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Ancient DNA in Charred Wheats: Taxonomic Identification of Mixed and Single Grains

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We report the results of experiments in which ancient DNA analysis was used in attempts to make taxonomic identifications with mixed assemblages and single grains of charred wheats. In the first set of experiments we used PCRs directed at the intergenic spacer regions of the wheat ribosomal DNA loci. The D genome sequences have a 71-bp insertion that is absent from the A or B loci, which means that the sizes of PCR products from these loci can be used to determine if D genome DNA is present in an extract. This analysis had previously been shown to provide secure taxonomic information with modern wheat DNA, but with DNA from charred wheats the approach was unsuccessful, PCRs yielding only the smallest of the series of products needed to make a secure taxonomic identification. The absence of the diagnostic, larger PCR products was probably due to the highly fragmented nature of the DNA templates. In a second series of experiments we used a different PCR system, directed at the multiallelic glutenin subunit genes. Phylogenetic analysis of sequences obtained from these genes enables the genome of origin to be identified. Using this approach we were able to obtain genome-specific allele sequences from a mixture of 3000-year-old charred grains. The alleles that were identified in this mixed grain extract indicated that the assemblage contained hexaploid grains, which was compatible with the results of morphological examination. When examined individually, five of 100 grains from this assemblage gave PCR products, but in three out of four cases the taxonomic affinities of the DNA sequences did not agree with the morphological identification of the grains.

Keywords: Ancient DNA, Charred plant remains, PCR, Wheats

INTRODUCTION

The initial discovery of preserved DNA in archaeological plant remains was made by Rogers and Bendich (1985) who used gel electrophoresis to detect nucleic acids in a range of mummified and herbarium specimens. Rollo *et al.* (1987, 1991, 1994) extended this work by hybridisation, PCR and sequencing experiments which showed that the DNA in mummified maize is species-specific rather than microbial in origin, and that the ancient DNA molecules are highly fragmented. A similar conclusion was reached by O'Donoghue *et al.* (1996b) who found short pieces of ancient DNA in 1400-year-old, desiccated radish seeds from Qasr Ibrim, Upper Egypt. Although fragmented, there was enough

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polymeric DNA and RNA in these seeds to be detectable by gas and liquid chromatography/mass spectroscopy (GC/MS and LC/MS; O'Donoghue *et al.*, 1994, 1996a), and exceptionally well-preserved fatty acids and sterols were also present (O'Donoghue *et al.*, 1996b).

Although desiccated remains appear to contain the best preserved biomolecules, specimens of this type are relatively infrequent in the archaeological record. Charring is by far the commonest form of preservation and the potential of ancient DNA in archaeobotany will be realised only if techniques can be developed for analysis of charred specimens. The first evidence for DNA in charred remains was published by Goloubinoff *et al.* (1993) who obtained PCR products and sequences from burnt maize cobs, and similar experiments were reported for a 2000-year-old charred spelt wheat from Danebury, UK (Allaby *et al.*, 1994). However, neither of these two papers contained the detailed information now considered to be a requirement for authentication of an ancient DNA detection, and in view of the apparent absence of polymeric lipids and proteins in similar charred seeds (R.P. Evershed and N. Tuross, personal communication) the DNA reports were looked on as controversial. In more recent experiments we have obtained the supporting evidence needed to authenticate the existence of ancient DNA in at least some assemblages of charred seeds (Allaby *et al.*, 1997). We demonstrated that ancient DNA detections with charred material were reproducible by carrying out extractions with replicate samples and performing PCRs directed at two separate loci in the wheat nuclear genome. We showed that the DNA from charred seeds includes alleles not present in modern wheats used in our laboratory. We discovered that the DNA in charred seed extracts is extensively fragmented and has undergone base degradation, as expected for ancient DNA, and we used hybridisation analysis to obtain independent, non-PCR evidence for the presence of DNA in charred seeds. These results gave us sufficient confidence to begin exploiting ancient wheat DNA to answer archaeobotanical questions.

In the short term the most significant application for ancient wheat DNA will almost certainly be in the secure identification of remains that lack sufficient morphological integrity for categorisation by conventional methods. Wild and cultivated wheats exist as diploid (genomes AA), tetraploid (AABB and AAGG) and hexaploid (AABBDD and AAAAGG) forms (Miller, 1987). Morphological features can be used to assign an intact plant to the correct ploidy level, but problems often arise with identification of fragments or explants such as dehusked grain. In archaeobotany this is a particular problem with the naked wheats, whose chaff is only infrequently preserved, as criteria such as length and breadth measurements of the grain are extremely imprecise. This means that agriculturally important varieties such as *Triticum turgidum*, *T. durum* (AABB tetraploids) and *T. aestivum* (an AABBDD hexaploid) are often impossible to distinguish in the archaeological record. Hexaploids do not exist in the wild and the origins, evolution and changing importance of AABBDD forms are central questions in the archaeology of prehistoric wheat (e.g. Sallares, 1991), questions that cannot be answered without secure identifications of the ploidy levels of archaeobotanical remains.

The objective of the work described in this paper was to devise a method that enables the ploidy of an archaeological wheat to be identified by PCR analysis, and to apply the method to taxonomic identification with mixed assemblages of charred grain and with individual charred seeds.

RESULTS

PCRs Directed at the *Nor* Loci

Description of the PCR System

In a previous paper (Sallares *et al.*, 1995), we described how the ploidy of modern wheats can be determined by PCRs directed at the intergenic

spacer (IGS) regions of the wheat ribosomal DNA (*Nor*) loci. The published sequences (Lassner and Dvorák, 1986; Lassner *et al.*, 1987) indicate that the spacers within the *Nor-D3* locus of the D genome have a 71-bp insertion absent from the spacers of the *Nor-B1* and *Nor-B2* loci of the B genome (Fig. 1). PCRs directed at this region therefore give products of diagnostic lengths that enable D+ plants (containing the D genome) and D− plants (lacking a D genome) to be distinguished.

As shown in Fig. 2, we designed the upstream primer (primer 1) to anneal within a subrepeat array so that multiple PCR products would be obtained from each genome. We anticipated that the ladder of bands resulting from the multiple upstream priming sites would be required to guarantee secure ploidy identifications with the fragmentary DNA molecules preserved in archaeological remains. If the upstream primer had a single, non-repetitive annealing site then the product sizes would be *a* bp (B genome) and *a*+71 bp (D genome). After electrophoresis of the PCR products, an AABB tetraploid would give a single band at the *a* bp position, and an AABBDD hexaploid would give two bands at *a* bp and *a*+71 bp. However, if the ancient DNA in a hexaploid specimen was highly fragmented then it is possible that PCR of the AABBDD specimen would give a single band at the *a* position as target molecules long enough to yield the *a*+71 bp product might be absent. A hexaploid in which the ancient DNA is highly degraded would therefore give a 'tetraploid' result. The multiple upstream priming sites in our PCR system avoid this problem as the expected bands are *a* bp, *b* bp, *c* bp, etc. for tetraploids, and *a* bp, *a*+71 bp, *b* bp, *b*+71 bp, *c* bp, *c*+71 bp, etc. for hexaploids. The presence of the higher order bands means that those specimens in which the ancient DNA is too degraded for ploidy identification can be discarded, so that all of the identifications that are made are secure. The results of typical PCRs with modern wheats are shown in Sallares *et al.* (1995).

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B:CGCCATGGAAAACTGGGCAAAACCATGTACGTGGCACACGCCG
D:CGCCATGGAAAACTGGGCAAAAACACGTACGACGCACACACA-
  Primer 1

B:CGTACACGGA----------
D:CGTACACGGACCCGTGAACGGCTGCACGTGCACGGACCGTTAC


B:----------CCCGT
D:ACGTACACGGACCCGTGAACGGGCGGTACGTGGACACGCACGT


B:ACACGGACCCGTGAACGGGTATGAGAGGTCCGGG--AAAAAAT
D:ACACGGACACGTGAACGGGTACGAGAGGTCCGGGAGAAAAAAA
                  Primers 4/5/6

B:GGCCCATACACCATGCGAACCGGCTCAAAACCAGCTAATGATG
D:GGCCCATACGCCATGGAAACCGGGTCAAAACTAGCTAATGATG


B:GTCAACAAACGGTGCCATGGCAGCGAAAACATGTCTCATGGCA
D:GTCAAGAAACGGTGCCATGGCAGCGAAAACATGTCTCATGGCA
                  Primer 3

B:AAAAAACGCTGCCACGGCAGCGTTTCAAAACAGTGTACCC
D:GAAAAACGCTGCCACGGCGGCGTTTCAAAACAGTGTACCC
                  Primer 2
```

FIGURE 1 Partial nucleotide sequences of the non-transcribed spacer regions of the *Nor-B2* (B; the third nucleotide in the sequence shown is position 1 in Fig. 6 of Lassner and Dvorák, 1986) and *Nor-D3* loci (D; the first nucleotide is position 1788 in Fig. 1 of Lassner *et al.*, 1987). Dashes indicate deletions. The annealing sites for primers 1–6 are indicated.

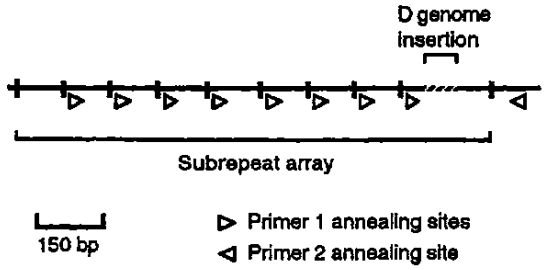

FIGURE 2 Diagrammatic representation of the *Nor-D3* spacer region showing the eight annealing sites for primer 1 within the upstream subrepeat array. There are nine units in the subrepeat array, but in the published sequence there is no clear annealing site for primer 1 in the first unit.

PCRs with Extracts of Charred Seeds

We carried out PCRs using primers 1 and 2 with extracts of two charred wheat samples, a 2000-year-old assemblage from Danebury, England, and a 3000-year-old assemblage from Assiros Toumba, Greece. We had previously demonstrated that ancient DNA is present in both samples (Allaby *et al.*, 1994, 1997). Extracts of both assemblages gave PCR products but in all cases only the smallest (225 bp) band was seen (Allaby *et al.*, 1997). The higher-molecular-weight PCR products (297 and 357 bp) required for secure ploidy identifications were absent, presumably because the ancient DNA was too fragmented to give these larger products.

In an attempt to solve this problem we replaced primer 2 (the downstream primer) with primer 3, the latter annealing to an internal site 41 bp upstream of primer 2 (Fig. 1). According to the published data (Lassner and Dvorák, 1986; Lassner *et al.*, 1987), and our previously reported experiments with modern wheats (Sallares *et al.*, 1995), PCR with primers with 1 and 3 will give a ladder of products with AABB and AABBDD wheats, but not with AA diploids. The three smallest products obtained with an AABBDD hexaploid, which are sufficient for a secure ploidy identification, are 184, 256 and 316 bp. However, when this PCR system was used with extracts of charred seeds only the smallest, 184 bp band, was seen. We therefore devised a third PCR system, designed to amplify even smaller products. Because of sequence heterogeneities the third system was more complex, utilising a family of three primers (4/5/6) which anneal to equivalent positions of the *Nor-A*, *Nor-B* and *Nor-D* loci (Lassner and Dvorák, 1986; Lassner *et al.*, 1987; Sallares and Brown, 1998; annealing positions indicated on Fig. 1). Secure ploidy identification after PCR with primers 1 and 4/5/6 requires that products of 100, 171 and 232 bp are obtained. The PCR system was optimised with modern DNA but, once again, failed to produce secure ploidy identifications with extracts of charred seeds.

Explanation for the Failure of the Nor PCR Systems with Extracts of Charred Grains

Our failure to amplify products other than the shortest one in any of the *Nor* PCR systems is presumably due to the highly fragmented nature of the DNA in charred seeds. Although it has proved possible to amplify products of up to 243 bp from charred seed DNA in PCRs directed at the *Nor* and other loci, we have shown (Allaby *et al.*, 1997) that the majority of the amplified molecules of this length are constructed by PCR jumping, the template DNAs being much shorter than the products. From the chimaeric structures of the amplified molecules we have inferred that the average length of the DNA molecules in the Assiros seeds is only 50–70 bp (Allaby *et al.*, 1997). According to computer based modelling of the *Nor* PCRs (Allaby *et al.*, in preparation), with such highly fragmented templates PCR jumping results in a bias of the amplification process so that only the shortest product is synthesised to any extent. This probably results from a combination of two factors. First, synthesis of a long product requires more jumps than synthesis of a short product, so full-length short products appear in the reaction at an earlier cycle than full-length long products. Exponential amplification of the short products therefore begins at a relatively early stage in the PCR, biasing the overall reaction towards these products and away from the longer ones. A second factor is that the initial, incomplete extension product obtained when primer 1 anneals to an upstream unit within the repetitive array might, in the next PCR cycle, reanneal to the repeat unit furthest downstream. Once again the result is that only the shortest product would be formed.

PCRs Directed at the Glutenin Loci

Description of the PCR System

The glutenins of wheat are members of a complex group of seed storage proteins called prolamins. There are two subfamilies of HMW glutenin

subunits (x- and y-type) which are thought to have arisen from a gene duplication event in the species ancestral to the A, B, D and G genomes of cultivated wheats. Single copies of the x- and y-type genes, *Glu-1-1* and *Glu-1-2* (Thompson *et al.*, 1983; Harberd *et al.*, 1986), occur at two tightly linked multiallelic loci on homeologous chromosome 1 (Payne *et al.*, 1982). We have previously used the glutenin loci as targets for PCRs in experiments that established techniques for analysis of ancient DNA from charred plant remains (Allaby *et al.*, 1994, 1997), and have also obtained sequences from modern wheats in order to study the evolution of the *Glu* loci. We found that a neighbour joining tree constructed from the sequences of 42 alleles from various wheat species comprises eight clades, each containing alleles from a different locus (Allaby *et al.*, 1998). This means that the genomic constitution of a wheat can be determined by identifying which clades its glutenin allele sequences fall in to.

PCRs with Extracts of Charred Seeds

We carried out a PCR with the glutenin primers described in Allaby *et al.* (1994) with an extract of twenty grains from the 3000-year-old assemblage from Assiros. The sample from which the grains were taken contained chaff fragments (glume bases and spikelet forks) which are relatively easy to identify morphologically, as well as grains which are not. All the chaff fragments (and grains) were identified on morphological criteria to one of the glumed varieties of wheat; no grains or chaff fragments (e.g. rachis internodes) of free-threshing wheats (either tetraploid or hexaploid) were found. Chaff of the tetraploid *T. dicoccum* type (AABB) was overwhelmingly predominant as were grains of a shape normally associated with *T. dicoccum*. Much smaller quantities of the diploid *T. monococcum* type (AA) chaff and hexaploid *T. spelta* type (AABBDD) chaff were also present.

PCR products obtained from the mixed assemblage were cloned and nineteen sequences were obtained. These sequences were chimaeric as a result of PCR jumping (Allaby *et al.*, 1997) but it was possible to reconstruct the sequences of five authentic glutenin alleles (details in Allaby *et al.*, 1998). After phylogenetic analysis four of these alleles were allocated to the A and B clades, whereas the fifth fell in a D clade. This indicated that the assemblage contained AABBDD hexaploid grains. Independent verification of the utility of the glutenin PCR system in ploidy identification of charred wheats has been provided by Schlumbaum *et al.* (in press) who carried out similar experiments with extracts of charred wheats from prehistoric sites of the Swiss alpine foreland.

Single Grain Analyses

The Importance of Single Grain Analysis

Our original methodology for ancient DNA extraction from charred wheat used 500 mg of starting material, equivalent to 20–40 grains. This approach was used in the experiments described above. The weakness of the mixed grain approach is that, as is the case with the Assiros assemblage, samples of archaeological grain are often heterogeneous, containing grains representing different species and phenotypic variants of the same species. Even when the grains all have similar phenotypes a consideration of prehistoric farming practices implies that genetic homogeneity cannot be assumed. Exploitation of ancient DNA analysis in archaeobotany therefore demands a refinement of our extraction methodology so that single grains can be analysed. The ability to utilise single grains is also necessary if ancient DNA studies are to encompass the earliest agricultural sites, which generally provide only sparse assemblages, and is a prerequisite for other types of genetic study such as analysis of genetic diversity within populations, for which it would be essential to consider one grain at a time.

Analysis of Single Grains from the Assiros Assemblage

To determine if it is possible to carry out ancient DNA studies with single charred grains, we

prepared an extraction from each of 100 grains from the Assiros assemblage. These grains had been identified on morphological criteria with varying degrees of certainty. Ten grains were processed per day, with a blank extraction carried out after each fifth and tenth grain extraction. Glutenin PCRs were then performed with each extraction. All twelve extractions prepared on the first day (ten grains and two blanks) gave PCR products: these results were discarded on the assumption that contamination had occurred. None of the remaining extraction blanks gave PCR products and none of the water blanks (sixteen in all) gave products. Five of the remaining 90 grains (numbers 17, 20, 54, 60 and 68) gave PCR products of the expected size. Four of these (numbers 20, 54, 60 and 68) were of a shape typical for a tetraploid glume wheat; number 17 was of a shape more characteristic of the hexaploid *T. spelta*. None of the other 85 grains gave PCR products of any size.

The PCR results therefore suggested that five out of 90 grains contained ancient DNA. To obtain further information on the authenticity of this result, the PCR products from the five positive grains were cloned and individual clones sequenced. No results were obtained for grain 17 due to technical error. Sequences were obtained for each of the four other grains (Fig. 3), and in each case the data showed that the target region of the wheat glutenin gene had been amplified. Phylogenetic analysis of the sequences obtained from the four charred grains gave the following results:

1. The five sequences obtained from grain 20 were identical (except for two nucleotide differences that can be ascribed to copying errors occurring during PCR) and represented

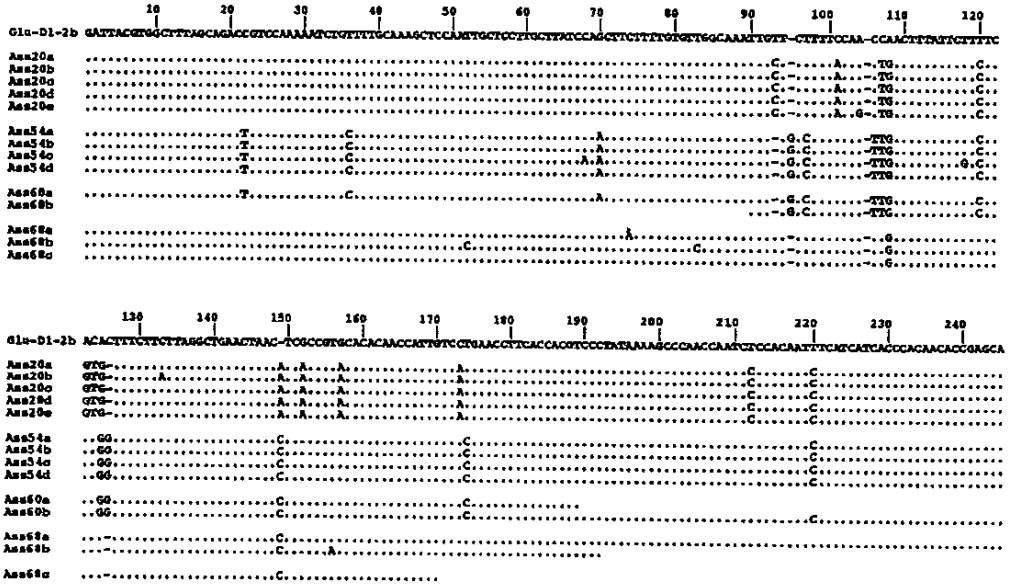

FIGURE 3 Sequences obtained from single grains of charred wheat. The sequences were obtained from cloned PCR products from single grains of the Assiros assemblage. The *Glu-D1-2b* allele is used as the reference sequence. Ass20, Ass54, Ass60 and Ass68 are four of the five DNA-containing grains from the Assiros assemblage. Sequences of individual clones are designated a, b, etc. Dots indicate identity with the *Glu-D1-2b* allele, dashes indicate deletion/insertion points, and spaces are regions that have not been sequenced.

an allele of the *Glu-A1-2* locus. All cultivated wheats contain the A genome so this result is compatible with what is known about the grain, but non-informative with regards to taxonomic identification.

2. The three sequences from grain 68 were also identical except for PCR errors and represented an allele of the *Glu-D1-2* locus. This result is incompatible with the morphological identification, grain 68 having the typical shape of a tetraploid glume wheat.
3. Grains 54 and 60 also gave identical sequences (four from grain 54, two from grain 60) except for PCR errors, these being for an allele of the *Glu-G1-2* locus, suggesting that grains 54 and 60 were from a AAGG tetraploid wheat similar to *T. timopheevi*. Cultivated AAGG wheats are thought to have had a restricted geographical range and were therefore not expected in the Assiros assemblage, but in some other samples from Assiros unusual chaff fragments have been encountered which might be from an AAGG wheat (Jones *et al.*, submitted). No such chaff fragments were found in the sample used in the DNA analysis (grains of AABB and AAGG tetraploids are morphologically indistinguishable), and so again the DNA results are incompatible with the morphological identification.

CONCLUSIONS

The results reported in this paper illustrate both the potential and the limitations of ancient DNA studies with charred seeds. Our initial strategy for ploidy identification, based on a system for typing size variations in the *Nor* loci present on different genomes, which works effectively with modern DNA, was unsuccessful with extracts of charred seeds, probably because the ancient DNA templates were highly fragmented. Our second strategy, involving phylogenetic analysis of sequences obtained after PCR of the glutenin loci, enabled us to identify genome-specific allele sequences in a mixed assemblage of charred grains. The alleles that were identified indicated that the assemblage contained hexaploid grains, which was compatible with the morphological examination of this sample. We further showed that it is possible to obtain PCR products from extracts of single grains, but that only a small proportion of the grains in an assemblage contain amplifiable DNA. The results of the DNA analyses of single grains did not, however, show extensive agreement with the morphological identifications. Of four grains that contained amplifiable DNA, one gave a result that was compatible with the morphological features of the grain, whereas three did not. These results indicate that caution is still necessary in the interpretation of ancient DNA studies with charred wheats.

MATERIALS AND METHODS

Archaeological Plant Material

The charred specimens were 2000-year-old *T. spelta* from Danebury, U.K. (Jones, 1984; previously used for DNA studies by Allaby *et al.*, 1994), and a 3000-year-old mixed assemblage from Assiros Toumba, Greece (Jones *et al.*, 1986; previously used for DNA studies by Allaby *et al.*, 1997, 1998). The Danebury samples were part of material excavated by B. Cunliffe (Jones, 1984), taken from well-sealed assemblages that were removed *en bloc* and immediately enclosed within aluminium foil. The Assiros material was excavated by K.A. Wardle (University of Birmingham, UK).

DNA Extraction and Analysis

For mixed assemblages, nucleic acids were prepared from 500-mg samples by the method previously described by O'Donoghue *et al.* (1996b). Extracts dissolved in 100 µl water were further purified by electroelution at 6.66 V cm^{-1} for 1 h after fractionation in a 1.3% agarose gel

(Towner, 1991) or by silica adsorption (Höss and Pääbo, 1993). Extracts of the Assiros assemblage were incubated at 37°C for 2 h with 0.01 vol 10 mg ml^{-1} DNase-free RNase A. All extracts were finally reprecipitated with 3 vol ice-cold absolute ethanol, washed with 70% (v/v) ethanol, and redissolved in 100 µl water.

Single grains were individually ground in a 1.5 ml microfuge tube, using a second microfuge tube of 0.5 ml as the pestle. The powder was mixed with 500 µl of pre-heated extraction buffer (2% w/v cetyl trimethylammonium bromide, 100 mM Tris-HCl pH 8.0, 20 mM Na_2EDTA, 1.4 M NaCl) and incubated for 42 h at 60°C, with mixing. The seed debris was removed by microcentrifugation (16 000 × *g*, 30 min) and the supernatant liquid extracted once with 24:1 v/v chloroform + isoamyl alcohol. Two volumes of pre-cooled precipitation buffer (1% w/v cetyl trimethylammonium bromide, 50 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA) were added to the final aqueous layer and the mixture maintained at 4°C overnight. Nucleic acids were collected by centrifugation as before, dried *in vacuo*, resuspended in 500 µl guanidium thiocyanate buffer (6 M guanidium thiocyanate, 100 mM Tris-HCl pH 6.4, 40 mM Na_2 EDTA pH 8.0) and incubated for 30 min at 60°C. After addition of 40 µl activated silica (Höss and Pääbo, 1993), the mixture was incubated for 10 min at 60°C and then centrifuged at 16 000 × *g*, for 10 min. The silica pellet was washed twice with 500 µl 6 M guanidium thiocyanate, 100 mM Tris-HCl pH 6.4, then washed with 500 µl absolute ethanol followed by 500 µl acetone. The DNA in the silica pellet was eluted into 60 µl 10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA.

PCRs used the 'hot-start' strategy (Erlich *et al.*, 1991). PCRs directed at the glutenin loci (Allaby *et al.*, 1994) were carried out in 100 µl reaction mixes containing 10 µl extracted nucleic acid (approximately 10 ng for modern samples), 10 µl buffer (Boehringer-Mannheim), 50 µM each dNTP, 200 ng each primer and 2.5 units *Taq* DNA polymerase (Boehringer-Mannheim), using the primers described by Allaby *et al.* (1994) either with or without 5′ extensions (5′-CTCTGGATCC-) containing a restriction site for cloning purposes and using the cycle conditions described by Allaby *et al.* (1994). PCRs directed at the intergenic spacer regions of the wheat rDNA loci were carried out as described by Sallares *et al.* (1995) using the primers shown in Fig. 1. PCR products (25 µl aliquots) were fractionated in 3% NuSieve or MetaPhor agarose gels (FMC BioProducts), and cloned and sequenced as described by O'Donoghue *et al.* (1996b). Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and subjected to principal coordinate analysis (Higgins, 1992). Neighbour-joining trees were obtained using CLUSTAL W and the topology verified by the maximum likelihood method with PHYLIP 3.57 (Felsenstein, 1989).

Precautions were taken to minimise the risk of contaminating ancient material with modern plant DNA: ancient extracts were prepared in a separate room not used for handling modern plant DNA or PCR products; PCR mixes were set up in a third laboratory in a laminar flow cabinet (HEPA filter, Class 100, conforming to BS 5295 and 5726) used for no other purpose; the procedure included standard precautions regarding pipette types, sterilisation of solutions by autoclaving and ultraviolet irradiation; control experiments were run to check for contamination of solutions used in DNA extractions and in PCRs; and all ancient DNA amplifications were accompanied by an extraction blank (extraction carried out with no seeds) and a water blank (PCR set up with no DNA).

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References

Allaby, R.G., Jones, M.K. and Brown, T.A. (1994) DNA in charred wheat grains from the Iron Age hillfort at Danebury, England. *Antiquity* **68**, 126–132.

Allaby, R.G., O'Donoghue, K., Sallares, R., Jones, M.K. and Brown, T.A. (1997) Evidence for survival of ancient DNA in charred wheat seeds from European archaeological sites. *Ancient Biomol.* **1**, 119–129.

Allaby, R.G., Banerjee, M. and Brown, T.A. (1998) Evolution of the high-molecular-weight glutenin loci of the A, B, D and G genomes of wheat. *Genome* in press.

Erlich, H.A., Gelfand, D. and Sninsky, J.J. (1991) Recent advances in the polymerase chain reaction. *Science* **252**, 1643–1651.

Felsenstein, J. (1989) PHYLIP – Phylogeny Inference Package (Version 3.20). *Cladistics* **5**, 164–166.

Goloubinoff, P., Pääbo, S. and Wilson, A.C. (1993) Evolution of maize inferred from sequence diversity of an *adh2* gene segment from archaeological specimens. *Proc. Natl. Acad. Sci. USA* **90**, 1997–2001.

Harberd, N.P., Bartels, D. and Thompson, R.D. (1986) DNA restriction-fragment variation in the gene family encoding high molecular weight (HMW) glutenin subunits of wheat. *Biochem. Genet.* **24**, 579–596.

Higgins, D.G. (1992) Sequence ordinations: a multivariate analysis approach to analysing large sequence data sets. *Cabios* **8**, 15–22.

Höss, M. and Pääbo, S. (1993) DNA extraction from pleistocene bones by a silica-based purification method. *Nucl. Acids Res.* **21**, 3913–3914.

Jones, G., Wardle, K.A., Halstead, P. and Wardle, D. (1986) Crop storage at Assiros. *Scientific American* **254**(3), 95–103.

Jones, G., Valamoti, S. and Charles, M. A new glume wheat from northern Greece. *Veget. Hist. Archaeobot.* submitted for publication.

Jones, M.K. (1984) The plant remains. In, *Danebury: An Iron Age Hillfort in Hampshire* (B. Cunliffe, Ed.), pp. 483–495, Council for British Archaeology, London.

Lassner, M. and Dvorák, J. (1986) Preferential homogenization between adjacent and alternate subrepeats in wheat rDNA. *Nucl. Acids Res.* **14**, 5499–5512.

Lassner, M., Anderson, O. and Dvorák, J. (1987) Hypervariation associated with a 12-nucleotide direct repeat and inferences on intergenomic homogenization of ribosomal RNA gene spacers based on the DNA sequence of a clone from the wheat *Nor-D3* locus. *Genome* **29**, 770–781.

Miller, T.E. (1987) Systematics and evolution. In, *Wheat Breeding – its Scientific Basis* (F.G.H. Lupton, Ed.), pp. 1–30. Chapman and Hall, London.

O'Donoghue, K., Brown, T.A., Carter, J.F. and Evershed, R.P. (1994) Detection of nucleotide bases in ancient seeds using gas chromatography/mass spectrometry and gas chromatography/mass spectrometry. *Rapid Comm. Mass Spectrom.* **8**, 503–508.

O'Donoghue, K., Brown, T.A., Carter, J.F. and Evershed, R.P. (1996a) Application of high performance liquid chromatography – mass spectrometry with electrospray ionisation to the detection of DNA nucleosides in ancient seeds. *Rapid Comm. Mass Spectrom.* **10**, 495–500.

O'Donoghue, K., Clapham, A., Evershed, R.P. and Brown, T.A. (1996b) Remarkable preservation of biomolecules in ancient radish seeds. *Proc. R. Soc. Lond. ser. B* **263**, 541–547.

Payne, P.I., Holt, L.M., Worland, A.J. and Law, C.N. (1982) Structural and genetic studies on the high-molecular-weight subunits of wheat glutenin. 3. Telocentric mapping of the subunit genes on the long arms of the homeologous group 1 chromosomes. *Theor. Appl. Genet.* **63**, 129–138.

Rogers, S.O. and Bendich, A.J. (1985) Extraction of DNA from milligram amounts of fresh herbarium and mummified plant tissues. *Plant Mol. Biol.* **5**, 69–76.

Rollo, F., LaMarca, A. and Amici, A. (1987) Nucleic acids in mummified plant seeds: screening of twelve specimens by gel electrophoresis, molecular hybridization and DNA cloning. *Theor. Appl. Genet.* **73**, 501–505.

Rollo, F., Venanzi, F.M. and Amici, A. (1991) Nucleic acids in mummified plant seeds: biochemistry and molecular genetics of pre-Columbian maize. *Genet. Res.* **58**, 193–201.

Rollo, F., Asci, W. and Sassaroli, S. (1994) Assessing the genetic variation in pre-Columbian maize at the molecular level. In, *Conservation of Plant Genes II: Utilization of Ancient and Modern DNA* (R.P. Adams *et al.*, Eds), pp. 27–35, Missouri Botanical Garden, St. Louis.

Sallares, R. (1991) *The Ecology of the Ancient Greek World.* Duckworth, London.

Sallares, R. and Brown, T.A. (1998) PCR-based analysis of the intergenic spacers of the *Nor*-loci of the A genomes of *Triticum* diploids and polyploids. *Genome* in press.

Sallares, R., Allaby, R.G. and Brown, T.A. (1995) PCR-based identification of wheat genomes. *Molec. Ecol.* **4**, 509–514.

Schlumbaum, A., Neuhaus, J.-M. and Jacomet, S. Coexistence of tetraploid and hexaploid naked wheats in a neolithic lake dwelling of Central Europe. Evidence from morphology and ancient DNA. *J. Archaeol. Sci.* in press.

Thompson, J.D., Higgins, D.C. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**, 4673–4680.

Thompson, R.D., Bartels, D., Harberd, N.P. and Flavell, R.B. (1983) Characterisation of the multigene family coding for HMW glutenin subunits in wheat using cDNA clones. *Theor. Appl. Genet.* **67**, 87–96.

Towner, P. (1991) Recovery of DNA from electrophoresis gels. In, *Essential Molecular Biology: A Practical Approach*, Vol. 1 (T.A. Brown, Ed.), pp. 127–141, IRL Press at Oxford University Press, Oxford.