30 min at room temperature using 2 ml of phenol-based mixture. The tubes were removed from the wheel, the medium replaced with 1 ml of fresh mixture and seeds left to soak overnight at 4 °C in a refrigerator. For nucleic acids extraction, soaked seeds were put in a sterilized mortar, together with 1 ml fresh phenol mixture and homogenized with a pestle. The homogenate was mixed with an equivalent volume of water-saturated phenol, then extracted and precipitated as described above.

Invariably, ancient nucleic acids fractions appeared brown-coloured, a result previously experienced with other ancient seeds of different origin and age (Rollo *et al.* 1987) and with some new plant and soil samples (F. Rollo, unpublished observation).

To eliminate the brown contaminants, a simple protocol was devised. Nucleic acids were fractionated on low-temperature agarose using a minigel system. When observed under UV light, the brown contaminants produce a light-blue fluorescence which can be easily distinguished from the red fluorescence of the ethidium-bromide-stained DNA and RNA. Typically, modern (high-molecular-weight) nucleic acids are seen to migrate more slowly than the bluish fluorescence, whereas old (depolymerized) nucleic acids, migrate faster.

A portion of the gel can then be excised in correspondence with the reddish fluorescence and the nucleic acids extracted by treating the melted agarose with phenol.

(iii) *Anti-DNA antibodies*

Blood samples were obtained from the Institute of Internal Medicine, Perugia. Systemic lupus erythematosus (SLE) sera were from women who fulfilled the American Rheumatoid Association criteria for disease. The presence and titre of circulating auto-antibodies against double-stranded DNA were tested both by immunofluorescence utilizing *Crithidia luciliae* as the substrate, and by Farr's liquid-phase assay (Smeenk *et al.* 1988) using bacterial radiolabelled DNA. Monoclonal mouse IgM (mouse hybrid clone AC-30-10) specific for single and double-stranded DNA were purchased from Pharmacia (Uppsala, Sweden).

(iv) *Immunoprecipitation of nucleic acids*

Radioactivity labelled modern and ancient nucleic acids (approx. 0.1 μg, specific activity 10^7 cpm/μg) were resuspended in 0·2 ml TBS buffer (TBS: 10 mM Tris-HCl pH 8·0, 150 mM-NaCl) and incubated by rocking for 8 h at 23 °C with the following insoluble immuno-matrices: (i) 30 μl of protein A sepharose 4B-CL packed beads (Pharmacia) coupled with 0·4 mg of total IgG from SLE patients; (ii) 30 μl of anti-mouse IgM-u chain-specific agarose (Sigma) coupled with 2 μg of monoclonal anti-DNA IgM. Control matrices were made by coupling 0·6 mg of IgG from healthy human serum (i) or with uncoupled anti-mouse IgM (ii). After repeated washing in TBS buffer containing 0·02% Tween 20 the beads were resuspended in 0·1 ml of 2% SDS, 5% β-mercaptoethanol, 0·125 M Tris-HCl pH 6·8 and boiled for 5 min. After centrifugation, the eluted radioactivity was monitored by liquid scintillation counting. The specificity of antibody binding was expressed as the ratio of immuno (signal) to control (noise) recovered radioactivity.

(v) *HPLC analysis*

Total nucleic acids were hydrolysed by formic acid and analyzed by HPLC as reported by Paabo (1985) using a Beckman Ultrasphere ODS column.

(vi) *Reverse Southern blotting*

Two-μg plasmid pBG35 DNA (Goldsbrough & Cullis, 1981) were cut with the restriction enzyme *Bam*H I to excise the ribosomal DNA insert from the vector, then fractionated on 1% agarose. The gel was denatured and blotted on to nylon according to standard techniques (Southern, 1975). DNA was fixed to the membrane by UV crosslinking.

Total nucleic acids (about 100 ng) isolated from ancient maize seeds as described above, were terminally labelled using polynucleotide kinase and 50 μCi [^{32}P]ATP (Maniatis *et al.* 1982). The membrane was pre-hybridized for 4 h at 40 °C in 6 × SSC, 5 × Denhardt's, 0·5% SDS containing 1 mg/ml herring sperm DNA. The hybridization was carried out for 2 h in a fresh medium containing radiolabelled nucleic acids from mummified maize seeds. After hybridization, the membrane was washed twice in 3 × SSC at room temperature and once in 3 × SSC, 0·5% SDS at 45 °C. Autoradiography was carried on at −75 °C using Amersham's βMax film.

(vii) *DNA amplification*

The reaction mixture for DNA amplification through polymerase chain reaction (PCR: Saiki *et al.* 1988), contained 16·6 mM-$(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8·8 at 25 °C, 6·7 mM-$MgCl_2$, 10 mM β-mercaptoethanol, 200 μM each of dATP, dCTP, dGTP, dTTP, 0·05% (v/v) detergent W-1 (Bethesda Research Laboratories, Githersburg, MD); 0·3 μg each of

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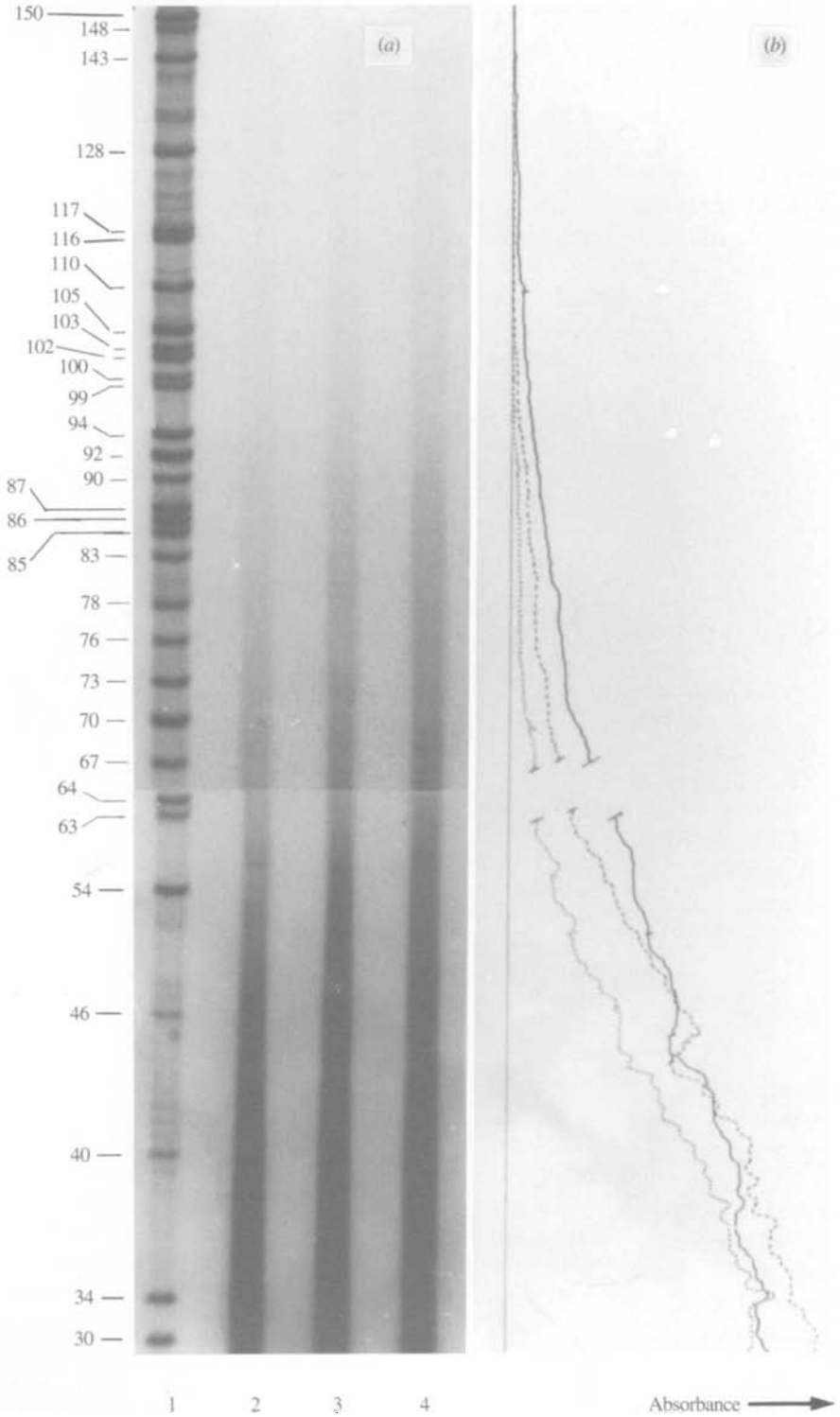

Fig. 1. Analysis by polyacrylamide gel electrophoresis of end-labelled nucleic acids from pre-Columbian maize. (*a*) Autoradiograph of the gel: 1, 'T'-sequencing pattern of M13mp8 phage DNA used as a size standard (figures indicate the length of the major fragments in bases); 2–4, nucleic acids from three different maize seeds. (*b*) Densitometric profiles of the nucleic acid patterns.

(iv) *Molecular hybridization*

End-labelled nucleic acids from ancient maize seeds were used as a probe in a reverse southern blotting experiment where cloned plant ribosomal RNA genes (rDNA) represented the DNA linked to the membrane. The results (Fig. 3) show that the probe derived from the Huari maize strongly hybridizes with the 8·6 kb plant rDNA insert of plasmid pBG35, whereas no signal is produced in correspondence with the vector moiety. As the same probe gave only very weak hybridization signals (not shown) when tested using a high repetitive maize sequence we conclude that a major proportion of the seed nucleic acids is

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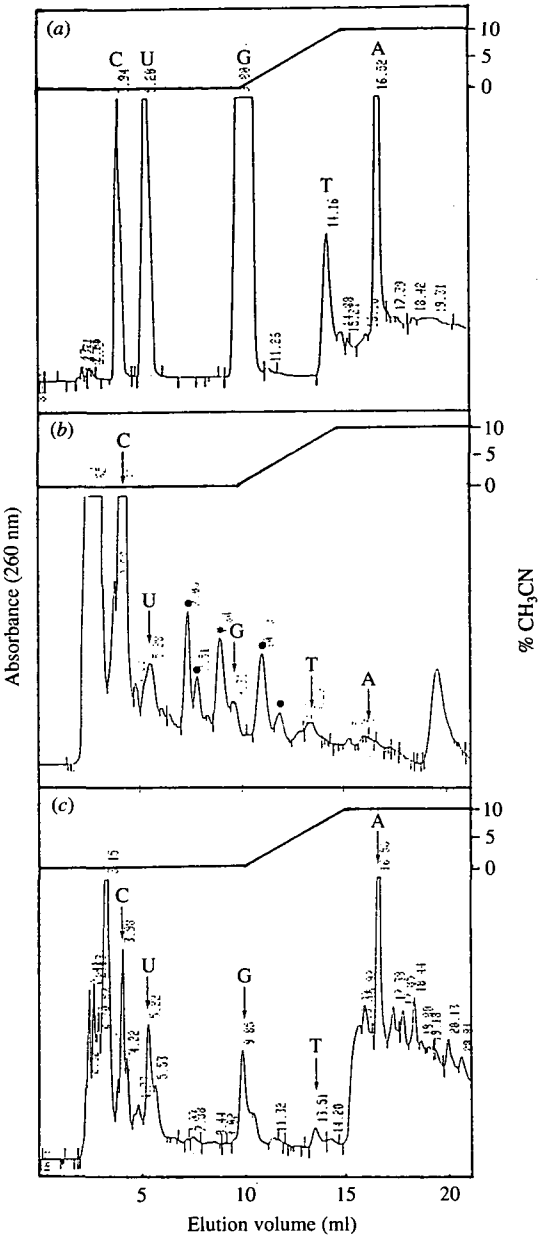

Fig. 2. HPLC analysis of the acidic hydrolysate of the nucleic acid fractions isolated from mummified seeds. (*a*) HPLC standards; (*b*) pre-Columbian maize; (c) 3300-year-old cress seeds from Thebes. Dots indicate unidentified peaks. Elution volumes of the U peaks are respectively 5·20 (HPLC standard), 5·28 (pre-Columbian maize) and 5·22 ml (3300-year-old cress). Elution rate: 1 ml/min.

represented by rRNA. On the other hand, no appreciable hybridization signal was obtained when the nucleic acids isolated from poorly preserved, soil-contaminated, ancient maize seeds from a different archaeological site (Moche culture, Sipan Pyramid, Peru, approx. fifth century) were tested against the plant rDNA sequence.

(v) *DNA amplification*

Previous experiments performed on the maize seeds from the Huari archeological site had shown that

Table 1. *Immuno-precipitation of nucleic acid fractions isolated from mummified plant seeds*

	Specific radioactivity recovered (% of total)	
Antigen	A	B
Control DNA	30·5	52·0
Control RNA	0·0	0·0
Control DNA + nucleic acids from maize seeds	22·4	—
Nucleic acids from maize seeds	0·2	0·3
Nucleic acids from cress seeds	< 0·1	0·1

A, anti-DNA IgG (SLE); B, anti-DNA IgM (mouse).

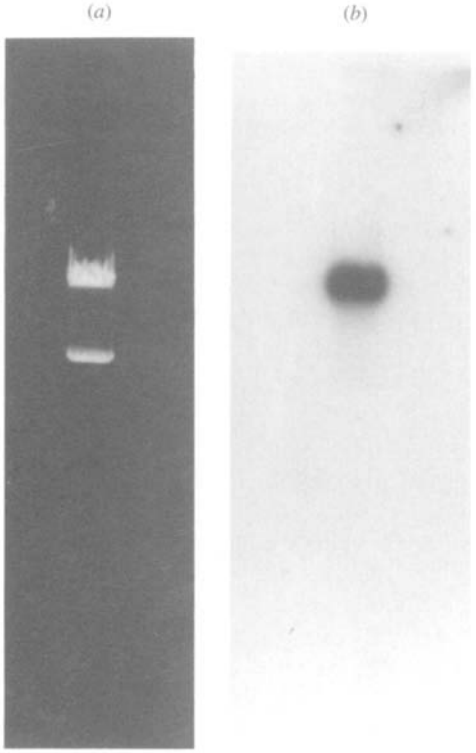

Fig. 3. Reverse-southern blot analysis of the nucleic acids isolated from pre-Columbian maize. (*a*) UV picture of *Bam*H I-cut plasmid pBG35 fractionated on agarose: the upper and the lower bands represent respectively, the 8·6 kbp plant rDNA insert and the vector (pAT153) moiety.

short (110–130 bp) nuclear and mitochondrial DNA sequences were amplified using PCR, while longer segments were not (Rollo *et al.* 1988). However, even the amplification of the shorter segments took place in one out of five nucleic acid preparations only (F. Rollo, unpublished results), a result that was attributed to differences in the state of polymerization of DNA present in the different seeds. For this reason the length of the amplification systems was further reduced to 90 bp. Mitochondrial DNA has proved an invaluable system for analysing human and animal

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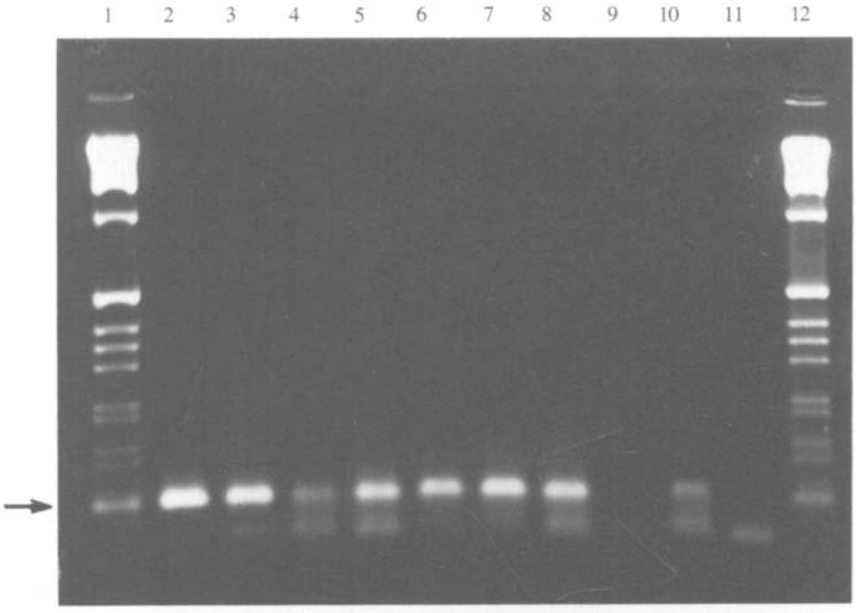

Fig. 4. Agarose gel electrophoresis (UV picture) of the amplification products of the nucleic acids fractions isolated from single seeds of pre-Columbian maize, using the 90 bp long Mu-1 terminal system. 1, 12, molecular standards (1 μl of '1 kb ladder', BRL, + 2 μl of amplified seed DNA); 2–10, amplification products (10 μl) of single seed DNA; 11, control (no DNA). Arrowhead indicates 90 bp amplification products.

remains (Paabo *et al.* 1988; Higuchi *et al.* 1984, 1987), however, plant mitochondrial DNA is known to evolve rapidly in structure, through numerous internal rearrangements but slowly in sequence. Recent estimates suggest that the point mutation rate in plant mitochondrial DNA can be 100 times slower than in that of animals (Wolfe *et al.* 1987; Palmer & Herbon, 1988). Sequence analysis of short mitochondrial DNA segments (such as those which can be recovered from our pre-Columbian maize samples) is, thus, not expected to show any polymorphism when evolutionarily related plants are compared (Rollo, 1989). These considerations prompted us to look for a system which could lead to a molecular genetic characterization of the ancient maize samples, avoiding the constraints imposed by the poor state of preservation of the seed DNA and by the need of utilizing sequence data comparisons. A possible system of this sort is based on the assessment of the relative amounts of different Mu-type elements components in single maize kernels. Mu elements are present in about 40 copies per genome in non-mutator maize stocks (Chandler *et al.* 1986). They have been shown to be structurally diverse and distributed throughout the genus *Zea*. All Mu-type elements share conserved terminal repeats which flank different internal sequences (Talbert *et al.* 1989).

Two PCR systems were designed: the first encompassing a 90 bp segment of the terminal inverted sequence of Mu1, known to be conserved in Mu4, Mu5 and Mu8 elements, the second spanning an equivalent length of the internal sequence of Mu5. The first system was preliminarily tested on the nucleic acid fractions isolated from 9 different kernels from the Huari site. The results (Fig. 4) showed that amplification took place, more or less efficiently, in 8 seeds out of 9 while no amplification signal was obtained in the absence of DNA. A further control experiment, was set up using DNA purified from soil through phenol-extraction and electrophoresis on low-gelling agarose, as a reaction substrate. In this case too, no specific amplification band was obtained (not shown). To confirm the specificity of the reaction, the amplification product of one seed was purified and cloned into plasmid pUC13. Subsequently 36 recombinant plasmids were purified and 6 of them, bearing inserts of appropriate size, further analysed by sequencing. Of these, 1 plasmid was found to carry multiple oligonucleotide dimers only, and 1 other a 72-bp-long sequence made up by the two amplification oligonucleotides separated by a central 32-bp-long sequence, showing no homology with the target Mu sequence.

However, the nucleotide sequences carried by the remaining 4 plasmids (Fig. 5) were clearly recognized as Mu1, Mu4 and Mu8 element components (right or left terminal inverted repeat), though differing by some substitutions (transitions and transversions) from the published sequences (Talbert *et al.* 1989; Fleenor *et al.* 1990). At present, we do not know whether the observed substitutions should be attributed to the possible presence, in the Huari maize lines, of variant Mu elements, or to mere amplification artifacts. The ratio transversions/transitions observed when ancient and modern sequences are compared (approx. 1·3) is significantly higher than the ratio obtained when modern Mu sequences only are considered (approx. 0·5), which seems to suggest that

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(*a*)

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7H      TAGAGAAGTACGCGACAGCAAAACTCTAAAACGGACACAA
Mul R   ------C------------C--------------------

11H     TAGAGACGCAGACGACAGACAAACTCTAAAATGGATACGA
Mu4 L   ----------C----------------------------

13H     TAGAGACGCAGTCGAGAGACATACTCCAAAATGGATACGG
Mu4 L   ----------C---C-----A----T------------A

16H     TGGAGACGCAGACGACAGCCAATCGCTAAAACGGAAAGGT
Mu8 R   ----------G----------A---C------------A
Mu8 L   ----A-----------------A---C------------A
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(*b*)

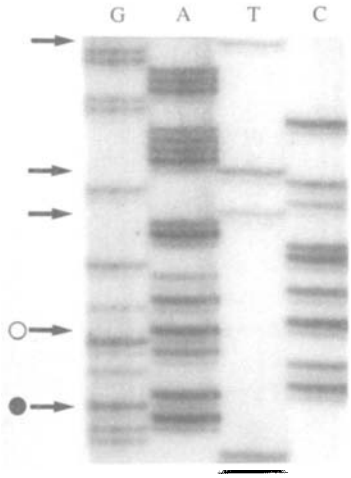

Fig. 5. Nucleotide sequences (40 bp) of four Mu-type element components from Huari maize. Sequences were obtained through cloning into pUC13 of ancient maize seed DNA which had been amplified using the Mu1 (terminal) system, followed by sequence analysis of individual clones. (*a*) Alignment of the nucleotide sequences of the four clones (7H, 11H, 13H and 16H) with those of known Mu-type element components (right and left terminal repeats of Mu1, Mu4 and Mu8: only the best-fitting alignments are reported; the sequences of the two amplification oligonucleotides have been omitted; dashes indicate identity with the published sequences). (*b*) Sequencing gel of the 16H clone, evidencing (arrowheads) the substitutions found when the clone sequence is compared with the terminal repeats of Mu8. The circles indicate the two repeat-specific substitutions (○, right-repeat; ●, left repeat), the remaining three substitutions are common to both repeats.

the second hypothesis may, at least in part, be correct. A definitive answer to this point will come from sequence analysis of large sets of clones obtained from independent amplification reactions. This should enable us to derive a reliable consensus sequence for each of the different Mu-type elements found in the Huari maize.

In a further experiment, nucleic acid preparations obtained from 10 pre-Columbian maize seeds were

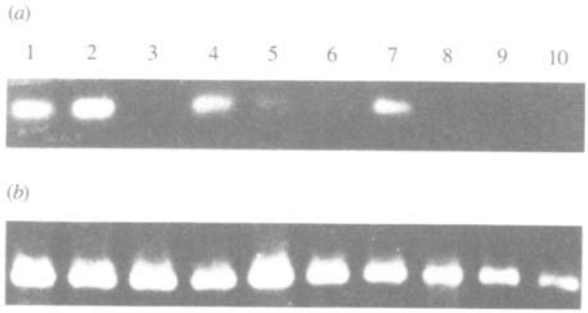

Fig. 6. Gel electrophoresis (UV picture) of the amplification products of the nucleic acid fractions isolated from single seeds (1–10) of pre-Columbian maize. (*a*) Amplification products of the Mu5 internal system. (*b*) Amplification products of the Mu1 terminal system.

submitted to enzymatic amplification using both Mu1 (terminal) and Mu5 (internal) systems. Results are shown in Fig. 6. One can observe that, in this case, all seed samples produce sharp amplification bands with the Mu1 system, while, when the Mu5 system is used, the same nucleic acid preparations produce sharp or weak amplification bands, or no band at all, depending on the sample. As the two amplification systems are of the same length (90 bp), the different results given by the Mu5 probing system should not depend on a difference in the state of preservation (degree of polymerization, relative amount, presence of inhibitors) of the seed DNA. If this had been the case, these factors would have affected both systems to the same extent.

A possible explanation is that the different amplification signals produced by the Mu5 system reflect a polymorphism in the relative amount of Mu5 elements present in the seed genome. In this case too, further work is required to confirm this conclusion. At the moment, we are considering the use of the sole Mu1 (terminal) amplification system, united to temperature gradient gel electrophoresis (TGGE) fractionation of amplified DNA, as a possible way of assessing the relative amount of Mu element components, in ancient maize seed preparations.

4. Conclusions

The first indirect evidence about the possible presence of DNA in ancient plant seeds from archaeological excavation was suggested by the work of Hallam (1973). His analysis of the ultrastructure of emmer wheat (*Triticum dicoccum*) grains from Thebes in Egypt dated 5000-4000 yr BP on archaeological evidence, and other grains from the silos at Fayum, ^{14}C dated 6441 ± 180 yr BP, shows an amazing amount of fine structure still preserved. In particular, nuclei, nucleoli and chromatin are all recognizable. A biochemical analysis performed by Osborne (Osborne *et al.* 1974; Osborne, personal communication) on the same material, in fact showed the presence of extremely degraded DNA and RNA.

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Very short nucleic acids fragments were isolated by Rollo (1985) from 3300-year-old cress (*Lepidium sativum* L.) seeds found in Thebes. Fragments were characterized by polyacrylamide-gel electrophoresis and molecular hybridization. They were shown to be ribonuclease-sensitive and to bind to modern plant DNA, but not to bacterial (plasmid) DNA. Using a nucleic acid extraction procedure, based on the CTAB (cetyltrimethylammonium bromide) separation of nucleic acids from enzyme-inhibiting polysaccharides, Rogers & Bendich (1985) obtained DNA from a number of mummified plant seed/embrios remains (from 500 to > 45000 years old) belonging to the *Encelia*, *Eschscholtzia*, *Lycium*, *Juniperus*, *Opuntia* and *Symphoricarpos* genera. The DNA presented a maximum length ranging from 1·5 to 30 kb in the different samples. Unfortunately, Rogers and Bendich's results cannot be regarded as conclusive, as no specific hybridization test was applied to assess whether the DNA was endogenous to the seeds or due to contaminations.

Analysis of *Rubus* sp. and *Vitis vinifera* seeds from a Lombard archaeological site in Northern Italy (approx 1200 years BP), as well as that of *Ceratophyllum* sp., *Cirsium* sp., *Ficus* sp., *Ranunculus* sp., *Rubus* sp., *Vitis vinifera*, *Zannichellia* sp. and *Zostera marina* sp. from the site of the submerged sacred spring at Pizzica Pantanello (Metaponto, southern Italy) (Carter *et al.* 1985) led Rollo *et al.* (1987) to conclude that most, if not all, the nucleic acids present in those mummified plant remains were due to contamination. In fact, while most of the seed samples were found to contain prominent RNA bands and polymerized DNA (up to approx. 10 kbp), RNA was shown to be of procaryotic origin (16 and 23 srRNAs), and cloned ancient DNA did not hybridize to the DNA isolated from modern plants, but, rather to bacterial and fungal DNA.

However, this may not be the rule. Goloubinoff *et al.* (1991) reported the extraction of DNA from 600 and 2000 years old maize remains from the South-western United States and the successful amplification of single-copy nuclear and organellar gene sequences. In addition, recent data suggest that very particular environmental conditions such as those found in cold, anaerobic hypolimnia can indeed preserve high molecular weight plant DNA for an extremely long time. Thanks to these particular conditions, Golenberg *et al.* (1990) were able to isolate DNA from a fossil *Magnolia* leaf from the Miocene Clarkia deposit (17-20 Myr old) and to obtain quite long (820 bp) amplification products of the chloroplast ribulose diphosphate carboxylase (Rubisco) gene subunit which were subsequently sequenced and compared with the gene sequences of the modern representatives of the genus *Magnolia*. It must be reported, however, that theoretical considerations on the rate of spontaneous depurination of DNA in aqueous solution, as well as some experimental observations on fossil leaf samples from the same deposit, performed by different authors (Paabo & Wilson, 1991) seem now to challenge this achievement.

The present results, and those previously obtained by Osborne *et al.* (1974) and Rollo (1985), show that, frequently, while it may be possible to find endogenous nucleic acids in mummified seeds which have been collected in ancient times and kept out of indirect contact with the soil (either stored in silos to be used as a food reserve, or put in tombs for ritual purposes), these are extensively depolymerized.

The present results also indicate that a consistent fraction of the nucleic acids found in the ancient seed remains is represented by rRNA. This is not so surprising since rRNA, due to its highly constrained secondary structure, modified nucleotides, and strong association with cellular proteins, is a relatively stable molecule (Venanzi & Rollo, 1990). In addition, plant seed reserve tissues are particularly rich in ribosomes (Bewley & Black, 1982). The presence of rRNA does not exclude that of chemically modified DNA. At least in the case of pre-Columbian maize, HPLC analysis of nucleic acid hydrolysates evidences several unidentified peaks which may correspond to chemically modified bases, as reported by Paabo (1985) for mummified human remains. Moreover, on a speculative basis, one cannot exclude that the observed uracil peaks stem in total or in part, from deamination of cytosine, nor exclude that the RNAse-sensitivity of the ancient nucleic acid fractions is due to abasic sites in chemically modified DNA. However, the first hypothesis is challenged by the presence of sharp cytosine peaks in the HPLC chromatograms, while the second is inconsistent with the immuno-reactivity of the ancient nucleic acid fractions.

A phenomenon which has been pointed out by several authors, is the reduced amplificability displayed by ancient, compared to modern, DNA (Rollo *et al.* 1988; Paabo *et al.* 1988; Paabo, 1989; Rogan & Salvo, 1990). This phenomenon has been attributed to the presence of structural damage, such as modified bases, purineless sites and crosslinks, affecting the double helix (Paabo, 1989). In the case of our maize samples the effect of these factors cannot, obviously, be excluded; however it seems to us that the extremely reduced availability of DNA molecules of a suitable length can primarily account for the strong, inverse correlation observed between amplification efficiency and size of the amplification product, observed. Despite the problems posed by the poor state of preservation of seed DNA, the present analysis indicates that a certain amount of genetic information can, nevertheless, be retrieved from the ancient seed remains by use of appropriate molecular strategies which take into account the particular organization and mode of evolution of the plant genome.

As a matter of principle, figures for the occurrence of the different Mu-type elements, and possibly other repeated genomic components, might be derived from any ancient seed stock. This could offer a new key to

the genetic characterization of pre-Columbian maize populations.

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