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Molecular Analysis of Ancient DNA Extracted from 3250-3450 Year-old Plant Seeds Excavated from Tepe Sagz Abad in Iran

H. Mahmoudi Nasab[1], M. Mardi[2], H. Talaee[3], H. Fazeli Nashli[3], S. M. Pirseyedi[2], A. Hejabri Nobari[4*], and S. J. Mowla[1*]

ABSTRACT

To determine ancient seed species, 3250-3450 year-old charred seeds obtained from different Iranian archaeological sites were studied using Scanning Electron Microscopy (SEM) and molecular analysis. SEM analysis of ancient seeds revealed that the surface of the seeds was damaged. Therefore, we could not accurately identify their species. Molecular analysis on ancient specimens was done on different samples obtained from Masjede Kabood (Tabriz), Tepe Rahmat Abad (Pasargad) and Tepe Sagz Abad (Qazvin plain). The specific primer pairs were designed based on a part of the promoter region of the High Molecular Weight (HMW) glutenin gene and a short fragment of the *vrs*1 gene were verified on samples of modern wheat and barley varieties, respectively. The designated primers failed to amplify ancient DNAs (aDNAs) obtained from Masjede Kabood and Tepe Rahmat Abad, but successfully amplified the aDNA obtained from Tepe Sagz Abad. This finding was expected since the latter seeds had a better morphological preservation in comparison to the former ones. The accuracy of the amplified products was further proved by cloning and sequencing.

Keywords*:* aDNA, Ancient DNA, Barley, Molecular analyses, Seed, Wheat.

INTRODUCTION

DNA remains in archaeological specimens open up a new field of research known as molecular archaeology (Pääbo, 1989) which can track approximate dates of domestication events, identifying the wild ancestors of species and the spread of agriculture.

Although chemical and physical factors degrade the DNA of ancient organic remains over a long period of time, it is still possible to study ancient DNA (aDNA) by PCR-based molecular methods. There are three major problems associated with aDNA studies: (1) the poor quality and quantity of extracted aDNA, (2) the existence of inhibitors of *Taq* DNA polymerase in ancient samples and (3) the risk of cross-contamination of ancient samples with modern DNA (Hofreiter *et al*., 2001; Kaestle and Horsburgh, 2002; O'Rourke *et al*., 2000; Sykes, 1991). Therefore, the authenticity of the aDNA results must be carefully verified to be used in archaeological and anthropological studies (Kolman and Tuross, 2000; Poinar, 2003; Yang *et al*., 2004).

[1] Department of Genetics, Faculty of Basic Science, Tarbiat Modares University, P. O. Box: 14115-175, Tehran, Islamic Republic of Iran.
[2] Department of Genomics, Agricultural Biotechnology Research Institute, Karaj, Islamic Republic of Iran.
[3] Department of Archaeology, Faculty of Letters and Humanities, Tehran University, Tehran, Islamic Republic of Iran.
[4] Department of Archaeology, Faculty of Humanities, Tarbiat Modares University, Tehran, Islamic Republic of Iran.
* Correspondence authors; e-mail: sjmowla@modares.ac.ir, or e-mail: hejebri@modares.ac.ir

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The fragmented nature of aDNA along with various structural modifications usually poses some difficulties during PCR amplification. Furthermore, the preservation and extraction of aDNA from plant remains is more difficult than human and animal remains (Brown, 1999) because the forms of preservation for plant remains are different. "Charring" or "carbonization" is the commonest form of preservation for archaeological plant specimens. Plant remains of this type are thought to have undergone burning or baking and hence to have been exposed to high temperatures (possibly 250°C for several hours) (Boardman and Jones, 1990) which would be expected to degrade DNA.

Nevertheless, there are several reports of molecular analyses of aDNA of plants. The 17,310±310 B.P. (before present) site in Korea (Chungbuk National University, South Korea) revealed the first ancient rice (*Oryza sativa*) seed remains with extractable amount of aDNA (Suh *et al*., 2000). Allaby *et al*. extracted aDNA from 1000 year-old charred British spelt (Allaby *et al*., 1994) and 3000 year-old mixed Greek grain (Allaby *et al*., 1999). aDNA was also extracted from 1600 year-old millet (*Panicum miliaceum*) (Gyulai *et al*., 2006). In 1993, Goloubinof *et al.* extracted aDNA from 600 year-old maize cobs (Goloubinof *et al.*, 1993); 600 year-old aDNAs have also been extracted form medieval millet (*Panicum miliaceum*) (Lágler *et al*., 2005). The extraction of aDNA was reported from 5000 year-old charred hexaploid wheat seeds in Switzerland (Schlumbaum *et al*., 1998). The only study conducted in Near Asia with a closer attention on wheat domestication and early agriculture was reported by Bilgiç (Bilgiç, 2002).

The main objective of this study was to extract and amplify aDNA from ancient charred seeds collected from three archaeological sites in Iran. Among these, molecular analysis of samples of 3250-3450 year-old charred seeds collected from Tepe Sagz Abad succeeded.

MATERIALS AND METHODS

Ancient and Modern Seeds

Charred seeds were excavated in Tepe Sagz Abad (Qazvin plain; 3250-3450 years old), Masjede Kabood (Tabriz; ~3500 years old) and Tepe Rahmat Abad (Pasargad; ~6000 years old) (Figure 1). As a control, modern wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) samples of Iranian cultivars Sardari and Afzal,

Figure 1. Geographical location of Masjede Kabood, Tepe Sagz Abad and Tepe Rahmat Abad.

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respectively, were used for comparative analyses.

Contamination Precautions

To avoid cross-contamination of samples during the extraction and amplification steps, reactions were carried out in two separate laboratory work spaces, where all equipments were treated with 10% commercial bleach and subjected to UV irradiation using a laminar flow cabinet. All reagents were handled in sterile conditions. PCR reactions were set up in a laminar flow cabinet. Each set of DNA extraction was accompanied by a blank control extraction where no seed was included, to monitor any potential risk of contamination of reagents and equipments used in the extraction and amplification steps. Each set of PCR also contained a negative control "water blank" in which distilled water was used instead of DNA samples for further monitoring of any potential risk of contamination in PCR reactions. For non-disposable tools, 10% commercial bleach solution was used for cleansing. The extraction and amplification of aDNA and modern DNA were performed in different laboratories.

SEM Analysis

The surface ornamentations of modern and ancient charred seeds were examined by SEM (Gyulai *et al*., 1992; Başli *et al*., 2008; Güner *et al*., 2008) by using a Philips scanning electron microscope (Philips model XL30, Netherland) located in the Faculty of Engineering of Tarbiat Modares University.

DNA Extraction

DNA was extracted using a silica based method with minor modifications (Mohandesan *et al.,* 2008). 0.1-0.25 g charred seeds were ground into a fine meal with liquid nitrogen. Five ml extraction buffer (0.45 M EDTA pH 8, 0.25 mg ml^{-1} Proteinase K) was added to the powdered seeds, followed by vortexing and incubation in the buffer overnight at room temperature in a dark room with continuous vertical rotation. After phase separation by centrifugation at 4,000 rpm for two minutes, the supernatant was transferred to a 50 ml tube and mixed with 18 ml of L2 buffer (5 M guanidinium thiocyanate (GuSCn), 0.05 M Tris/HCl pH 8, 0.25 M NaCl) and 100 µl silica suspension, and rotated at room temperature in the dark for three hours. DNA fractions bound to silica suspension (Boom *et al*., 1990) were precipitated by centrifugation at 4,000 rpm for one minute. The silica pellet was washed twice: first with 1 ml of L2 buffer and then with 1 ml of new wash buffer (51.3% EtOH 99%, 125 mM NaCl, 10 mM Tris/HCl pH 8, 1 mM EDTA pH 8). DNA fractions were eluted from silica by incubation in 100 µl TE (10 mM Tris/HCl pH 8, 1 mM EDTA) at room temperature and separated with centrifugation at 13,000 rpm for 10 minutes. The resulting DNA was directly used for PCR.

PCR Amplification

As the amount of extracted aDNA is very low, the internal primers were designed to perform a nested and a semi-nested PCR. The amplification reactions contained 2 µl of extracted aDNA, 1 unit *Taq* DNA polymerase (Boehringer Mannheim, Germany), 2.5 mM of each dNTPs (Cinagene, Iran), 1X PCR buffer (10 mM Tris/Hcl pH 8.3, 50 mM KCl, gelatin solution 1%), 20 pmol of each specific primers in a 25 µl total volume.

The wheat-specific primer pairs were designed on the basis of a part of the promoter region of the HMW glutenin subunit gene and barley-specific primer pairs based on a short fragment of *vrs*1 gene (Table 1). The second semi-nested and nested PCR were performed with 2 µl of the

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Table 1. Sequence data of the designed primer pairs used to amplify specific segments in wheat and barley genomics DNA and in the ancient DNA samples.

Name	Sequence	Size of product
External wheat primers	5' GATTACGTGGCTTTAGCAGAC 3' 5' TGCTCGGTGTTGTGGGTGAT 3'	243 bp
Internal wheat primers	5' GATTACGTGGCTTTAGCAGAC 3' 5' GTGTGCACGACGAAGGTTAG 3'	160 bp
External barley primers	5' CTGTGTGGGAAGGGGAAAAC 3' 5' ATGAACTAGGGTTGGACATG 3'	117 bp
Internal barley primers	5' ATCATCACCAACGCTTCCTC 3' 5' TGGTGCTGGTGAATATGTTG 3'	62 bp

product of the first round of PCR, using the corresponding internal primers.

Cycling conditions for both rounds of PCR were: 2.5 minutes at 94°C; 35 cycles with: 1 minute at 94°C, 1 minute at 62.5°C (for wheat primers) 62.2°C and 56.5°C (for external and internal barley primers, respectively), 1 minute at 72°C; and finally 9 minutes at 72°C. The efficiency and reliability of PCR reactions were monitored by two parallel control reactions: an extraction control to confirm the lack of contamination of the mock DNA extraction with no seed added and amplification control to confirm the purity of the PCR reagents with no DNA added. In order to check the amplified fragments sizes and their quality, PCR products were resolved in 1.5% agarose and/or 8% polyacrylamide gels using GeneRuler™ 100 bp DNA size marker (Fermentase, Lithuania).

Cloning and Sequencing

PCR products obtained from ancient and modern seed samples were cloned using p-GEM-T easy Vector Kit (Promega, Madison, USA). Plasmid DNA of the three positive clones were extracted and purified using the AccuPrep Plasmid Extraction Kit (Bioneer, South Korea) and sequenced by the MWG-Biotech AG Company (Ebersberg, Germany). Multiple sequence alignment of sequences was analyzed using the ClustalW program (Thompson *et al*., 1994).

RESULTS

Morphological Analysis of Ancient Seeds

All the ancient seeds were charred and brittle with a black color. The seed sizes were also smaller compared to the modern samples when viewed by stereomicroscope or with the naked eye (Figure 2). SEM examination of the seeds revealed that the microscopic surface ornamentations of the seeds has been lost (Figure 3). Thus, the seeds are not suitable for accurate morphological determination, as noted by Dr. Fatemeh Zarinkamar (Iran), and Dr. Ferenc Gyulai (Gödöllő, Hungary).

Confirming the Specificity of Designed Primer Pairs

The specificity of the designed primers was first tested on modern wheat and barley samples (Figure 4). The wheat-specific primer pairs amplified a 243 base pair fragment in modern wheat, but not in modern barley and rice DNA samples. A similar result was obtained with barley-specific primer pairs, which exclusively amplified a 117 base pair fragment in modern barley.

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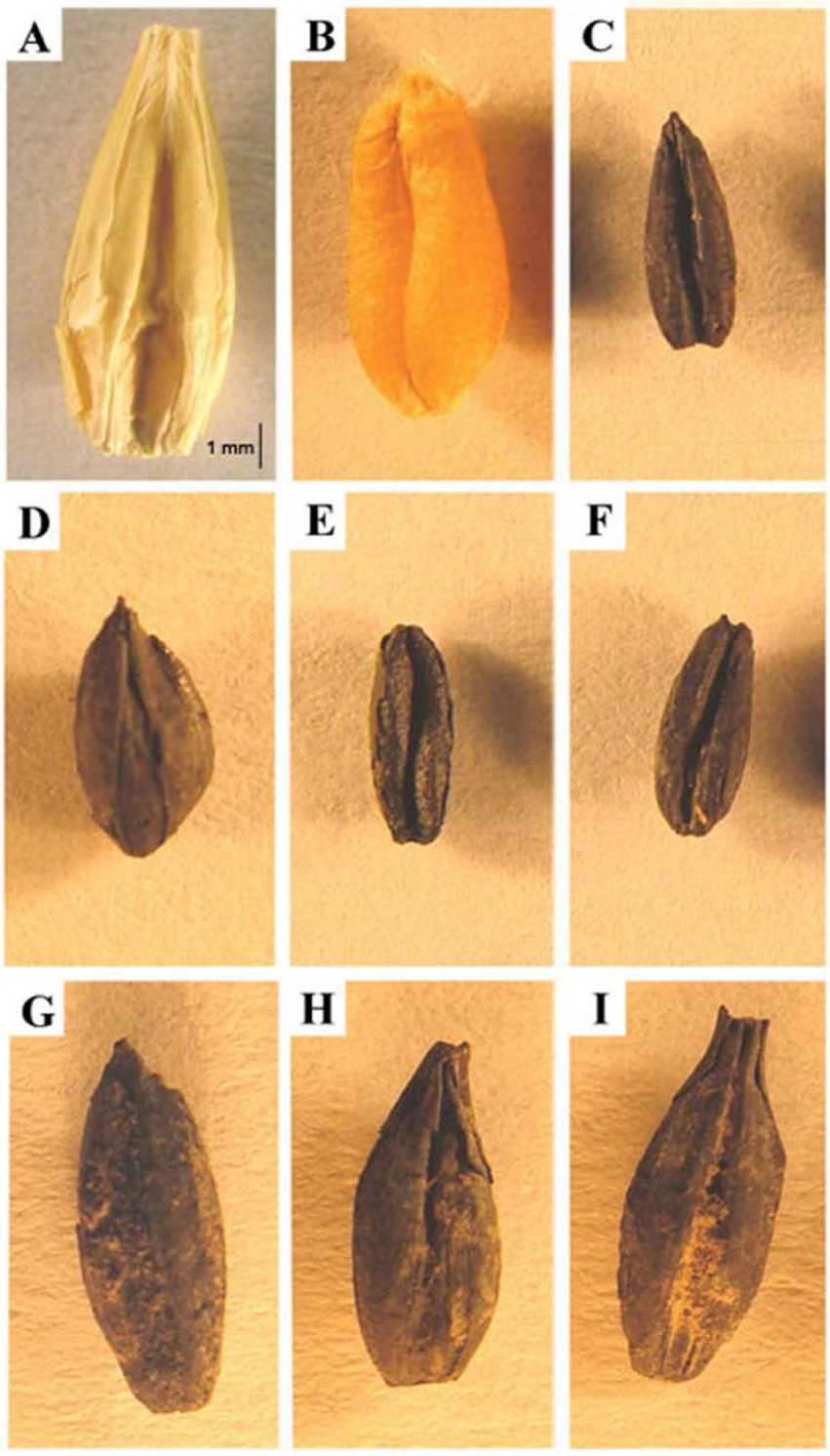

Figure 2. (A) Pictures of modern barley (*Hordeum vulgare* L.), (B) Wheat (*Triticum aestivum* L.) and (C-I) Ancient charred barley (*Hordeum* ssp) seeds obtained from Tepe Sagz Abad.

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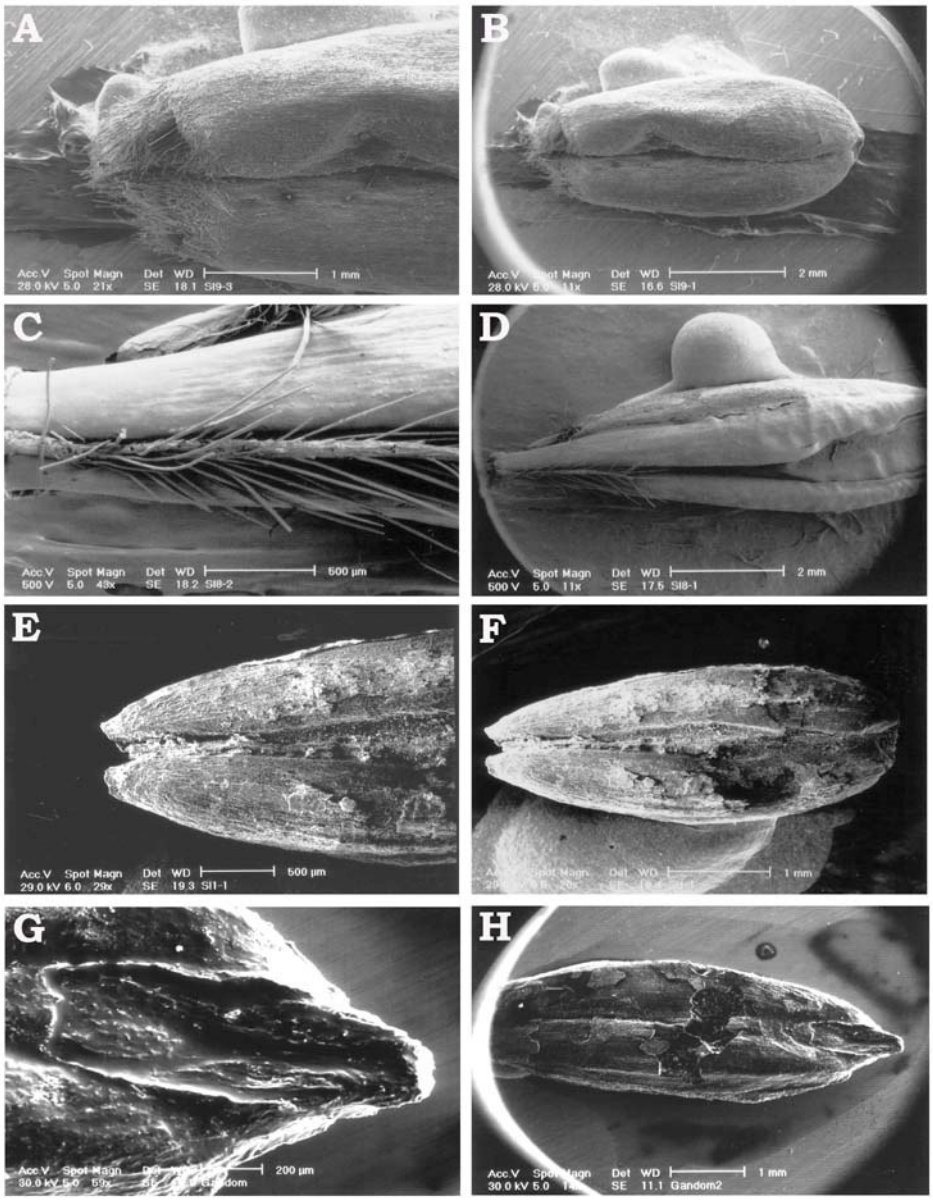

Figure 3. Scanning electron microscopy analysis of modern wheat (*Triticum aestivum* L.) (Sardari, A and B), barley (*Hordeum vulgare* L.), (Afzal, C and D) and ancient charred barley (*Hordeum* ssp) seeds (E-H) obtained from Tepe Sagz Abad.

Successful Extraction and Amplification of aDNA Fragments from Tepe Sagz Abad

Bread wheat is an allohexaploid species (*Triticum aestivum* L. 2*n*=6*x*=42), consisting of three sets of highly related genomes (*A*, *B*, and *D*). The glutenin genes are located on chromosome 1 of the wheat genomes *A*, *B* and *D* and are subdivided into *X* and *Y* types (Payne, 1987). The primers hybridize to two highly conserved sequences that flank a polymorphic region and thus amplify all six homoeologous genes present in hexaploid

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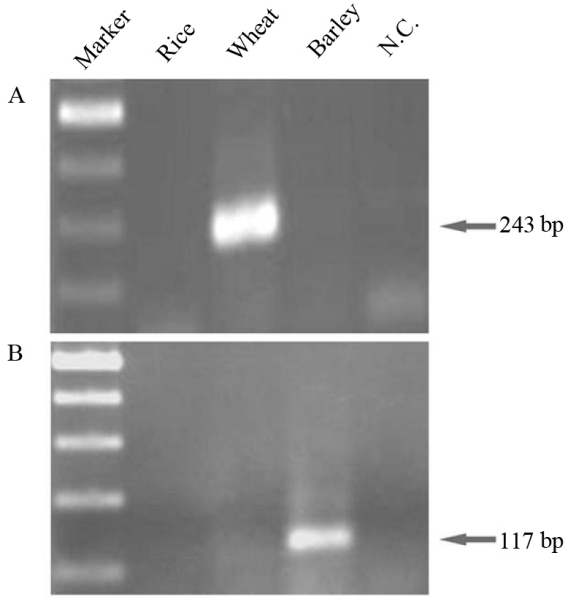

Figure 4. The specificity of primer pairs designed for: (A) Wheat and (B) Barley were tested on modern DNA samples of Wheat and Barely. Rice and N.C. (negative control without DNA) represent controls.

wheat, as well as the allelic variants (Allaby *et al*., 1994).

The wheat primers successfully amplified fragments of aDNA extracted from 3250-3450 year-old charred seeds collected from Tepe Sagz Abad and genomic DNA from modern Iranian seeds. The same primer pairs failed to amplify any detectable band on aDNA samples extracted from Tepe Rahmat Abad (Figure 5). The result was consistent with the very poor preservation of these samples compared with the seeds obtained from Tepe Sagz Abad. Some sort of contamination was detected in our first attempts at aDNA amplification of the barley (Figure 6) but, with changing reagents and optimizing the conditions for aDNA extraction and PCR, the aforementioned contamination was eliminated in further replicates of the experiments (data not shown). The samples obtained from Masjede Kabood had the least quality and we failed to extract any detectable aDNA from these samples.

The identity of the PCR products was confirmed by cloning the PCR products in a T/A vector followed by sequencing. The sequence alignment comparison of the sequences obtained with that of modern DNA sequences confirmed the true identity of the PCR products as wheat and barley DNA. A 160 base pair amplified segment of wheat aDNA and a 117 base pair amplified segment of barley aDNA had 91% and 100% homology with that of their modern counterparts, respectively (Figure 7).

DISCUSSION

In this study, we have attempted to isolate and amplify aDNA from ancient plant seeds excavated from three different archaeological sites in Iran. Plant tissues are usually richer in primary or secondary metabolites, such as carbohydrates and phenolic compounds (Ziegenhagen *et al*., 1993). Moreover, the best-preserved plant parts, wood and charred seeds, are rich in PCR inhibitors (Gugerli *et al*., 2005). For these reasons, technical adjustments to DNA isolation protocols for plant fossil remains

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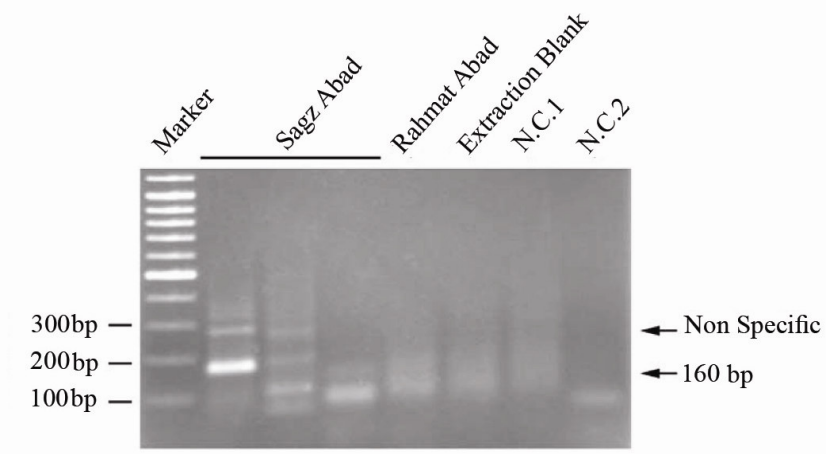

Figure 5. Wheat-specific fragment amplification in aDNA of 3250-3450 year-old charred cereal seeds excavated in Tepe Sagz Abad (Iran). The designed specific wheat primer pairs amplified a part of the promoter region of the high molecular weight (HMW) glutenin gene. Note the lack of any specific band in samples from Tepe Rahmat Abad and no DNA in extraction blank (extraction control of ancient samples) N.C. 1 (negative control 1, water blank in the first round of PCR) and N.C. 2 (negative control 2, water blank in the second round of PCR.

are needed, to ensure removal of these kinds of inhibitors prior to PCR amplification (Banerjee and Brown, 2002). Due to the low quantity and poor quality of extracted aDNA, there is a chance for preferential amplification of rare contaminant modern DNA sequences, which could be present in the samples or laboratory environment. There are several guidelines that could assist researchers to surmount the problem of contamination and investigate the authenticity of ancient DNA work (Yang and Watt, 2005; Pääbo *et al*., 1989; Cooper and Poinar, 2001).

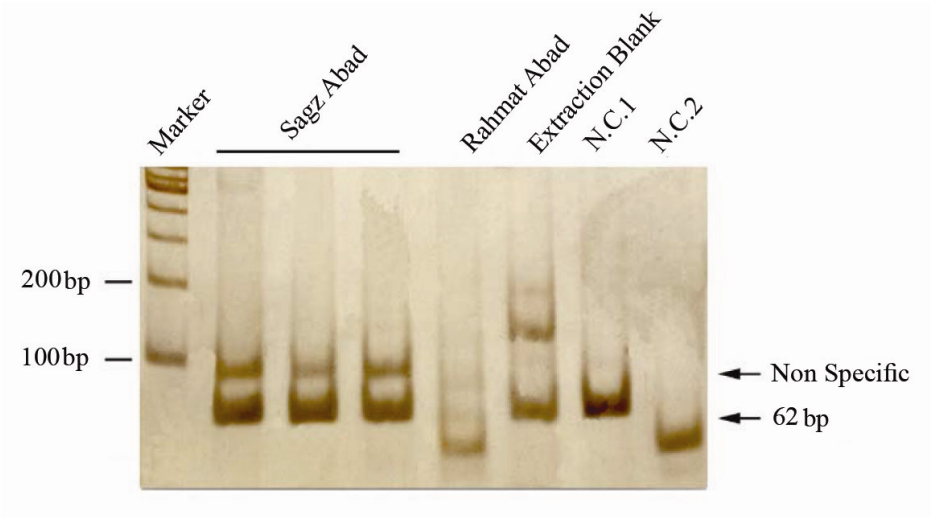

Figure 6. Barley-specific short fragment amplification in aDNA of 3250-3450 year-old charred cereal seeds excavated in Tepe Sagz Abad (Iran).The designed barley-specific primer pairs amplified a short fragment of *vrs*1 gene. The contamination have been seen in two extraction blank (extraction control of ancient samples) and negative control 1 (water blank in the first round of PCR) but not in N.C. 2 (water blank in the second round of PCR and aDNA extracted from Tepe Rahmat Abad.

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Figure 7. Sequence alignment of modern (upper row, red) and ancient charred wheat seeds (lower row, black) sequences of the promoter region of the high molecular weight (HMW) glutenin gene amplified with 91% homology. Asterisks indicate the consensus sequences.

To avoid PCR amplification of modern contaminant DNA molecules, the following precautious and controls were included in our study: (1) Different steps of extraction and amplification of modern DNA were carried out in separate and distant laboratories. (2) Extraction of aDNA was performed in a newly opened lab with no previous history of DNA work. (3) Ancient specimens were stored separately from modern reference specimens to prevent any potential cross-sample contamination. (4) All of the instruments and work places underwent a special kind of decontamination, as described previously (Mohandesan *et al*., 2004; Mohandesan *et al*., 2008). (5) The possibility of contamination from modern DNA was monitored during PCR amplification through the inclusion of extraction blanks and PCR negative controls in each experiment.

Generally, DNA recovered from ancient specimens is in a poor condition and is mostly damaged and broken into small pieces no longer than 200 nucleotides (Pääbo *et al*., 2004). Therefore, in order to determine the species of ancient seeds via molecular analysis, we designed pairs of specific primers that amplify short fragments of DNA. In our study, the wheat-specific primer pairs amplified a part of the promoter region of the high molecular weight (HMW) glutenin gene and specific barley primer pairs amplified a short fragment of the *vrs*1 gene of aDNA charred seeds and modern Iranian wheat and barley cultivars, Sardari and Afzal, respectively. This part of the promoter region of the HMW glutenin gene locus has already been shown to be suitable for wheat species identification (Allaby *et al*., 1999; Waines and Payne, 1987; Fernandez-Calvin and Orellana, 1990).

Using a silica-guanidinium-thiocyanate method, published for aDNA extraction of animal bone and human bone remains, we succeeded in extracting amplifiable aDNA from charred seeds excavated from Tepe Sagz Abad. The same conditions failed to extract any amplifiable aDNA from Tepe Rhmat Abad specimens. The finding was consistent with a poor preservation of seeds in Tepe Rhmat Abad compared to the ones obtained from Tepe Sagz Abad. The lack of any detectable PCR amplified DNA in these samples is further a good evidence to confirm the authenticity of the aDNA extracted from Tepe Sagz Abad.

In order to confirm the authenticity of the PCR products, they were cloned and

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sequenced. The sequence of the amplified products was identical in ancient and modern barley and was partially similar (more than 91% homology) to that of the modern wheat DNA sequence. The difference in sequences of ancient seeds from modern seeds is probably higher from that expected to happen during a short time evolution. Anyway, the novel allele obtained in this study support the ancient origin of the amplified DNA. Probably most of these differences occur subsequently in ancient seeds as a result of DNA alteration by chemical damage or they reflect polymerase errors (Pääbo *et al*., 2004).

In general, while our data suggest that aDNA can provide valuable information on approximate dates of wheat and barley domestication or identifying their wild ancestors in Iran, the achievement of aDNA is still a challenge due to the notorious DNA contamination and weakness of DNA preservation. The fact that we failed to extract amplifiable aDNA from two other archaeological sites suggests that the physical and chemical conditions of burial sites are very critical in getting amplifiable aDNA from archaeological remains. Also, the consideration of strict criteria for ancient DNA authenticity and contamination precautions are major factors for aquiring reliable data for any future attempts to work in this area of research.

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تجزیه مولکولی DNAی باستانی استخراج شده از بذرهای ۳۴۵۰-۳۲۵۰ ساله بدست آمده از تپه سگز آباد ایران

ح. محمودی نسب، م. مردی، ح. طلایی، ح. فاضلی نشلی، س. م. پیرسیدی، ع. ر. هژبری نوبری و س. ج. مولا

چکیده

به منظور تعیین گونه بذر های باستانی، بذر های سوخته ۳۴۵۰-۳۲۵۰ ساله از محل‌های حفاری شده مختلف در ایران جمع آوری شدند. ابتدا نمونه ها با استفاده از میکروسکپ الکترونی اسکنینگ و سپس با روش مولکولی مورد مطالعه قرار گرفتند. در بررسی میکروسکپ الکترونی مشخص شد که سطح بذر ها به شدت صدمه دیده و به این وسیله نمی توان گونه آنها را مشخص نمود. آنالیز مولکولی بر روی نمونه های باستانی متفاوت بدست آمده از مسجد کبود (تبریز)، تپه رحمت آباد (پاسارگاد) و تپه سگزآباد (دشت قزوین) انجام شد. اختصاصی بودن جفت پرایمرهای طراحی شده برای قسمتی از ناحیه پروموتر ژن پروتئین گلوتنین با وزن مولکولی زیاد (HMW) و قطعه کوچکی از ژن *vrs*1 بر روی نمونه های امروزی گندم و جو به ترتیب بررسی شد. پرایمرهای طراحی شده aDNA ی بدست آمده از نمونه های مسجد کبود و تپه رحمت آباد را تکثیر نکردند اما aDNA ی بدست آمده از نمونه های تپه سگزآباد با موفقیت تکثیر شد. بذرهای باستانی بدست آمده از تپه سگزآباد از لحاظ مورفولوژیکی نسبت به نمونه های دیگر بهتر محافظت شده بودند بنابراین این نتایج مورد انتظار بودند. برای تایید صحت توالیهای تکثیر شده، قطعات مربوطه کلون و تعیین توالی شدند.

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