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DNA analysis in charred grains of naked wheat from several archaeological sites in Spain

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ABSTRACT

In the present work we attempt to recover endogenous ancient DNA from cereal grains preserved under different conditions: charred, partially charred and waterlogged. A total of 126 grains from naked wheat and 18 from barley from different sites on the Eastern Iberian Peninsula ranging from the beginning of agriculture in the region to the turn of the Common Era, were studied. Two different extraction protocols were used, a standard phenol–chloroform method and a silica-based DNA extraction procedure implemented for artificially charred seeds. Amplifications were directed to three markers: the large subunit of ribulose 1,5 biphosphate carboxylase (rbcL) and the microsatellite WCT12 in the chloroplast genome and the x and y subunits of the high molecular weight glutenin gene (*Glu-1*) in the nucleus. The first two were used to assess the preservation status of the samples, while with the third we tried to identify the wheat grains at species level. It was possible to obtain eleven positive amplifications in 8 partially charred seeds but only two amplifications of the *Glu-1* gene from a single sample of the Early Bronze age were genome-specific. Different contamination sources were identified and reported. Cloning and alignment of sequenced clones showed a correspondence of the amplified fragment to modern wheat D genome haplotypes. This result suggests that the sample corresponds to hexaploid wheat (*Triticum aestivum* L.), thus being the first ancient DNA evidence to date for the cultivation of hexaploid wheat in the prehistoric agriculture of the Iberian Peninsula. Moreover, obtained results highlight contamination problems associated to the study of ancient archaeobotanical charred seeds suggest that the combination of a silica-based extraction method together with the amplification of specific targets is a good strategy for recovering endogenous ancient DNA from this kind of material.

1. Introduction

Wheat has been one of the most important crops in the Old World since the Neolithic. In spite of the spread of cereal agriculture in Europe being associated with cultivation of free-threshing wheat, methodological limitations prevent clear assignment of the prominent species(s) cultivated; either the hexaploid bread wheat (*Triticum aestivum* L.) or the tetraploid durum wheat (*Triticum turgidum* L. ssp. *durum* [Desf.] Husn.). For example, Maier (1996) concluded that tetraploid naked wheat spread from its point of origin in the Middle East via a Mediterranean route to southwest Europe. However, bread wheat has been identified as part of the cultivation assemblage at the Neolithic site of La Draga (Girona Province, Spain), one of the earliest sites in the Western Mediterranean where agriculture has been reported (Antolín and Buxó, 2011). Other studies have even suggested the coexistence of tetraploid and hexaploid naked wheat as far back as the early Neolithic near the Fertile Crescent (Fairbairn et al., 2002) and central Europe (Schlumbaum et al., 1998).

Distinguishing between bread and durum wheat from the morphology of archaeobotanical remains is not easy. Only when the rachis is recovered it is possible to ascertain with a certain degree of confidence which of the two species is present (Antolín and Buxó,

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2011; Fairbairn et al., 2002). However, the rachis is not among the most frequently recovered cereal remains that are encountered in archaeological sites and therefore it is common to refer to naked wheat as *T. aestivum*/*durum* (Van Zeist and Bakker-Heeres, 1982) or *T. aestivum*/*durum*/*turgidum* (Jacomet and Schlichltherle, 1984) in archaeological studies of agriculture. Ancient plant DNA analyses from archaeobotanical remains and the use of molecular markers may represent an alternative way to identify the naked wheat specie(s) cultivated (Oliveira et al., 2012; Palmer et al., 2012a; Schlumbaum et al., 1998).

The possibility of recovering ancient endogenous DNA from plant remains has been widely discussed in the literature (Brown, 1999; Gugerli et al., 2005). In an archaeological context, botanical evidence could survive through one of the following preservation methods: desiccation, waterlogging, charring (complete or partial) and mineralisation. Among them, desiccated remains are the most suitable for ancient DNA preservation due to rapid water exclusion causing a stop in hydrolysis reactions. However, this kind of preservation is restricted to very specific environments. Successful DNA recovery has been reported from a variety of desiccated plant tissues from different species (Palmer et al., 2012a). This is the case for olive pits (Elbaum et al., 2006), maize grains, cobs, kernels and rusks (Freitas et al., 2003; Goloubinoff et al., 1993; Jaenicke-Després et al., 2003; Lia et al., 2007; Rollo et al., 1987, 1994), radish seeds (O'Donoghue et al., 1994, 1996), wheat (Allaby et al., 1994; Blatter et al., 2002; Oliveira et al., 2012), cotton (Palmer et al., 2012b), sorghum grains (Deakin et al., 1998) and fruit rinds of bottle gourd (*Lagenaria siceraria*) (Erickson et al., 2005). Preservation through waterlogging is based on oxygen exclusion during the time of deposition. However, submerged remains are subjected to hydrolysis reactions causing a rapid decay in DNA. Ancient DNA survival in this case seems to be limited to hard tissues such as fruit stones (Elbaum et al., 2006; Pollmann et al., 2005) or seeds (Cappellini et al., 2010; Manen et al., 2003; Schlumbaum et al., 2012).

The great bulk of plant material found in archaeological contexts corresponds to charred or partially charred evidence. Studies of DNA decay in aqueous solution suggest that DNA is not able to survive at temperatures higher than 250 °C, like the ones used for cooking and baking, which are the two main methods involved in the charring process (Boardman and Jones, 1990). However, experiments performed with artificially charred seeds have evidenced DNA survival after a charring period of 5 h at temperatures up to 250 °C (Threadgold and Brown, 2003). Studies on ancient charred material also support DNA survival, but recovery rates are significantly lower than for desiccated and waterlogged remains (Allaby et al., 1994, 1997, 1999; Blatter et al., 2002; Boscato et al., 2008; Brown et al., 1994, 1998; Goloubinoff et al., 1993; Mahmoudi Nasab et al., 2010; Manen et al., 2003; Schlumbaum et al., 1998). The fact that combustion experiments *in vitro* only monitor DNA degradation during the charring process while DNA preservation in the archaeological background depends mainly on soil, environmental conditions and taphonomic changes, could explain the differences in DNA recovery between both approaches. Nevertheless, all charring experiments agree on the level of oxygen during the charring process being a key factor in DNA survival. In an archaeological deposit, this would vary from largely anoxic environments found in sealed storage vessels or pits to aerobic seed concentrations. Moreover, these studies indicate that not all seeds from an assemblage contain ancient DNA (Allaby et al., 1994, 1997). Depending on the location of the grains in the storage system, different seeds can suffer from differential exposure to fire or oxygen. Differences in seed morphology could also account for a differential preservation of DNA content once the charring process is finished.

Ancient DNA analysis of charred plant remains may be of great importance, for example, in the identification of wheat species, especially the free-threshing kind. In this case, the threshing process results in naked grains that are impossible to identify merely by morphological traits. Moreover, the charring process may alter the shape of not only the seeds, but also other cereal remains, complicating their identification at species level. Thus, as a way of distinguishing between the two most agronomically important species of free-threshing domesticated wheat, genetic detection of durum wheat can be achieved by determining the ploidy level. While durum wheat is tetraploid (AABB), bread wheat resulted from amphidiploidization between the tetraploid wheat, *Triticum dicoccum* Schrank. (AABB), and the diploid goat grass, *Aegilops tauschii* Coss. (DD). The genetic characterisation of genes or genetic markers characteristic of the D genome would allow the distinction between either wheat species.

The amplification of specific regions of the intergenic spacer (IGS) of ribosomal DNA (rDNA) produces products of different sizes between the A, B and D genomes, allowing differentiation between hexaploid and tetraploid wheat (Brown et al., 1998). This approach has been used in a recent study on naked wheat from desiccated grains. The authors demonstrated the presence of both durum and bread wheat in a naked grain assemblage from a pre-Hispanic grain silo on Gran Canaria island (Oliveira et al., 2012). However, these authors failed to amplify DNA from charred grains.

The glutenin locus 1 (*Glu-1*) encodes for a seed storage protein of high molecular weight (HMW) that is involved in bread quality. The *Glu-1* gene is located on wheat chromosome 1 of genomes A, B and D and it is divided into two paralogous genes named X and Y that code for two different glutenin subunits (Glu-x and Glu-y) (Payne et al., 1987). These genes are genome specific and multiallelic. The study of short regions of *Glu-1* has been also employed to identify the ploidy level of ancient charred wheat seeds with different rates of success (Allaby et al., 1997, 1999; Brown et al., 1994, 1998; Mahmoudi Nasab et al., 2010; Palmer et al., 2012a; Schlumbaum et al., 1998). However, the potential and the difficulties in this field that are related to DNA preservation and to risks of contamination have to be highlighted, and this may even raise concerns about the conclusions attained by older studies (Gugerli et al., 2005).

One of the key factors influencing the recovery of endogenous ancient DNA from charred plant material is the extraction protocol. The most popular DNA extraction protocols used with archaeobotanical material are the CTAB/DTAB methods and the silica-based ones using commercial kits (Lister et al., 2008; Yang et al., 1998) or home-made solutions (Höss and Pääbo, 1993; Poinar et al., 1998). Experiments performed with artificially charred seeds have suggested that silica-binding protocols are more efficient than traditional CTAB/DTAB extraction protocols (Giles and Brown, 2008). Compared to other extraction methods, this protocol is quick, easy to reproduce and sensitive enough to obtain DNA from single grains. However, in a recent study the extraction efficiency of commercial DNA (Lambda DNA, Fermentas) using different extraction protocols was compared: the standard CTAB, Giles and Brown (2008) and combinations of both were used (Oliveira et al., 2012). Examination of DNA extracts in agarose gels showed that the recovery of Lambda DNA was very limited when using the Giles and Brown (2008) protocol. Efficiencies of the other three protocols tested were high, but the CTAB extracts were highly inhibited by Maillard like products that were produced during the extraction process. Thus, a combination of CTAB-based extraction buffer with commercial silica columns was chosen as the most suitable combination for the analysis of ancient archaeobotanical DNA.

In the present work we evaluate the usefulness of the protocol of Giles and Brown (2008) in the extraction of genuine ancient DNA from charred cereal seeds from six archaeological sites from the Iberian Peninsula. As the main objective, we aim to infer the ploidy