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RESEARCH ARTICLE

Ancient DNA (aDNA) extraction and amplification from 3500-year-old charred economic crop seeds from Kaymakçı in Western Turkey: comparative sequence analysis using the 26S rDNA gene

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Abstract Ancient DNA (aDNA) from 3500–4000 years old seeds of *Triticum aestivum* L. or *Triticum durum* Dest., *Vicia ervillia* (L) Willd., *Cicer arietinum* L. and *Vitis vinifera* L. excavated from the archaeological site of Kaymakçı was successfully extracted using various isolation methods. The genomic DNA of each species was amplified with respect to the 26S ribosomal DNA (rDNA) gene further using the aDNA of the seeds. The reasons for successful DNA extraction and amplification are likely due to (1) preservation of certain ancient seed specimens in good conditions and (2) use of improved DNA extraction and amplification methods. The results indicate that all seeds were identified correctly by the DNA sequence data from the 26S rDNA gene. Specifically, a morphologically unidentified wheat seed from Kaymakçı was characterized by DNA sequence data as bread wheat (*Triticum aestivum*). Comparative sequence analysis revealed that specific base positions in the ancient 26S rDNA gene were either lost or substituted with different DNA bases in contemporary seeds, most likely due to continued domestication and breeding activities. Attaining high amounts and a good quality of amplified genomic DNA from ancient seeds will further allow the investigation of the extent of genetic change between ancient seeds and their contemporary species in genetic diversity studies.

Keywords Ancient DNA · 26S rDNA gene · Comparative sequence analysis · Genomic DNA · Kaymakçı · Domestication

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Introduction

Genetic characterization of plant species and their past genetic histories can be made from their fossils and charred forms (wood, seeds, etc.) as well as from living tissues using molecular methods developed for ancient specimens (Parducci and Rémy 2004; Gugerli et al. 2005; Rogers and Kaya 2006). Studying interspecific as well as intraspecific ancient DNA

(aDNA) from archaeological materials provides valuable information for palaeobotany, ethnobotany, population genetics, and phylogenetics, which may enhance our knowledge of past evolutionary processes, domestication, and social life (Tani et al. 2003; Gugerli et al. 2005; Nasab et al. 2010; Özgen et al. 2012). The presence of aDNA in charred seeds was first reported in the early 1990 s (Allaby et al. 1994). Allaby et al. (1997) and Brown (1999) reported only one charred grain with amplifiable DNA in twenty archaeological samples owing to the low amount and quality of DNA. Paabo et al. (2004) demonstrated that hydrolytic and oxidative damage will degrade aDNA to short fragments no longer than 200 nucleotides. Selecting the appropriate molecular markers in ancient DNA study is essential to address a particular question in the use of highly degraded DNA. Because the extracted DNA from most ancient plant samples is generally degraded, DNA amplification studies based on cpDNA, mtDNA, and ribosomal DNA sequences that are of small size and high copy number per cell produce the best results with more reliable amplification (Gugerli et al. 2005). Ancient DNA studies in archaeology are now using more and more robust techniques, resulting from rapid changes in the development of "next generation" sequencing (NGS) methods in archaeological specimens (Palmer et al. 2012; Bunning et al. 2012; Nistelberger et al. 2016; Mascher et al. 2016). These methods offer powerful ways to explore biological materials from excavations in detail and to answer unclear and new archaeobotanical and archaeological questions.

The region of the 26S rDNA gene sequence, which meets the important criteria of more reliable amplification of ancient DNA with small size and high copy number per cell, is widely used because of its fast evolving character (Kuzoff et al. 1998). Early aDNA studies from fossils and archaeological remains indicated that the authenticity of aDNA and its degradation were major concerns (Deguilloux et al. 2003; Tani et al. 2003; Gugerli et al. 2005; Rogers and Kaya 2006; Özgen et al. 2012). Thus, in aDNA studies, it is important to have contamination-free material as well as less degraded DNA (Rogers et al. 2004; Rogers and Kaya 2006). Fruitful collaboration between archaeologists and geneticists is thus essential in aDNA studies, throughout the processes of excavation, sample recovery and preparation, and interpretation of results.

Turkey is rich in archaeological sites that provide ample animal and plant materials for aDNA studies. Mikic (2016) reported archaeological findings that confirm the importance of vetches in the primeval agriculture of Europe and its adjacent regions. Remains from archeological sites such as Neolithic Çatalhöyük and Bronze Age Arslantepe indicate, for example, that chickpea and bitter vetch were important cultivated legumes in ancient times (Sadori et al. 2006; Fairbairn et al. 2007). The oldest wheat DNA sequenced to date (approximately 8400 years old) comes from a seed from Çatalhöyük and demonstrated genetic connections to early wheat in the Fertile Crescent (Bilgiç et al. 2016). Mascher et al. (2016) reported considerable genetic overlap between ancient seeds and present-day domesticated lines from the region.

Recent studies suggest, however, that the Fertile Crescent is not the only region of domestication in the eastern Mediterranean. Studies of ancestral connections reveal that populations in western Anatolia show closer relations with those in Europe, rather than communities along the eastern Mediterranean Levant (Kılınç et al. 2016). Brown et al. (2008) also demonstrate that human activities occurring concurrently in the arc of the Fertile Crescent have resulted in complex evolutionary trajectories. The Late Bronze Age in western Anatolia, roughly 1700/1650–1200 B.C.E., represents an auspicious period in Anatolian cultural history. The leaders of the central Anatolian Hittite state vied for control of territories in western Anatolia, especially those with access to trade routes and diplomatic connections to the coveted areas of the Aegean, Levant, and Egypt (Bryce 2005). One of the most important nodes of transportation between the central Anatolian plateau and the Aegean coast was (and remains) the Gediz Valley (Fig. 1). The Marmara Lake basin of the middle Gediz Valley contained a number of small settlements from the beginning of the Middle Bronze Age (2000–1700/1650 B.C.E.) through the end of the Late Bronze Age (Luke et al. 2015). Kaymakçı was the largest among these settlements (Roosevelt et al. 2018; Luke and Roosevelt 2017; Roosevelt and Luke 2017).

This archaeological evidence corroborates understandings of historical geography known from written records. This region is the best candidate for the Seha River Land mentioned in Hittite texts, primarily from

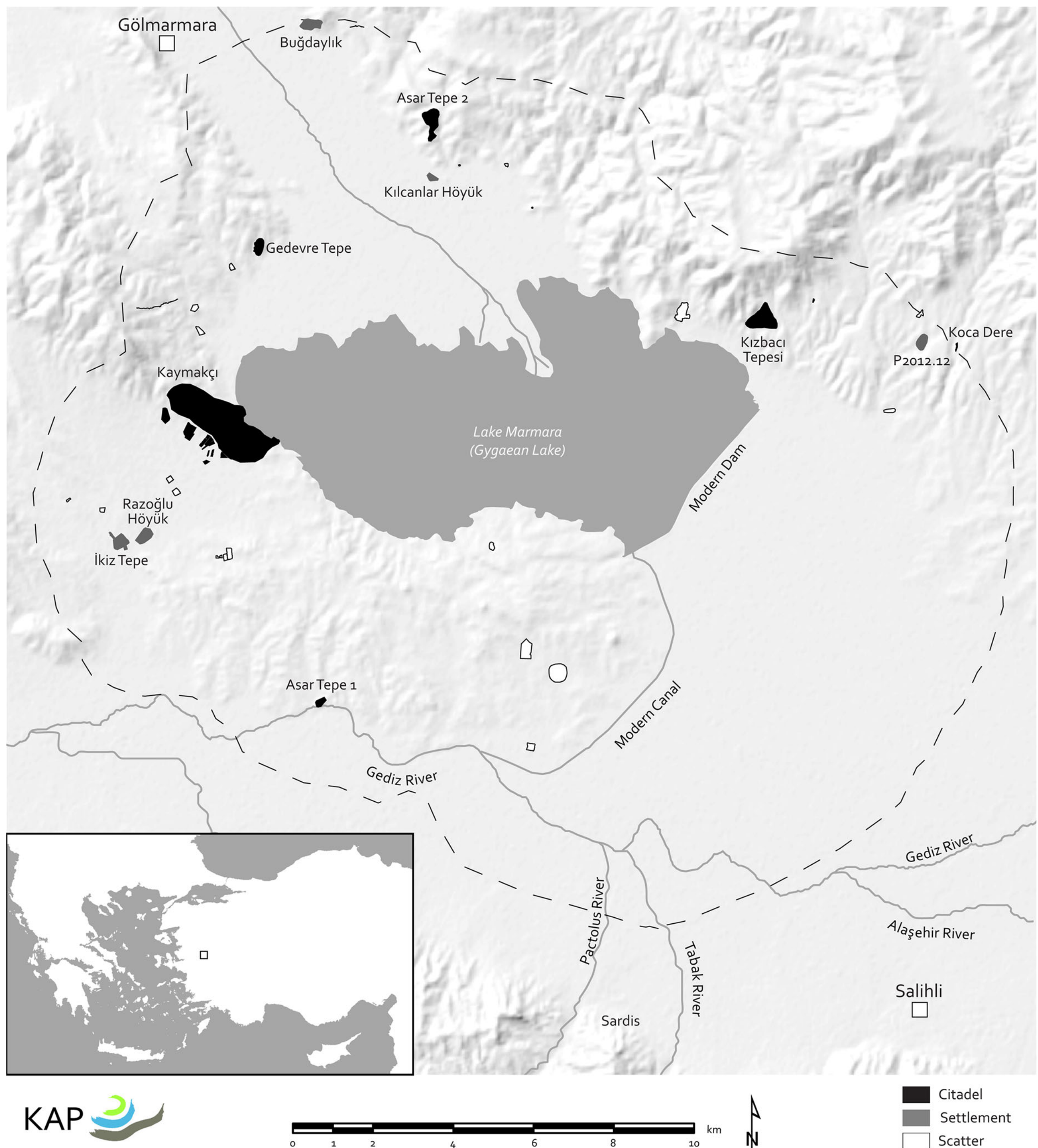


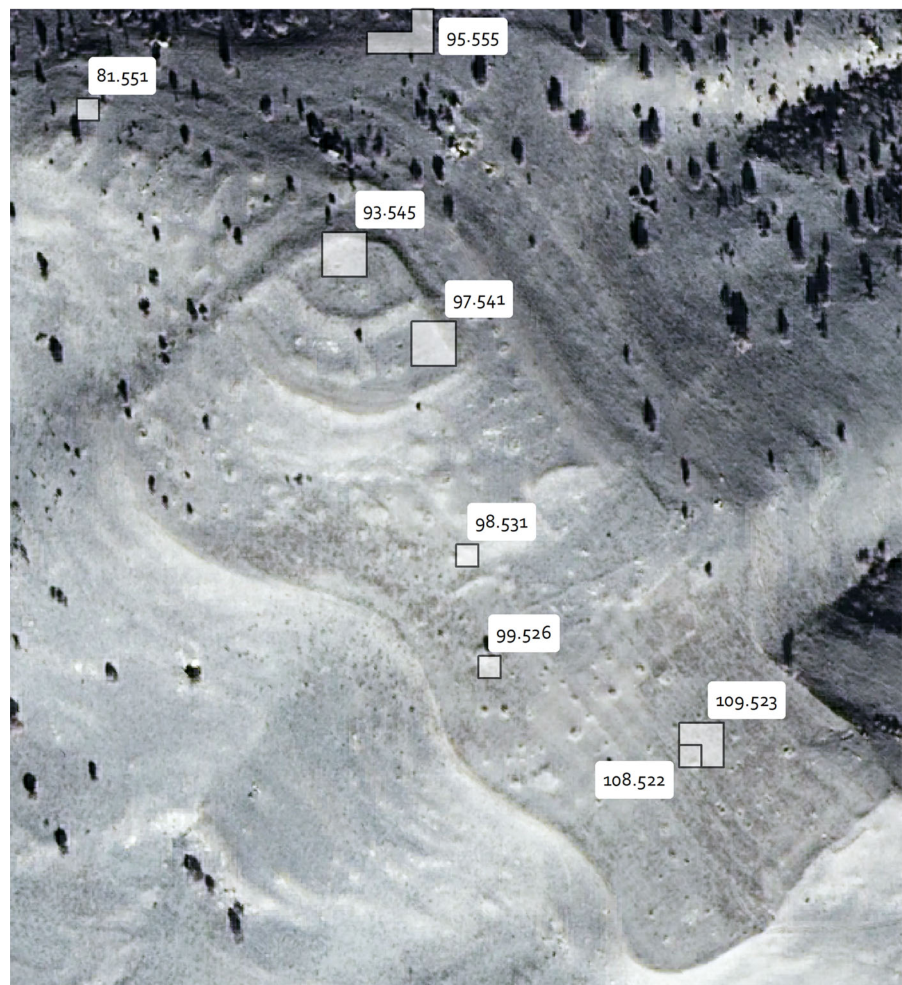
Fig. 1 Map showing the location of Kaymakçı and contemporary sites in the Marmara Lake basin of the Gediz River valley. Inset shows location in western Turkey (Courtesy of the Kaymakçı Archaeological Project)

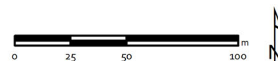
the 14th and 13th centuries B.C.E. (Hawkins 1998). Ceramic analyses and Accelerator Mass Spectrometry (AMS) radiocarbon dating of charcoal and seeds from Kaymakçı confirm activities at the site throughout the Middle Bronze Age (MBA) and Late Bronze Age (LBA). In fact, we can now further refine subphases of the LBA into at least two phases: Late Bronze (LB) 1 (17th–15th centuries) and LB 2 (14th–13th centuries). During this period, mixed agro-pastoral lifeways sustained many communities, yet the inhabitants of

larger settlements, and certainly citadels, practiced intensive agriculture of certain crops.

Excavations at Kaymakçı since 2014 (Fig. 2) have resulted in the discovery of houses, alleys, courtyards, and semi-subterranean circular features hewn into the natural rock and/or earth (Roosevelt et al. 2018). These latter features likely functioned as grain silos, as demonstrated at many contemporary sites in central and western Anatolia. Analysis of botanical samples from 263 archaeological contexts at Kaymakçı points to cultivation of crops for human consumption and probably also for animal husbandry (Roosevelt et al. 2018). Cultivation of cereals is indicated by barley (*Hordeum vulgare* L.), free-threshing wheat (*Triticum aestivum/durum*), emmer wheat (*Triticum turgidum* spp. *dicoccum* (Schrank) Thell.), and einkorn wheat (*Triticum monococcum* L.). In addition, abundant evidence of legumes includes bitter vetch (*Vicia ervilia*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris* Medik.), and grass pea (*Lathyrus sativus* L.). The only evidence of fruit cultivation is that of grape (*Vitis vinifera*). This dataset is similar to that known from contemporary sites in western and central Anatolia such as Troy and Gordion (Shin et al., in review; Fig. 3).

In the current study, palaeogenetic analysis of aDNA extracted from seeds of four plant species retrieved from Kaymakçı were carried out comparatively with contemporary DNA from the same species. The study aims to test the following: (1) if it is possible to extract good quality DNA successfully





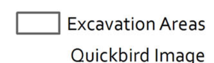


Fig. 2 QuickBird satellite image showing the locations of excavation areas at Kaymakçı (after Roosevelt et al. 2018)

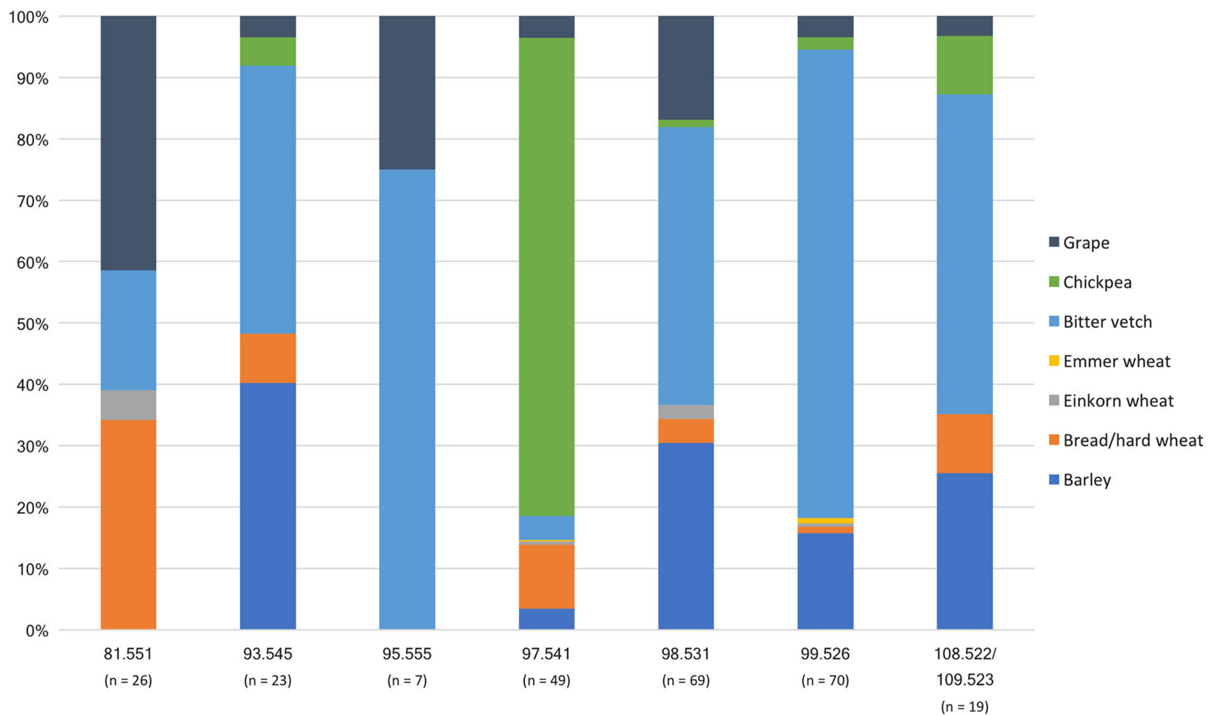


Fig. 3 Proportions of crop seeds recovered from excavations at Kaymakçı (after Roosevelt et al. 2018)

from charred seeds, (2) if the ancient plant specimens are identified correctly on the basis of morphological examination of charred seeds, (3) if the genetic composition of the 3500 year old ancient crop specimens compared to their contemporary counterparts changed with respect to 26S rDNA gene, and (4) if observable changes vary among studied ancient specimens as well as among ancient specimens and their contemporary counterparts. To address these issues, preserved portions of nuclear 26S rDNA gene sequences from both ancient seeds and their contemporary relatives were studied comparatively, and here we present these preliminary results.

Materials and methods

Botanical remains and contexts

Hundreds of ancient seeds have been recovered from Kaymakçı, yet only seven were chosen for this first study on aDNA (Table 1). The selected seeds were embedded in sealed archaeological stratigraphic contexts of associated ancient cultural sediment and carefully excavated cm by cm. Since Kaymaçı is positioned at a high elevation well above the shores of Lake Marmara, they were not exposed to wetland conditions. Furthermore, the stratigraphy of archaeological contexts atop the Kaymakçı ridge created favorable conditions for their preservation; the buried contexts were not directly exposed to external humidity and annual temperature shifts. Seeds were chosen from different contexts from two excavation areas, both with good preservation and relatively secure dating within the local Late Bronze Age (LBA) (see Roosevelt et al. 2018). With the exception of one seed (cf. *Vicia ervilia*), seven of the eight specimens retained enough of their plant structure to be identified to the species level. All seeds were fully carbonized excluding two pulse seeds (*V. ervilia* and cf. *V. ervilia*), which showed possible signs of partial carbonization (i.e., almost all, if not all, of each seed was carbonized) (Fig. 4).

Excavation area 97.541 at Kaymakçı (Fig. 5) is located on the lower, outer terrace of the inner citadel. Excavations here revealed the remains of at least seven circular features and three building complexes dating to the LB phases of the site. Among other remains, a grape seed (sample no: 8; 97.541.158.1) was recovered from a fill deposit located just inside the wall of

Table 1 **a** Ancient DNA sources. **b** Contemporary DNA sources

Codes	Sample number	Taxon	Weight (g)	Fraction size (mm)	Date	Age of the seeds
(a) Ancient DNA sources						
a-VE1	99.526.572.2	cf. *Vicia ervilia*	0.003	> 2	LB 1	
a-VE2	99.526.572.2	*Vicia ervilia*	0.009	> 2	LB 1	
a-CA1	97.541.118.5	*Cicer arietinum*	0.039	> 2	LB 2	
a-CA2	97.541.330.5	*Cicer arietinum*	0.032	> 2	LB 1	
a-VV1	99.526.178.2	*Vitis vinifera*	0.011	> 2	LB 1–2 transition	
a-VV2	97.541.158.1	*Vitis vinifera*	0.009	> 2	LB 1 or LB 2	
a-TA or a-TD	99.526.570.1	*Triticum aestivum/durum*	0.011	> 2	LB 1	

Codes	Taxon	Cultivar name	Source provider
(b) Contemporary DNA sources			
c-VE	*Vicia ervilia*	Farmer variety	Ankara University, Faculty of Agriculture, Ankara
c-CA	*Cicer arietinum*	Gökçe	Ankara University, Faculty of Agriculture, Ankara
c-VV	*Vitis vinifera*	Cultivar Chardannay	Ankara University, Faculty of Agriculture, Ankara
c-TA	*Triticum aestivum*	Bezostaja-1	Ankara University, Faculty of Agriculture, Ankara
c-TD	*Triticum durum*	Kunduru 1149	Ankara University, Faculty of Agriculture, Ankara

the northeastern building complex dating to late in the LB 1 phase or to the LB 2 phase. One of the chickpea seeds (sample no. 7; 97.541.330.5) was found in a contemporary fill deposit located in a lower level of the alley-like space between the northeastern and southeastern building complexes. The other chickpea seed (sample no. 3; 97.541.118.5) was recovered from a higher-level fill deposit in the same alley-like space and probably dates to later in the LB 2 phase.

Excavation area 99.526 at Kaymakçı (Fig. 6) is located near the southwestern edge of the southern terrace. Because of the depth of stratigraphy, this area exhibits the best preservation of botanical remains at the site. Current data suggests a LB 1 pre-architectural phase of use in this area before several later phases marked by the construction of buildings. The bitter vetch (sample nos. 1–2; 99.526.572.2) (sample no. 6; 99.526.572.2) seeds were recovered from a surface dating to the LB 1 phase. The wheat seed (sample no. 5; 99.526.570.1) was found in a contemporary fill deposit. The grape seed (sample no. 4; 99.526.178.2) comes from a later fill deposit dating to the earliest architectural levels of the area, at the transition between the LB 1 to 2 phases.

aDNA and contemporary DNA extraction and quantification procedures

DNA extractions from ancient seeds of four plant species (*V. ervilia, C. arietinum, Vitis vinifera, Triticum aestivum/ T. durum*) were performed with two different methods. These were the following: (1) the modified Cetyltrimethylammonium bromide (CTAB) method modified by Kistler (2012), and (2) the commercial *High Pure PCR Product Purification Kit* method (Roche). The latter was used successfully by Lister et al. (2008) and Oliveira et al. (2012) for DNA extraction from archeological wheat grains. DNA isolation and PCR studies were carried out in a specially designed and reserved laboratory for ancient DNA studies, where no previous study related with DNA isolation or Polymerase Chain Reaction (PCR) application to any materials had been conducted. Laboratory areas were cleaned with 20% bleach and absolute ethanol and DNA AWAY™ Surface Decontaminant solutions (Molecular Bioproducts, Inc. San Diego, CA). Prior to the DNA extraction, isolation equipment and suitable chemicals were sterilized with an autoclave. When starting isolation, materials were thoroughly cleaned with 70% ethanol and DNA

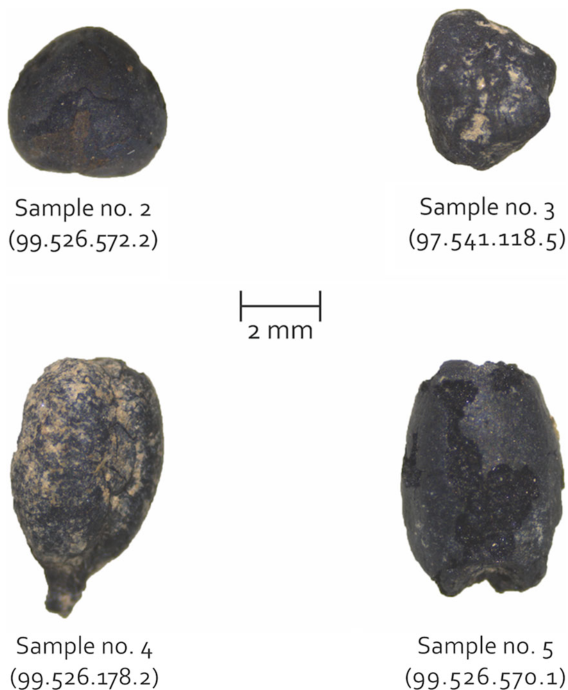


Fig. 4 Photographs of a selection of ancient seeds sampled for aDNA: *V. ervilia* (sample no. 2; 99.526.572.2), *C. arietinum* (sample no. 3; 97.541.118.5), *Vitis vinifera* (sample no. 4; 99.526.178.2)), and *T. aestivum/T. durum* (sample no. 5; 99.526.570.1) (Courtesy of the Kaymakçı Archaeological Project)

Fig. 5 Overhead view of excavation area 97.541 at Kaymakçı (north at top); White squares are 1 × 1 m on a side (Courtesy of the Kaymakçı Archaeological Project)

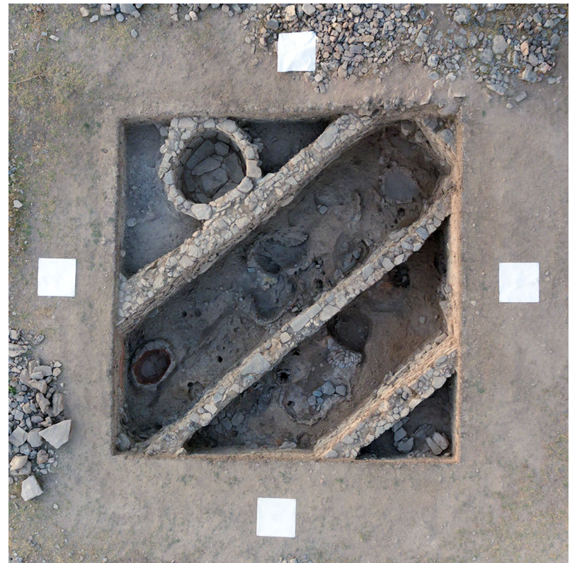


Fig. 6 Overhead view of excavation area 99.526 at Kaymakçı (north at top); White squares are 1 × 1 m on a side (Courtesy of the Kaymakçı Archaeological Project)

AWAY™ Surface Decontaminant solutions. To avoid possible contamination of ancient DNA from the lab environment, all equipment used for DNA isolation and PCR were subjected to UV treatment. Important precautions such as protective clothing, double gloving, and face masks were implemented while conducting the ancient DNA work. Frequent treatment with bleach, UV-irradiation, and changes of gloves were carried out during the experiment.

To understand the fidelity of two different DNA isolation procedures, the DNA of one ancient *Vitis vinifera* seed was isolated according to Kistler' modified CTAB method, and the DNA of the other *Vitis vinifera* seed was isolated via the commercial kit method (*High Pure PCR Product Purification Kit*, Roche), following the manufacturer's instructions. Comparison of the concentrations of isolated DNA from the two methods were undertaken after quantifying DNA amounts by spectrophotometer (Biodrop μLite 7141 V.1.0.4, Biological Sciences Department, METU); the results revealed that the commercial kit method yielded higher concentrations of DNA. Subsequently, DNA extraction from all remaining ancient seeds was performed using the *High Pure PCR Product Purification Kit* method (Roche). Extraction blanks were used as a negative control for DNA extraction of each seed.

Because the isolation of ancient DNA from ancient seeds produced still generally low concentrations of representative DNA, the *Illustra GenomiPhi HY DNA Amplification Kit* (Amersham, GE Healthcare, and UK) was used to increase the amount of whole genomic DNA from the ancient seeds, again following the manufacturer's instructions. And, once again, the concentration and quality of obtained ancient DNA and amplified genomes were determined with a spectrophotometer (Biodrop μLite 7141 V.1.0.4, Biological Sciences Department, METU).

To enable comparative sequence analysis of the ancient seeds with contemporary seeds using the 26S rDNA gene, DNA extractions were also carried out on seeds of contemporary *Triticum durum*, *T. aestivum*, *V. ervilia*, *C. arietinum*, and leaf tissues of contemporary *Vitis vinifera* obtained from the Field Crop Department of the Faculty of Agriculture, Ankara University. DNA extractions of the contemporary seeds also followed the same commercial kit methods described above except for the contemporary *Vitis vinifera*. Ancient DNA extraction from ancient seeds and contemporary DNA from young leaves of germinated *Vitis vinifera* seeds were conducted via the DNeasy Plant Mini Kit (QIAGEN, Germany), also following the manufacturer's instructions. Because two ancient seeds were available from *Vitis vinifera*, the *High Pure PCR Product Purification Kit* (Roche), and the *DNeasy Plant Mini Kit* (Qiagen) methods were initially compared for yield and quality of DNA extraction from ancient and contemporary seeds. After assessment of both kits, only the *High Pure PCR Product Purification Kit* method (Roche) was used in all further DNA extraction experiments due to good DNA yield and quality, but the ancient and contemporary DNA extracted from *Vitis vinifera* leaves were kept for use in further PCR amplification. To avoid contamination, both sets of contemporary DNA extractions were conducted in a laboratory that was separate from the one used for ancient DNA extractions.

Amplification of the 26S rDNA region

For the PCR technique, 26S rDNA primers synthesized by BM Labosis (Çankaya, Ankara) were used to amplify conserved fragments of nuclear rDNA genes both from the ancient DNA of the Kaymakçı seeds and from the contemporary DNA from the contemporary seeds described above. The sequences of the primers are 26S forward: 5'-*ttcccaaacaacccgactc*-3' and 26S reverse: 5'- *gccgtccgaattgtagtctg*-3'. Amplified sequences with 26S rDNA primers obtained from this process give information about phylogenetic relationships among species of a given genus (Alvarez and Wendel 2003).

The amplified whole genomes were diluted 50 times to be used for PCR reactions to prevent inhibition of amplification as a result of high amounts of template DNA. Each reaction was carried out in a 25 μl volume, consisting of 5 μl HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.5 μl of 200 nM forward and reverse primers, 5 μl template DNA diluted as 10 ng, and 14 μl water. To detect any contamination, negative and positive controls were performed. The negative control contained all PCR components except the template DNA, while the positive control included a distant plant species (*Salix alba*). The PCR protocol used for ancient and contemporary seeds was one cycle at 95 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 60 °C (Ta) for 30 s and at 72 °C for 1 min, following a final extension at 72 °C for 10 min.

PCR products were run on 3% agarose gels at 100 V for 45 min in electrophoresis. The bands were visualized under UV light (Vilber Lourmat, France) by considering bands of a low range DNA ladder (Fermentas, Generuler, EU). PCR products with the expected length of bands were sent for sequence analysis. The purification and sequencing procedures were performed by the BM Labosis (Çankaya, Ankara). An ABI 310 Genetic Analyzer (PE applied Biosystem) and ABI3730XL 96 capillary automatic sequencer were used for sequencing of amplified forward and reverse DNA products to get accurate sequences of ribosomal DNA. The chromatogram data visualization, BLAST search (Altschul et al. 1990), and CLUSTAL alignment (Thompson et al. 1994) were performed with MEGA 7.0.2 Software (Kumar et al. 2018). To identify homologies, the DNA sequences of all ancient and contemporary seeds were compared with the sequences of the same species from the NCBI database by BLAST analysis. The accession numbers of the contemporary seeds from different studies used for comparison in this study are available on Genbank and provided in Table 3.

Phylogenetic and molecular diversity parameters such as total nucleotide length (bp), GC content (%),

nucleotide deletion and insertion (indel), preserved and variable sites, parsimony informative sites, and nucleotide diversity of sequences were estimated comparatively between ancient, contemporary, and BLAST aligned DNA sources with the MEGA 7.0.2 Software (Kumar et al. 2018).

Results

Among the tested DNA extraction methods, the commercial kit method yielded higher concentrations of DNA than did the modified CTAB method. No DNA contamination from other sources was detected. The amount of recovered DNA concentration of seeds prior to the use of the *Illustra* amplification kit is given in Table 2.

The genomes of all seeds were amplified using the Illustra GenomiPhi HY DNA Amplification Kit (Amersham, GE Healthcare, UK). The results indicated that high concentrations of aDNA from the ancient seeds were obtained (Table 2). By using the amplified genomic DNA from ancient seeds as template DNA, the 26S rDNA regions were amplified successfully for both ancient and contemporary seeds. The expected band sizes of the amplified 26S rDNA regions for ancient and contemporary DNAs are presented in Fig. 7.

The ancient and contemporary sequences obtained from the chromatogram data were checked with a BLAST search to determine the genetic similarities between ancient seeds and their contemporaries. The sequences of the 26S rDNA gene, which are available from the NCBI database for the species in the current study, were also comparatively analyzed. The results of the comparison of ancient DNA sequences with the sequences from databases are given in Table 3, highlighting the likelihood of identity correctness given as a percentage. Sequence data for the respective ancient and contemporary seeds have been uploaded to the NCBI database to be accessible. The Genbank accession numbers for these samples are provided in Table 5.

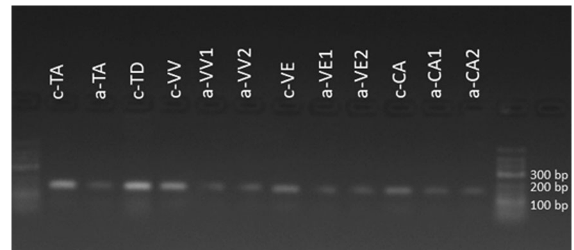


Fig. 7 Amplified band patterns of the 26S rDNA region for ancient and contemporary seeds. See Table 1 for codes

First, the 26S rDNA sequences of contemporary species of this study were compared with sequences of the same species available from the NCBI. The comparison indicated very high homology, as expected. Then, the 26S rDNA sequences of the ancient seeds were compared with the sequences of the same species available from the NCBI. The 26S rDNA ancient sequences also showed high levels of homology with the sequences from their contemporary species.

After the alignment of the sequences of the ancient seed morphologically identified as either *T. aestivum* or *T. durum* and its contemporaries, the length of the 26S rDNA region was found to be about 150 base pairs

Table 2 DNA concentrations of ancient seeds

Codes	Species	DNA concentration of seeds prior to the use of the *Illustra* amplification kit (µg/ml)	230/260 260/280 absorbance ratio	DNA concentration of negative control	DNA concentration of seeds after genome amplification by use of the *Illustra* amplification kit (µg/ml)	230/260 260/280 absorbance ratio
a-VE1	*cf. Vicia ervilia*	2	0.5/3.00	0	512	2.24/1.83
a-VE2	*Vicia ervilia*	28	0.62/2.75	0	601	2.13/1.79
a-CA1	*Cicer arietinum*	4	1/− 2.00	0	569	2.10/1.77
a-CA2	*Cicer arietinum*	2	0.78/− 2.00	0	526	2.00/1.72
a-VV1	*Vitis vinifera*	17	0.67/1.78	0	613	2.15/1.81
a-VV2	*Vitis vinifera*	4	0.72/1.82	0	537	2.09/1.78
a-TA/ a-TD	*Triticum aestivum* or *Triticum durum?*	14	0.91/2.00	0	647	2.24/1.85

Table 3 Ancient DNA samples, Genebank accession numbers of aligned sequences retrieved from the NCBI databases, and the results of identity comparisons. **a** Identity comparison of the 26S rDNA sequences of ancient seeds with the sequences from the database. **b** Identity comparison of the 26S rDNA sequences of contemporary seeds (or tissues) with the sequences from the database

Name of species having alignment with ancient seed	Genebank accession number of aligned species	Query cover (%)	E value	Identity (%)
(a) Identity comparison of the 26S rDNA sequences of ancient seeds with the sequences from the database				
a-TA versus *Triticum aestivum*	AY049041.1	98	1e−57	93
a-VE versus *Vicia villosa*	KT459256.1	96	2e−64	96
a-VE versus *Vicia faba*	X17535.1	90	3e−59	96
a-CA versus *Cicer arietinum*	XM_0127134181.1	96	2e−55	95
a-VV versus *Vitis vinifera*	AM474244	98	3e−62	97
(b) Identity comparison of the 26S rDNA sequences of contemporary seeds (or tissues) with the sequences from the database				
c-TD versus *Triticum aestivum*	AY049041.1	99	3e−67	98
c-VE versus *Vicia villosa*	KT459256.1	96	1e−75	99
c-VE versus *Vicia faba*	X17535.1	90	2e−70	99
c-CA versus *Cicer arietinum*	XM_0127134181.1	97	9e−73	99
c-VV versus *Vitis vinifera*	AM474244	100	2e−76	100

with 68.9% guanine-cytosine (GC) content. The nucleotide diversity, defined as the average number of nucleotide differences per site between sequences, of the *Triticum* sequences was found to be 0.031. The number of variable sites between sequences of ancient *Triticum* and its contemporaries (c-TA, c-TD, and aligned Blast sample AY049041.1) were found to be 10. Substitutions were observed at base positions 21, 22, 26, 38, 55, 56, 75, 87 113 and 126 (Table 5). Two of these variable sites were parsimony informative (Table 4). According to parsimony informative sites found at base positions 21 and 113, the ancient *Triticum* seed can be identified as *T. aestivum* (Table 5); the sequence of ancient and modern *T. aestivum* DNA in these two positions includes the same base (Cytosine), while *T. durum* contains a different base (Thymine). When the variable sites of ancient and contemporary *T. aestivum* (c-TA) sequences were compared, four substitutions were seen at the base positions 22, 38, 55, and 56. In addition to one Adenine insertion event at the base position 78, three base deletions were present at the base positions 75, 87, and 124 in the contemporary *T. aestivum* (c-TA) sequence compared to the ancient *T. aestivum* sequence (Table 5).

The sequences of 26S rDNA for two ancient *Vicia* seeds were found to be same. When comparing the sequences from ancient *Vicia* seeds (a-VE1, a-VE2) with its contemporaries (c-VE and aligned Blast samples KT459256.1, KT459255.1, X17535.1), the length of the 26S rDNA was 152 bases long with 67.3% GC content. Nucleotide diversity was found to be 0.014. None of the six variable sites for six *Vicia* sequences were parsimony informative (Table 4). One base deletion in the studied *Vicia* sequences occurred at the 78th base position. Adenine base was absent in this position in the ancient *Vicia* sample compared to contemporary samples of species (Table 5).

Two ancient *Cicer* seeds have the same sequence of the 26S rDNA region. The length of the studied region was found to be 153 base pairs long with 67.1% GC content for two ancient *C. arietinum* seeds and its contemporaries (c-CA and aligned Blast sample XM_0127134181.1). Nucleotide diversity for four sequences was found to be 0.018. Four variable sites among the *Cicer* sequences were found, but none of these were parsimony informative (Table 4). Three deletion events were observed in the sequences of contemporary *Cicer* seeds at base locations 78, 114, and 128 when compared to ancient *Cicer* seeds (Table 5).

The 26S rDNA region has the same sequence for two ancient *Vitis* seeds. The length of the 26S rDNA region was determined as 152 bases long with 68.6% GC content for the ancient seeds morphologically identified as *Vitis vinifera* and their contemporaries.

Table 4 Estimated molecular diversity parameters based on 26S rDNA sequences of ancient seeds from Kaymakçı and contemporary seeds of the same species

	Triticum a/d	*Vicia ervilia*	*Cicer arietinum*	*Vitis vinifera*
Number of 26S rDNA sequences*	4	6	4	4
Sequence length (bp)	150	152	153	152
GC content (%)	68.9	67.3	67.1	68.6
Conserved sites	140	146	146	148
Variable sites	10	6	4	1
Parsimony informative sites	2	0	0	0
Number of indels	5	1	3	3
Nucleotide diversity	0.031	0.014	0.018	0.004

*The "number of sequences" include ancient and contemporary examples of the same species and the 26S rDNA sequences of the same species or close relatives of the studied species available from the NCBI database

Estimated nucleotide diversity was estimated as 0.004. Only one variable site was present, but it was not parsimony informative (Table 4). The base substitution was observed at the 22nd base position (Table 5). Three bases were found to be deleted in the sequences of contemporary *Vitis* (c-VV and aligned Blast sample AM474244). These deletions were located at the base positions 78, 114, and 128, where *Vitis* contemporaries lost the bases present in the ancient *Vitis* (Table 5).

Discussion

The majority of ancient plant DNA studies have been performed with generally cultivated species found in archaeological sites (Gugerli et al. 2005). Several studies describe the isolation, amplification, and analysis of DNA from charred seeds of wheat, barley, chickpea, bitter vetch, and grape from archaeological sites in Europe (Cappellini et al. 2010; Jovanovic et al. 2011; Li et al. 2011; Bilgiç et al. 2016; Mascher et al. 2016; Medovic et al. 2011; Mikic et al. 2015). Because of different environmental eroding factors, such as sun, rain, wind, and frost, the quantity and quality of DNA from charred ancient seeds are typically low and variable, rendering them unsuitable for the extraction of amplifiable DNA. In the current study, extraction of high quality and good amounts of aDNA was obtained. Also, amplification of 26S rDNA from charred seeds was successfully carried out. The reasons for successful DNA extraction and amplification are likely due to (1) the good preservation of certain ancient seed specimens and (2) the use of improved DNA extraction and amplification methods.

The site of Kaymakçı was abandoned by its community and no other community permanently settled in this location after the Late Bronze Age. For this reason, the deepest stratigraphic contexts remained undisturbed by later cultural activities until excavations began. Through careful excavation strategies, following a born-digital protocol developed for Kaymakçı, and among the only such recording systems in Turkey (Roosevelt et al. 2015), seeds were excavated from their exact contexts, usually indicated by changes in soil color and texture. This is done through a process of context-by-context removal of soil and then screening of the sediment and final identification in a laboratory. In some cases, archaeological contexts with botanical remains are found in proper storage containers, such as ceramic jars or pits, yet in the case of Kaymakçı, the charred botanical remains were embedded in the daily contexts of domestic spaces (i.e., houses, walkways, and potential ovens) and the depositional fills that covered them (Roosevelt et al. 2018).

Furthermore, the high ground of the site of Kaymakçı, along a ridge top, and the pristine condition of the site contributed to the good preservation of the studied seeds and their genetic materials. Desiccation plays an important role for the preservation of DNA. The relatively low humidity and high temperature in western Turkey is certainly a factor for preservation, yet most important for Kaymakçı is the carbonized nature of the seeds and the undisturbed

Table 5 Sequence alignments of the ancient seeds and their contemporaries

Triticum Spp.	1	20	21	22	25	26	36	38	51	52	55	56	57	75	76	77	78	87	113	114	120	124	126	128	145	147
a-TA/D (MK413185)	T	G	C	A	C	A	G	T	G	G	A	C	G	T	C	C	_	C	C	C	G	G	T	C	G	C
AY049041.1(Blast)	.	.	T	T	.	G	.	G	.	.	C	G	.	C	.	_	_	T	T	.	.	G	C	.	.	.
c-TA (MK413186)	.	.	C	T	.	A	.	G	.	.	C	G	.	_	.	C	A	_	_	.	.	_	.	.	.	.
c-TD (MK413187)	.	.	T	T	.	A	.	G	.	.	C	G	.	_	.	C	A	_	T	.	.	G	.	.	.	.
Vicia spp.	1	20	21	22	25	26	37	38	51	52	55	56	57	75	76	77	78	87	113	114	120	124	126	128	145	147
a-VE1 (MK413188)	T	G	C	A	C	A	A	T	G	G	A	C	G	T	C	C	A	C	C	C	G	G	T	C	G	C
a-VE2 (MK413189)			.	A	.	.	A	.	.	G	.	.	G	.	.	.	A	.	.	.	G	.	.	.	G	.
KT459256.1(Blast)	.	.	.	C	.	.	G	.	.	A	.	.	A	.	.	.	_	.	.	.	G	.	.	.	A	.
KT459255.1(Blast)	.	.	.	C	.	.	G	.	.	A	.	.	A	.	.	.	_	.	.	.	G	.	.	.	A	.
X17535.1	.	.	.	C	.	.	G	.	.	A	.	.	A	.	.	.	_	.	.	.	A	.	.	.		
c-VE (MK413190)	.	.	.	C	.	.	G	.	.	A	.	.	A	.	.	.	_	.	.	.	G	.	.	.	G	.
Cicer Spp.	1	20	21	22	25	26	36	38	51	52	55	56	57	75	76	77	78	87	113	114	120	124	126	128	145	147
a-CA1 (MK413191)	T	G	C	T	C	A	G	T	G	G	A	C	G	T	C	C	A	C	C	T	G	C	C	C	C	G
a-CA2 (MK413192)	.	.	.	T	.	.	.	.	.	G	.	.	G	.	.	.	A	.	.	T	.	.	.	C	.	G
XM_0127134181.1(Blast)	.	.	.	C	.	.	.	.	.	A	.	.	A	.	.	.	_	.	.	_	.	.	.	_	.	A
c-CA (MK413193)	.	.	.	C	.	.	.	.	.	A	.	.	A	.	.	.	_	.	.	_	.	.	.	_	.	G
Vitis Spp.	1	20	21	22	25	26	36	38	51	52	55	56	57	75	76	77	78	87	113	114	120	124	126	128	145	147
a-VV1 (MK413194)	T	G	C	A	C	A	G	T	G	G	A	C	G	T	C	C	A	C	C	T	G	C	C	C	C	G
a-VV2 (MK413195)	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	C	.	.
AM474244(Blast)	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	_	.	.
c-VV (MK413196)	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	_	.	.

The “_” and “.” Indicate the deleted and the same bases, respectively

contexts. Similarly, several studies reported that archaeological plant remains have preserved genomic DNA by help of desiccation, waterlogging, charring, or mineralization (Schlumbaum et al. 2008). Long-term storage capability of the studied plants with protective coverings of their seeds such as *Vicia, Vitis,* and *Cicer* are also likely a contributing factor for good preservation of DNA in charred materials.

The commercial extraction kit method and the Illustra GenomiPhi HY DNA Amplification Kit yielded not only high quality but also a respectable amount of DNA from the ancient seed specimens. Comparison of ancient DNA extraction methods in the current study demonstrates that commercial extraction kit methods improved DNA extraction compared to the modified CTAB method. It appears that commercial extraction kit methods effectively eliminated a caramel-like substance which occurs after alcohol precipitation that acts as a PCR inhibitor using the CTAB method (Oliveira et al. 2012). By using the Illustra GenomiPhi HY DNA Amplification Kit, the amplifiable amount and quality of genomic DNA were increased further such that it has been possible to study well-preserved states of aDNA in ancient seeds. This successful result will permit us to explore these archaeological materials in more depth by studying other well-preserved and informative genome regions of the species represented by the ancient seeds from a number of different archaeological contexts.

Molecular genetic studies increasingly play a more and more important role for understanding unanswered archaeological questions. Analysis of ancient DNA from archaeological plant remains and its comparison with DNA extracted from extant wild or cultivated plants have advanced our understanding of patterns of phylogenetic relationships and speciation. When studying genetic structures of any ancient species, the target sequences of ancient DNA should be short and informative for comparing the ancient species with its contemporaries. The analysis of intra-specific genetic diversity in archaeological plant remains should be performed by using multiple, well-characterized, and highly polymorphic genetic loci (Gugerli et al. 2005).

Here we have studied the highly polymorphic and well-preserved 26S rDNA region from ancient seeds and compared it with that of contemporary species. The goals of this comparison were to identify ancient specimens correctly and to determine genetic changes in ancient vs contemporary species in the 26S rDNA gene. We did not intend to get detailed information on the history of domestication and evolution of these crops. Thus, the preliminary results from the current study will contribute to archaeobotany with understanding of changes in the genomic 26S rDNA region over the last 3500 years.

For the amplified and sequenced 26S rDNA region, molecular diversity analysis revealed that the ancient seeds maintain high levels of GC content. This result indicates that there was high genomic variation in the studied sequences. For the four sequences of *Triticum* species, 10 variable sites were observed. The variable site positions in the ancient *T. aestivum* 26S rDNA sequences had correct DNA base calls and no sign of DNA degradations, especially when compared to contemporary sequences. The changes at the variable sites are not likely to have resulted from DNA degradation. Grains have undergone continual evolutionary change as a result of human interventions, notably the process of domestication, resulting in their gradual improvement as nutritional resources. By looking at the genetic distance between the modern cultivated and wild ancestors of wheat, this high rate of nucleotide substitution can be explained by intensive domestication and breeding activities over roughly 3500 years and thus provides a future baseline for comparison of ancient and contemporary samples.

Vicia and *Cicer* belong to the Fabaceae family and had four and five variable sites, respectively. The sequences of 26S rDNA from the two ancient *Vicia* seeds were found to be the same, as were those from the two ancient *Cicer* seeds. A higher number of variable sites in the sequences of *Vicia* and *Cicer* compared with the sequence of *Vitis* could be explained with reference to higher mutation rates seen in sexually reproducing plants and herbs with shorter generation times, compared to trees and shrubs which have relatively long generation times (Smith and Donoghue 2008; Baer 2008). For example, Smykal et al. (2014) extracted aDNA from carbonized pea seeds from deposits at Hissar in southeast Serbia and reported that using chloroplast DNA loci placed the ancient sample between extant cultivars and wild pea species with respect to number of variable sites (see also Mikic et al. 2015).

For ancient and contemporary *Vitis vinifera* samples, only one variable site was observed among the compared sequences. The sequences of 26S rDNA from the two ancient seeds were found to be the same. Low genomic variation between ancient specimens and contemporary ones could again be explained by the observed lower mutation rates in trees and shrubs, which have relatively long generation times compared to herbaceous plants (Smith and Donoghue 2008). Analysis of many molecular phylogenies have also revealed positive correlations between substitution rates and the number of species in studied plant families (Barraclough and Savolainen 2001; Duchene and Bromham 2013). High levels of substitution in *Vicia*, *Cicer*, and *Triticum*, which belong to the largest families of flowering plants (Fabaceae and Poaceae, respectively), compared with the *Vitis* member of the Vitaceae family, which includes fewer species, could also explain the findings of this study.

From the studied ancient specimens representing four species, it appears that directed selection and adaptation to changing environmental conditions certainly had impact on the genetic composition of contemporary species. But the domestication and breeding issues of crop species need to be further explored at Kaymakçı with different genomic regions involved in agronomic traits.

Conclusions

A large amount and good quality of genomic DNA was obtained from charred ancient seeds with the help of a commercial extraction kit method. The amplification of sequences of the 26S rDNA gene from ancient seeds demonstrates that nuclear DNA was preserved in good condition. The sequences of 26S rDNA from ancient seeds of *Triticum*, *Vicia*, *Cicer,* and *Vitis* showed high levels of homology with their contemporary relatives. The results of comparative sequence analysis revealed that the specific base locations in the ancient DNA were either lost or substituted with different DNA bases in the contemporary DNAs. The effects of continued domestication and breeding activities on these changes need to be studied further. The amplifiable DNA in the studied seeds encourages future works to be carried out with multiple, well-characterized, and highly polymorphic genetic loci involved in agronomic traits in detail. Successful genetic analysis of microsatellite loci should enable clearer understandings of the extent of genetic change (e.g., allelic loss, bottlenecks) between ancient seeds and their contemporary varieties. Integration between fragment and sequence data will provide important information about indigenous cultivation in the past and the origins and distribution of studied species. The large amount and good quality of genomic DNA obtained from charred ancient seeds allow high-throughput sequencing of ancient DNA that can generate abundant genetic data for answering important archaeobotanical and archaeological questions.

Compliance with ethical standards

Conflict of interest No known or potential conflicts of interest exist for any author.

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