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DNA in wheat seeds from European archaeological sites

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Abstract. We have used hybridization analysis to detect ancient DNA in wheat seeds collected from three archaeological sites in Europe and the Middle East. One of these samples, carbonized *T. spelta* dated to the first millennium BC, has yielded PCR products after amplification with primers directed at the leader regions of the HMW (high molecular weight) glutenin alleles. Sequences obtained from these products suggest that the DNA present in the Danebury seeds is chemically damaged, as expected for ancient DNA, and also indicate that it should be possible to study the genetic variability of archaeological wheat by ancient DNA analysis. Finally, we describe a PCR-based system that enables tetraploid and hexaploid wheats to be distinguished.
Key words. Ancient DNA; archaeobotany; carbonized grain; DNA sequences; glutenin alleles; seed proteins; *Triticum*; wheat.

Wheat in prehistoric agriculture

Wheat was one of the first plants to be cultivated by the Neolithic farmers of the 'Fertile Crescent', a region of the Near and Middle East comprising the plains of Mesopotamia, the deserts of Syria, and some of the neighbouring mountainous areas of Anatolia. The earliest archaeological evidence for wheat cultivation comes from the site of Tell Mureybit, on the east bank of the Euphrates in northern Syria, where wild einkorn, *Triticum boeoticum* (classification of ref. 20), was grown in the eighth millennium BC and possibly earlier[2]. During the following 2000 years, there was a proliferation in the number of cultivated species of wheat with domesticated forms (i.e. those not known in the wild) appearing for the first time. The genetic transformations that occurred during this period led to phenotypic changes such as less fragile ears (beneficial to harvesting as the mature seeds remain attached to the plant rather than being dispersed) and a decrease in the size of the glumes enclosing the seeds (resulting in easier threshing). Domestication was also accompanied by an increase in chromosome number, the first hexaploid wheats appearing during the latter part of the eighth millennium BC, probably as a result of amphiploidy between the diploid *Aegilops squarrosa* and the tetraploid *Triticum dicoccum*[2,20].

This period of genetic diversification was accompanied by an increase in the geographical range of wheat. Farming became established in the Balkans soon after 6000 BC and subsequently spread throughout Europe via the Danube and Rhine valleys, and along the Mediterranean coast, arriving in northern Europe and Britain around 4000–3500 BC[24]. During roughly the same period similar trajectories are thought to have resulted in the spread of wheat cultivation into central and southern Asia and into the Nile valley[12]. This geographical expansion of farming can be looked on as a record of prehistoric population interactions, as transfer of agricultural technology from one place to another must have occurred through human contact, either by the farmers supplanting an indigenous population, or by a peaceful acculturation of the existing society. The subsequent waves of advance of new varieties of wheat in the millennia following the initial expansion of agriculture continues this record of human contacts across an extensive period of prehistory.

An examination of the types of wheat grown at different archaeological sites could therefore provide information relevant to an understanding of the changing relationships between human populations in the prehistoric Old World. Expressed simplistically, if closely related wheats are present at two or more contemporaneous sites then it could be proposed that these sites were in contact, whereas the presence of widely differing wheats might indicate cultural distinctiveness. Unfortunately, it is difficult to address these possibilities by conventional archaeobotanical techniques based on morphological examination of preserved material. Only with the 'glume wheats' (those with which the chaff tightly encloses the grain) can species and varieties be discriminated with any confidence from the morphology of those parts of the plants that are generally preserved on archaeological sites. The 'naked wheats', which include agriculturally important varieties such as *Triticum turgidum*, *Triticum durum* and *Triticum aestivum*, are usually impossible to distinguish. With these wheats the morphometric indicators are so ambiguous that it may not even be possible to determine the ploidy level of an archaeological specimen. This imprecision in genotype identification places severe limitations on the degree of confidence that can currently be assigned to archaeobotanical-based analyses of contacts between human populations.

Before we can exploit the potential of wheat as a means of studying interactions between prehistoric communi-

ties, we must develop more reliable methods for examining the genotypes of archaeological remains. The greatest accuracy in genotype analysis is provided by comparisons of nucleotide sequence polymorphisms, an approach that has revolutionized the study of extant populations of wheat and other plants, and could do the same for archaeological material if sufficient DNA is preserved in a form suitable for sequence analysis. The first indications that ancient DNA might be present in archaeological plant remains came from work with preserved maize ears[10,26,27]. The successful extraction and sequence analysis of ancient DNA from this type of material prompted us to attempt similar experiments with wheat.

Detection of ancient DNA in archaeological wheat remains

There are four common mechanisms by which plant remains are preserved in archaeological contexts: complete or incomplete reduction to carbon by heat; exclusion of water in a desiccating environment; exclusion of oxygen, often in a waterlogged environment; and partial mineralization with calcium phosphate, calcium carbonate or, less commonly, iron sulphate (pyrites). As yet we do not know enough about the factors that influence DNA degradation to be able to predict with confidence which, if any, of these preservation modes might be compatible with the presence of ancient DNA[19]. Intuition suggests that biomolecular decay might be retarded in dry and/or anoxic environments, a conjecture that is supported by the presence of ancient DNA in Miocene plant fossils from anoxic deposits at Clarkia, Idaho[8,9,30], and in maize cobs preserved by carbonization and/or desiccation[10,27]. Our first experiments were therefore directed at a small set of archaeological wheat remains encompassing three types of preservation–waterlogging, carbonization and desiccation (table). Extracts were prepared by a standard procedure previously shown to be applicable to dried material (cf. ref. 25, modified as described in ref. 3) and the presence of wheat DNA determined by dot blot hybridization analysis with wheat-specific probes. Hybridization signals were seen with all three extracts, indicating that wheat nucleic acids might be present in each type of preserved material.

Although archaeological wheat remains encompass the full range of preservation states, these various states display different geographical and temporal coverages. Rich assemblages of anoxically preserved and dry preserved cereals have been discovered, for example, in East Africa and around the lakes of Switzerland (e.g. refs 13 and 28), but by far the most complete spatial and temporal coverage is provided by carbonized grain. To be of general value in wheat archaeobotany it must therefore prove possible to extract and analyse DNA from carbonized material. In view of this, we have devoted the greater part of our work on the charred *Triticum spelta* seeds described in the table, from the Iron Age hillfort at Danebury, England[16,21], directly dated by the conventional radiocarbon method to the second half of the first millennium BC[5]. We have used extracts of the Danebury grain in PCR experiments targeted at a 246 bp segment of the leader regions of the genes for the HMW glutenin proteins. These are nuclear genes with a copy number of two per chromosome set, twelve copies present in a hexaploid set. The results of a successful PCR are shown in figure 1. Our experience is that a positive result is not obtained with every extract of the Danebury seeds: some extracts give no PCR products at all, others give a range of products possibly including a band of the expected size (but with many unexpected bands), and a few extracts give the result shown in figure 1. However, the authenticity of the positive results that we have obtained is supported by the results of control experiments. Figure 1, lanes 3 and 4, show that no amplification occurs in the 'water blank', in which the aliquot of extract is replaced with an aliquot of water, nor in the 'extract blank' prepared by carrying out the entire extraction procedure without the seeds. In addition, we follow the standard proce-

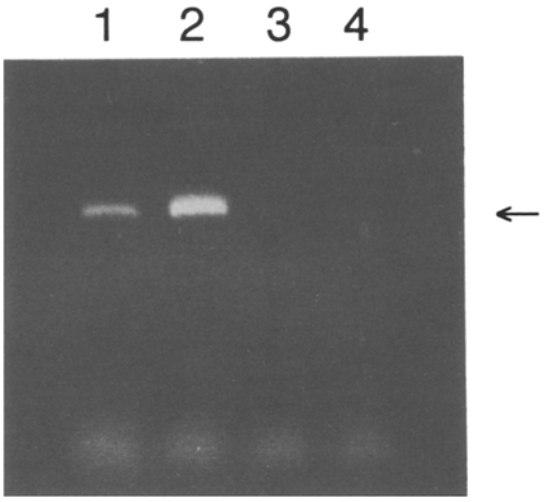

Figure 1. PCR analysis of an extract from the Danebury wheat seeds. Lanes: *1*, result of PCR with modern *Triticum aestivum* DNA; *2*, result of PCR with an extract from the carbonized *T. spelta* seeds from Danebury; *3*, result of PCR with an extract blank (extraction procedure carried out with no seeds); *4*, result of PCR with a water blank (PCR set up with no DNA). The arrow indicates the position corresponding to 246 bp, the expected size of the PCR product.

Wheat remains used in this study.

Species	Preservation	Age (BP)	Site
T. spelta	charred	2000 years	Danebury, UK
T. aestivum	waterlogged	700 years	Oxford, UK
T. dicoccum	desiccated/ mineralized	1000 years	Tell Es Sa'idiyeh, Jordan

Abbreviation: BP, before present.

dures designed to prevent cross-contamination of samples, and have taken the added precaution of only using seeds from sealed assemblages at Danebury, these assemblages being excavated en bloc and the material immediately wrapped in aluminium foil.

Why are PCR products of the expected size obtained from only a few extracts? Inspection of the carbonized Danebury grains shows that this material is by no means uniform in appearance. Some grains retain their original shape but many others are distorted, and the seeds display a range of colours, not all being the soot-black expected of complete carbonization. Our hypothesis is that only the undistorted seeds, those that retain their natural morphology, are sufficiently well preserved to contain ancient DNA. This idea is supported by our observation that the success rate for ancient DNA amplification is rather greater with a second sample of carbonized grain, from Assiros Toumba in Greece[14,15], in which a much greater proportion of the seeds retain their natural morphology.

Genetic analysis of the Danebury seeds

Our long term objective is to utilize ancient wheat DNA to examine population interactions in the prehistoric Old World. Implicit in this is the need to obtain sequence information from preserved material. To determine whether it will be possible to study polymorphic loci in ancient wheat DNA we have obtained nucleotide sequences from the PCR products amplified from the HMW glutenin leader regions. The glutenin genes, which code for seed storage proteins important in determining the viscoelastic properties of the dough prepared from the grain[6], exist as a number of allelic forms, various combinations occurring in a single genotype[4,23]. The sequences of six alleles have been published, each allele having a different sequence in the 246 bp region targeted for amplification (fig. 2). Initial experiments that we have carried out with a modern cultivar of wheat have shown that it is possible to reconstruct at least a partial allele set by cloning and sequencing these amplification products. Of the ten clones that we have studied so far, four have the same sequence as the published 5(X) allele, two others are identical to one another and have a single nucleotide difference from the 9(Y) allele, three others are identical to each other but different from any of the published sequences, and the tenth sequence is unique (different from the other sequences we have obtained and different from the pub-

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10(Y)  GATTACGTGGCTTTAGCAGACCGTCCAAAAATCTGTTTTGCAAAGCTCCAATTGCTCCTTGCTTATCCAGCTTCT
 9(Y)  ....................T.....................A.............................
12(Y)  ................................-.....................................
 1(X)  ....................T.....................................................
 5(X)  ....................T.....................................................
SILENT ..................................................................

10(Y)  TTTGTG-TTGGCAAATTGTT-CTTTTCCAACCAACTTTATTCTTTTCACACTTTCTTCTTAGG-CTGAACTAA-C
 9(Y)  ......C..........C.G.........TG..........C....G-...........-..A......C.
12(Y)  ......-...........-..........G..............-...........G.........C.
 1(X)  ......-........C..CG-..........G.T...G.....C..G.G.............-..A...A..C.
 5(X)  ......-........C..CG-..........G.T...G.....C..G.G.............-..A...A..C.
SILENT  ......-..........C.-.....A....TG..........C.TGTG-...........-.........-.

10(Y)  -TCGCCGTGCACACAACCATTGTCCTGAACCTTCACCACGTCCCTATAAAAGCCCAACCAATCTCCACAATTTCA
 9(Y)  -A.A.....................C.....................................................C...
12(Y)  -...T.....--.....................G.....................................................
 1(X)  -..A........G..G....G..............T..............T.GTT..C..T......C.T.
 5(X)  -..A........G..G....G..............T..............T.G....C..T......C.T.
SILENT A..A....A.............A.............................A.........C.......C...

10(Y)  TCATCACCCACAACACCGAGCA
 9(Y)  .....................
12(Y)  .....................
 1(X)  .....................
 5(X)  .....................
SILENT .....................
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Figure 2. Partial nucleotide sequences of the six published HMW glutenin alleles[1,7,11,31,32]. The region shown is a 246 bp segment that lies immediately upstream of the open reading frame. The sequence is given in full for the 10(Y) allele and in abbreviated form for the other alleles, a dot indicating an identical nucleotide and a dash indicating a deletion. Underlined segments are the PCR priming sites.

lished alleles). We can therefore describe with some confidence the sequences of the amplified regions of four of the glutenin alleles in this modern wheat, and by sequencing additional clones we will be able to determine if there are any further amplified alleles that we have not yet detected. Theoretically, twelve different allele sequences could be obtained from a hexaploid wheat, but in fact we do not anticipate this degree of polymorphism in the sequences amplified by PCR. It is unlikely that twelve different alleles are present in a single genotype, and there may be alleles that are not amplified by our primers. The latter point means that we cannot at any stage be certain that we have reconstructed the complete set of alleles present in a genotype, but we can reach a point where we have enough sequences to be confident that we have identified all the alleles amplified by the PCR system. This partial allele set will be diagnostic for the genotype under study, and comparisons with the allele sets obtained from other plants will enable us to make deductions about genetic variability.

Similar experiments with the Danebury extracts have produced encouraging results. Five sequences have been obtained to date, these falling into two groups. One group comprises a single sequence that has a one-nucleotide difference from the published 5(X) allele. The other four sequences are distinct from the published alleles (at least five nucleotide differences when compared with most closely related allele) but are very similar, though not identical, to one another. A possible interpretation is that these four sequences derive from a single allele, with the apparent variability being due to chemical damage suffered by the ancient DNA templates. Chemical modification (e.g. G deaminated to U, ref. 27) and the loss of nucleotide bases[22] could both result in misreadings when PCR products are sequenced. A similar argument has been put forward by Goloubinoff et al.[10] to account for variability seen in ancient maize sequences.

The important question is whether we can, by sequencing a sufficient number of clones, make a statistically valid determination of the real sequences of the alleles present in the Danebury seeds. To do this, we need to obtain a large enough number of sequences from each allele to be able to identify nucleotide variations that are due to the damaged substrate, and to be able to deduce the correct identity of the nucleotide present at each position in the undamaged allele. Goloubinoff et al.[10] found that fourteen sequences were sufficient for a confident reconstruction of a diploid allele pair. Reconstructing a multiple allelic system such as the glutenin loci will clearly be a more demanding task, especially as undamaged alleles may differ by just one or two nucleotides, requiring a fine degree of discrimination to be able to identify them with confidence. The feasibility, or otherwise, of this task will become clear only when we have sequenced a larger number of clones of the Danebury PCR products.

Ploidy analysis of archaeological remains

Difficulties inherent in using morphometric data to assign the correct ploidy level to wheat remains is a major hindrance in archaeobotany as agriculturally important varieties such as *T. turgidum*, *T. durum* (AABB tetraploids) and *T. aestivum* (an AABBDD hexaploid) are often impossible to distinguish in the archaeological record. Hexaploid wheats do not exist in the wild and the origins and subsequent spread of hexaploids are important questions (e.g. ref. 29) that can only be answered if secure identifications of ploidy level can be made. This is an area in which ancient DNA typing could have an immediate impact.

We have designed a PCR-based system that exploits sequence differences between the B and D genomes to identify D- (e.g. tetraploid AABB) and D + (hexaploid AABBDD) plants. This system is directed at the non-transcribed spacer regions of the wheat rDNA loci, as the D genome spacers have a 71 bp insertion absent from the B genome loci[17,18]. Amplification from primers that span this region (fig. 3) results in a single product for a D- genome and two products for a D + genome. We have had success with this system in examination of modern wheat DNA, and are currently applying it to archaeological material.

Conclusions

We believe that we have made a reasonably secure identification of ancient wheat DNA in the carbonized

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B:CGCCATGGAAAACTGGGCAAAACCATGTACGTGGCACACGCCG
D:CGCCATGGAAAACTGGGCAAAAACACGTACGACGCACACACA-

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

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

B:ACACGGACCCGTGAACGGGTATGAGAGGTCCGGG--AAAAAAT
D:ACACGGACACGTGAACGGGTACGAGAGGTCCGGGAGAAAAAAA

B:GGCCCATACACCATGCGAACCGGCTCAAAACCAGCTAATGATG
D:GGCCCATACGCCATGGAAACCGGGTCAAAACTAGCTAATGATG

B:GTCAACAAACGGTGCCATGGCAGCGAAAACATGTCTCATGGCA
D:GTCAAGAAACGGTGCCATGGCAGCGAAAACATGTCTCATGGCA

B:AAAAAACGCTGCCACGGCAGCGTTTCAAAACAGTGTACCC
D:GAAAAACGCTGCCACGGCGGCGTTTCAAAACAGTGTACCC
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Figure 3. Partial nucleotide sequences of the non-transcribed spacer regions of the B (top) and D (lower) rDNA loci[17,18]. Dashes indicate deletions. Underlined segments are the PCR priming sites.

Danebury seeds. The hybridization analyses indicate that DNA is present, amplification products of the expected size are obtained in experiments subject to the standard precautions and accompanied by controls designed to detect contaminating DNA, and the sequence information that we have obtained suggests that the DNA being amplified is chemically damaged, as would be anticipated for ancient molecules. We have demonstrated that the genetic variability of wheat can be studied by ancient DNA sequencing and have begun to develop a system for ploidy analysis of archaeological remains. We are currently expanding our work to cover a more diverse set of wheats in order to assess the extent to which ancient DNA is preserved in this important archaeological resource. We are still only at the beginning of this project and it may transpire that ancient DNA has no great role to play in wheat archaeobotany, but the initial results are encouraging and we are excited by the prospects that are opening up.

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