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Nucleic acids in mummified plant seeds: screening of twelve specimens by gel-electrophoresis, molecular hybridization and DNA cloning

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Summary. Twelve seed specimens of varying ages and from different archaeological sites were analyzed for the presence of polymerized DNA and RNA. Amongst the samples tested, one of *Vitis vinifera* from an archaeological site in Iran (2,000–3,000 B.C.) was found to be completely devoid of nucleic acids. *Zea mais* seeds of Precolumbial age from Peru (about 800 A.D.) contained depolymerized DNA and RNA. Samples of *Vitis vinifera* and *Rubus* sp. from a Lombard archaeological site (800 A.D.) as well as radiocarbon dated seeds from the site of the "Spring Sanctuary" near Metaponto (I–IV century B.C.) were found to contain polymerized DNA and rRNA bands. However the electrophoretic properties of the rRNAs in one case and hybridization experiments performed with cloned seed DNA in the other, clearly demonstrated that the polymerized nucleic acids were not of plant origin.

Key words: Mummified seeds – Molecular hybridization – DNA cloning – Exogenous DNA – rRNA

Introduction

In the past two years molecular geneticists have shown that polymerized DNA can persist in mummified animal (Higuchi 1984) and human (Paabo 1985) tissues. In a previous paper (Rollo 1985) we dealt with the isolation and detailed characterization of fragments of ribosomal RNA from cress seeds dated 3,300 years B.C. Our data was to a certain extent in contrast with that of Rogers and Bendich (1985) who reported the presence of high molecular weight DNA in seeds and embryos more than 45,000 years old.

In the present paper we discuss a survey performed on 12 seed specimens of varying origins and age aimed at evaluating the possible persistence of polymerized DNA in the seeds and thus their potential in studies of evolutionary plant genetics.

The present results confirm our previous data and contribute to a more accurate picture of the situation by describing obvious and less obvious cases of contamination by exogenous nucleic acids. In addition, simple strategies which can be conveniently applied to test ancient seed specimens for the presence of endogenous DNA and RNA are suggested.

Materials and methods

Plant material

Table 1 lists the seeds used in this study. Mummified seeds from the archaeological excavation of the "Spring Sanctuary" at Pizzica Pantanello, Metaponto, South Italy, were provided by Lorenzo Costantini. Two seed specimens (resp. *Ceratophyllum* sp. and *Zostera marina*) were radiocarbon dated and found to be respectively 2,320 ± 70 and 2,200 ± 60 years old. Seeds of *Zea mais* from Peru, which have been historically dated approximately 800 years A.D., were a gift of Giancarlo Ligabue. *Vitis vinifera* and *Rubus* sp. seeds from the Lombard archaeological site of Castelseprio (North Italy, IX century A.D.) were a gift of Lanfredo Castelletti.

DNA extraction and purification

Seeds were ground in a mortar with a pestle in the presence of a phenol-EDTA based mixture (Covey et al. 1983). An equal volume of buffer saturated phenol was added to the homogenate, mixed by a vortex and centrifuged at 13,000 r.p.m. for 3 min in an Eppendorf type centrifuge. The supernatant was recovered, diluted with an equal volume of phenol-chloroform mixture (1 : 1), mixed by a vortex and centrifuged as indicated above. The operation was then repeated using chloroform as an extraction medium. Total nucleic acids were precipitated

Table 1. Seeds used in this study

Species	Source	Estimated age (years)
Zea mais	1	1,200 a
Vitis vinifera	2	4,000–5,000 a
Rubus sp.	3	1,200 a
Vitis vinifera	3	1,200 a
Ceratophyllum sp.	4	2,200–2,300 b
Cirsium sp.	4	2,200–2,300 b
Ficus sp.	4	2,200–2,300 b
Ranunculus sp.	4	2,200–2,300 b
Rubus sp.	4	2,200–2,300 b
Vitis vinifera	4	2,200–2,300 b
Zannichellia sp.	4	2,200–2,300 b
Zostera marina	4	2,200–2,300 b

1 = unidentified Precolumbian site, Peru; 2 = Shahr-i-Sokhta, Iran; 3 = Castelseprio, Italy; 4 = Pizzica Pantanello, Metaponto, Italy
a = historically dated; b = radiocarbon dated

from the supernatant of the last centrifugation by the addition of a 2.5 volume of ethanol and 200 mM (final) Na-acetate. No commercial yeast or *E. coli* tRNA was added to facilitate the precipitation as we found some preparations (Boehringer Mannheim, FRG) to be contaminated by a small but clearly detectable amount of polymerized DNA. The precipitate was drained, vacuum desiccated and resuspended in a small volume of 10 mM Tris-HCl pH 8.0, 1 mM EDTA or in sterile distilled water. DNA was further purified by fractionation on low gelling agarose and reextraction from the gel (Maniatis et al. 1982).

Gel fractionation

Total nucleic acids were fractionated on 1.5% agarose slab gels in 40 mM Tris-acetate buffer, pH 7.6 and 10 mM EDTA.

Southern and dot-blots

DNA fractionated on agarose slab gel was transferred onto nitrocellulose membranes essentially as reported by Southern (1975). Dot-blots were performed as reported by Maule et al. (1983).

Radioactive probes were obtained by nick-translation of plasmid DNA as described by Maniatis et al. (1982). Hybridization was carried out in 3× SSC, 4× Denhardt's at 68 °C for 18 h. After hybridization, filters were washed three times for 30 min each in 2× SSC, 0.1% SDS at 68 °C. The buoyant density in CsCl of the rDNA of flax cloned in pBG35 was 1,709 $g \cdot cm^{-3}$ (Goldsbrough and Cullis 1981) which corresponds to a G + C content of 50%. From these data it can be calculated that the washings were performed at Tm-26 °C. In other words, assuming that a ΔTm of 1 °C for a hybridization indicates 1 to 1.5% mismatch, we were selecting for 26–39% mismatch. Films (Kodax X-Omat AR), were exposed at −80 °C with intensifying screens.

Cloning of seed DNA

Seed DNA (20–50 ng) which had been purified by electrophoresis on low gelling agarose and reextraction as indicated above was digested with 1 unit of Sau 3AI restriction endonuclease for 5–10 min. The reaction was stopped by heating (65 °C for 10 min) and the DNA mixed with 50 ng of Bam HI-cut, dephosphorylated pBR322 plasmid DNA, ligated with T4 DNA ligase (2 units, 4 °C, overnight) and subsequently used to transform Ca + +-treated *E. coli* (HB 101 strain). Cells were selected by their resistance to ampicillin and sensitivity to tetracyclin. Recombinant plasmids were isolated by the alkaline lysis method (Birnhoim and Doly 1979) and fractionated on agarose mini-gels before or after restriction with Sau 3AI.

Scanning electron microscopy

Seeds were dehydrated under vacuum for about 6 h before being gold-coated under vacuum. The observations were made with a Stereoscan 200 (Cambridge instruments).

Results

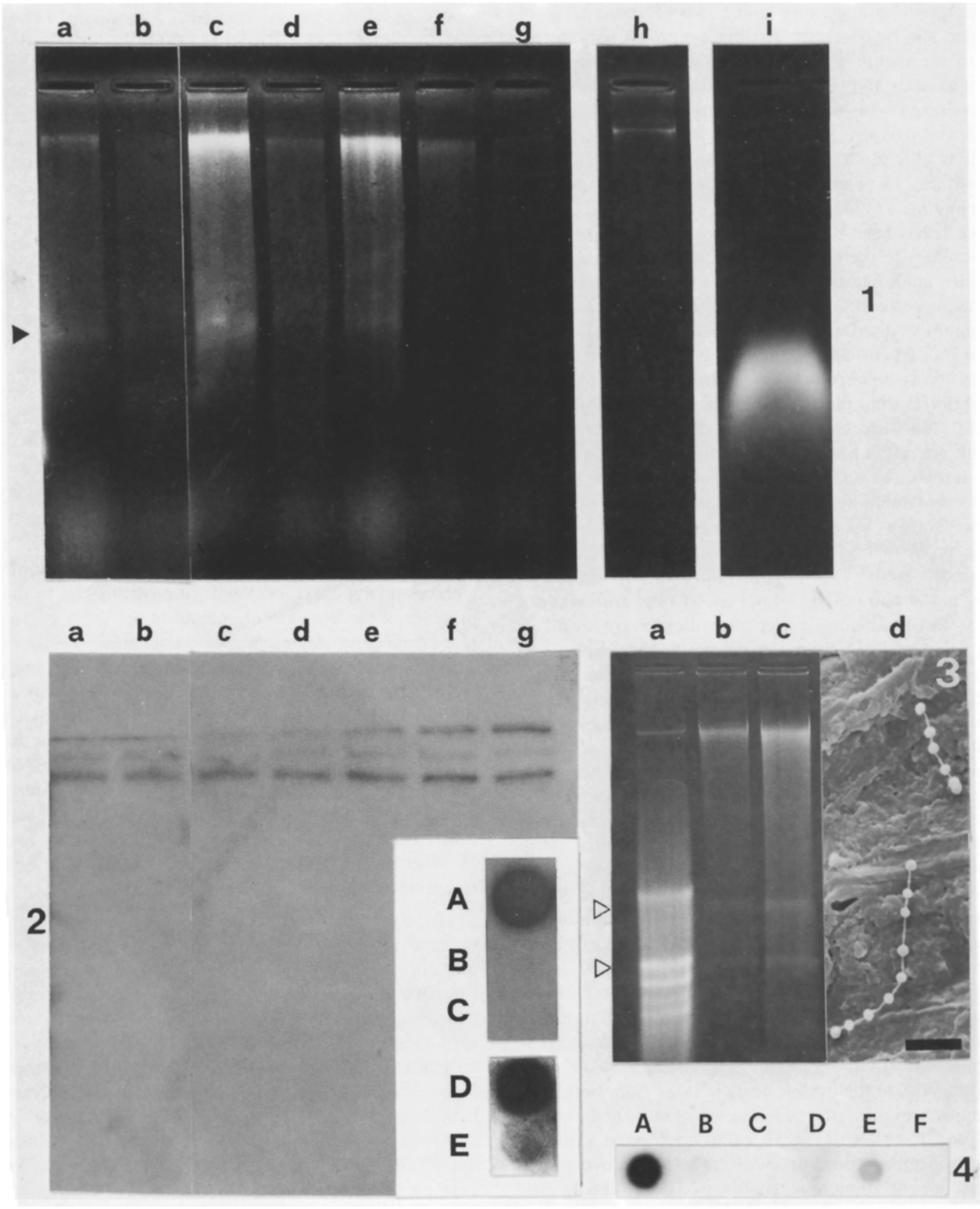
Nucleic acids were isolated by phenol from 100 mg of mummified seeds as described in the previous section. As reported above, no yeast or *E. coli* tRNA was added during the extraction steps as we found some commercial preparations to be contaminated by high molecular weight DNA. All the nucleic acid preparations obtained from the seeds were brown-coloured. The nucleic acids were analyzed by electrophoresis on 1.5% agarose and the results referring to the Metaponto seeds and to the Precolumbian *Zea mais* sample are shown in Fig. 1. It can be observed that all the seeds

Fig. 1. Agarose-gel electrophoresis of nucleic acids isolated from 0.1 g of mummified plant seeds. a = *Vitis vinifera;* b = *Zannichellia* sp.; c = *Ceratophyllum* sp.; d = *Cirsium* sp.; e = *Rubus* sp.; f = *Ranunculus* sp.; g = *Ficus* sp.; h = *Zostera marina;* i = *Zea mais;* a–h = Pizzica Pantanello site; i = Peru site. The arrowhead indicates the faint bands of ribosomal RNA

Fig. 2. Southern blot of the gel shown in Fig. 1; the blot was probed with nick-translated pBG35 DNA (25 and 18s rRNA flax cytoplasm genes); autoradiography was for one week at − 80 °C with intensifying screens. In insert: hybridization of plant rDNA excised from pBG35 with the DNA (0.2 μg) isolated from modern turnip (A) and *Rubus* sp. (D) seeds and from mummified *Ceratophyllum* sp. (B), *Rubus* sp. (E) seeds and *E. coli* (C) cells. Autoradiography was for three days under the conditions reported above

Fig. 3. a, b, c = characterization by agarose-gel electrophoresis of the rRNA present in the *Vitis vinifera* (b) and *Rubus* sp. (c) seeds from the Castelseprio site; a: control = turnip total rRNA; the migration of the 23 and 16s *E. coli* rRNAs is indicated by empty arrowheads; d = scanning electron micrograph of a *Rubus* sp. seed showing the presence of coccoidal structures; the bar represents 3 μm

Fig. 4. Hybridization of cloned DNA from mummified *Rubus* sp. seeds (Pizzica pantanello site) with various DNAs (2 μg each). A = fungus DNA; B = calf thymus DNA; C = maize DNA; D = datura DNA; E = turnip DNA; F = *Rubus* sp. DNA


a b c d e f g
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contain highly polymerized DNA, though in quite different amounts, with the only exception being given by the precolumbian maize which contains only low molecular weight nucleic acids. On the other hand, no fluorescence at all was observed in the case of the sample of *Vitis vinifera* from Iran. In some of the Metaponto samples very faint bands of ribosomal RNA could also be observed. To obtain a preliminary insight into the nature of the DNA isolated from the Metaponto seeds, the gel was blotted onto nitrocellulose and hybridized with nick-translated plasmid pBG35 DNA (25 and 18 s cytoplasmic rRNA of flax, Goldsbrough and Cullis 1981, courtesy of Noel Ellis, John Innes Institute, Norwich) under stringency conditions selecting for 61–74% homology (see Method and materials). It has been reported that the extent of nucleotide homology to the large rRNA subunits from *E. coli* and maize chloroplasts is 57% (Edwards and Kossel 1984) thus these conditions were expected to allow a certain amount of discrimination to appear between rDNAs of the prokaryotic and eukaryotic type. Under the same conditions this probe has been observed to cross-hybridize with fungal rDNA (not shown). After hybridization the blot was autoradiographed at −80 °C with intensifying screens. When the film was developed it turned out that appreciable hybridization signals were only obtained after one week of exposure (Fig. 2). This fact was taken as a circumstantial indication that the probe hybridized poorly with the DNA isolated from the seeds suggesting, therefore, that it was probably not of plant origin. However, as this result was not clear-cut to obtain a better quantification about 0.2 μg of DNA isolated from the mummified *Rubus* sp. and *Ceratophyllum* sp. seeds were dot-blotted onto nitrocellulose together with equal amounts of DNA isolated from *E. coli* cells and from modern *Rubus* sp. and turnip seeds. The blot was probed with cloned rDNA excised from pBG35 and autoradiographed for three days according to the conditions described above. The result (Fig. 2, insert) showed that the probe gave strong hybridization signals with the DNA isolated from modern turnip and *Rubus* sp. seeds but only a very weak signal with that isolated from mummified *Rubus* sp. seeds. The DNA isolated from the mummified *Ceratophyllum* sp. seeds and from *E. coli* did not show any relevant homology with the probe.

On the other hand, an evident example of exogenous contamination was given by the two samples of *Vitis vinifera* and *Rubus* sp. of medieval origin (Castelseprio site). In this case the fractionation by gel electrophoresis of the nucleic acids isolated from both seed samples revealed the presence of prominent bands of 23 and 16 s rRNA (Fig. 3 b, c). In addition, a scanning electron microscope survey of the seeds uncovered the presence of numerous chains of cocchi-like microorganisms (Fig. 3 d) (courtesy of Alessandro Valbonesi, Dept. of Cell Biology, Camerino).

In the case of the Metaponto seeds, the electron microscope failed to reveal any obvious presence of contaminating microorganisms, in addition, as said above, the fluorescent bands observed in some of the samples, which we interpreted as remnants of ribosomal RNA, were too faint to enable us to determine unambiguously whether they were of eukaryotic or prokaryotic type by gel-electrophoresis. On the other hand, the fact that pBG35 hybridized poorly with the DNA extracted from these seeds was not taken as a definitive evidence for an exogenous orgin of that DNA.

During the research work here described a major problem we experienced was that of the the small size of some of the seed samples available. This problem in some instances was rendered even more serious by the fact that the seeds appeared to contain very small amounts of nucleic acid. As we could not place any reliance on an additional supply of seeds of the same origin, at least within any brief period of time, we decided to proceed to the molecular cloning of the DNA isolated from the *Rubus* sp. sample. In this way we hoped to achieve two goals: (1) to free ourselves from the restriction imposed by the size of the samples (2) to obtain probes for plant specificity.

Preliminary experiments had shown that the DNA isolated from the Metaponto seeds was not digestible with restriction enzymes. We attributed this fact to the presence of the brown contaminant in the DNA preparations. Since in electrophoresis this pigment had been seen to migrate considerably faster than the high molecular weight DNA, we proceeded to the purification of the DNA isolated from the *Rubus* sp. seeds by fractionation on low gelling agarose, excision of the DNA band and reextraction by phenol. The DNA purified by this method was found to be readily digested by the restriction enzyme Sau 3AI and it was thus cloned in pBR322 as reported in Materials and Methods. In this way we were able to obtain about fifty recombinant clones.

To determine unambiguously whether the polymerized DNA present in the *Rubus* sp. seeds was or was not plant DNA, six clones bearing inserts ranging from 300 to 600 bp were selected amongst the recombinant clones obtained, labelled by nick-translation and used to probe the DNA isolated from modern *Rubus* sp. plants as well as other DNAs from different sources. In no case were we able to demonstrate a significant hybridization of the radioactive probes with the DNA of *Rubus* sp. plants but they were shown to hybridize appreciably with fungal and/or bacterial DNA. An example is shown in Fig. 4.

Conclusions

The studies about the persistence of DNA and RNA in biological specimens of palaeontological or archaeological origin may provide us with new clues with which to solve evolutionary problems. Promising results in this direction have been obtained by the cloning and sequencing of Quagga (Higuchi 1984) and human (Paabo 1985) mummy DNA.

The investigation into the possible persistence of nucleic acids in ancient plant specimens is particularly attractive as it lets us envisage the possibility of supporting the data currently available about plant breeding, domestication and, in general, man-directed plant evolution, with direct information about the organization of the genome of the ancestor plants. However, the achievement of such a goal might require screening tens and perhaps hundreds of seed specimens whose origin, age, and state of preservation is most variable.

The present data and that previously published (Rollo 1985) give a preliminarly picture of the situation and, at the same time, provides an operative protocol which, we hope, will be useful to those wishing to undertake similar investigations. In fact, our results show that when possible, the observation of the electrophoretic behaviour of the rRNA bands alone can allow a straightforward identification of the cases of contaminations to be made.

Where no rRNA band is visible, the use of cloned probes such as cytoplasmic plant rDNAs can give circumstantial evidence unless species-specific probes are available. If only very small seed samples are available (a relatively common occurrence), the molecular cloning of the DNA isolated from the ancient seeds and the use of randomly-selected clones as probes can furnish a relatively rapid means to determine the origin of the DNA and, at the same time, to save the DNA sample for further investigation. At our present state of knowledge, only very preliminary evidence is available about the persistence of nucleic acids in the seeds with regard to seed age. Rogers and Bendich (1985) reported the presence of highly polymerized DNA in seeds and embryos whose age ranges from 500 to more than 45,000 years, however no evidence has been given that the DNA found was endogenous to the seeds. Our results seem to put within 3,000–4,000 years the maximum age compatible with the presence of nucleic acids in seeds. In fact, 3,300 year old cress seeds from an Egyptian archaeological site were found to contain fragmented ribosomal RNA (Rollo 1985) while another seed sample from the same site was found to be completely devoid of nucleic acids (unpublished observation) and the same is true of the 4,000–5,000 year old *Vitis vinifera* seeds from Iran analyzed in the present study. The finding of only fragmented DNA and RNA in the 1,200 year old precolumbian *Zea mais* seeds from Peru seems to confirm previously reported data (Osborne et al. 1974) indicating that an extensive depolymerization of the nucleic acids takes place relatively soon in non-viable seeds. However, additional investigation is required to verify whether this is a general situation or whether some particular environmental conditions can allow this rule to be broken.

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