GENETIC CHARACTERIZATION OF ANCIENT CHARRED WHEAT FROM THE KAYMAKÇI ARCHAEOLOGICAL SITE

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ABSTRACT

GENETIC CHARACTERIZATION OF ANCIENT CHARRED WHEAT FROM THE KAYMAKÇI ARCHAEOLOGICAL SITE

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Wheat is one of the world's most essential crops, and understanding its evolutionary history is critical for improving its yield and nutritional quality. In this study, ancient DNA (*a*DNA) from wheat remains from Kaymakçı archaeological site, Manisa, Türkiye, was analyzed and compared to modern wheat varieties to investigate the effects of domestication on evolution of wheat. The 26s rDNA and ITS2 (Internal Transcribed Spacer) regions were used to make molecular identification of the 3,500 years old charred wheat seeds, while IGS (Intergenic Spacer) region was used to determine the genome type of the charred wheat seeds. These DNA barcodes revealed previously undetermined *Triticum aestivum/durum* ancient wheat seeds were *T. aestivum*. High Molecular Weight (HMW) subunits of wheat glutenin genes were investigated in this study since these genes are essential determinants of flour quality, and understanding their evolution is important for future improvement programs.. Results of this study revealed that the HMW subunits of wheat glutenin genes in ancient samples were highly conserved and that the genetic diversity of these genes has been preserved over thousands of years. These findings underscore the potential of *a*DNA research in elucidating the genetic history of wheat in Türkiye.

Keywords: wheat, ancient DNA, agriculture, archaeobotany, plant evolution

ÖZ

KAYMAKÇI ARKEOLOJIK ALANINDAN ALINAN ANTİK KÖMÜRLEŞMİŞ BUĞDAY TOHUMLARININ GENETİK KARAKTERİZASYONU

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Buğday, dünyanın en önemli tarımsal bitkilerinden biridir ve buğdayın evrimsel tarihini anlamak; verimini ve besin kalitesini iyileştirmek için kritik öneme sahiptir. Bu çalışmada, Türkiye'deki, Manisa, Kaymakçı arkeolojik sit alanından çıkarılan kömürleşmiş buğday kalıntılarından izole edilen antik DNA (*a*DNA) analiz edilmiş ve elde edilen genetik veriler modern buğday çeşitleri ile karşılaştırılmıştır. 26s rDNA ve ITS2 (Internal Transcribed Spacer) bölgeleri, 3,500 yıllık kömürleşmiş buğday tohumlarının moleküler tanımlamasını yapmak için kullanılmıştır, IGS (Intergenic Spacer) bölgesi ise kömürleşmiş buğday tohumlarının genom tipini belirlemek için kullanılmıştır. Buğday gluten genlerinin yüksek moleküler ağırlıklı (HMW) alt birimleri, un kalitesinin temel belirleyicileridir ve bunların evcilleşme geçmişini anlamak, ıslah programlarını iyileştirmek için kritik öneme sahiptir. Bu çalışmanın sonuçları, antik buğday örneklerinde buğday glutenin genlerinin HMW alt birimlerinin genetik çeşitliliğinin binlerce yıldır korunduğunu ortaya çıkardı. Bu bulgular, *a*DNA araştırmalarının Türkiye'deki buğdayın genetik tarihini aydınlatmada potansiyelini vurgulamaktadır.

Anahtar Kelimeler: buğday, antik DNA, tarım, arkeobotany, bitki evrimi

to my family

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ABBREVIATIONS

***a*DNA**	Ancient DNA
cpDNA	Chloroplast DNA
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid Disodium Salt
ETOH	Ethyl Alcohol
GEMA	Gediz Basin Erosion Control, Afforestation, Environment, and Development Foundation
HMW	High Molecular Weight
IGS	Intergenic Spacer Region
ITS	Internal Transcribed Spacer Region
LMW	Low Molecular Weight
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
SDS	Sodium Dodecyl Sulphate
SSR	Simple Sequence Repeat
TBE	Tris Borate EDTA Buffer

CHAPTER 1

INTRODUCTION

1.1. Wheat

Wheat is a type of grass that is grown for its seeds, which are ground into flour to make a variety of food, including bread, pasta, and pastries (Xu et al., 2019). Wheat is a significant source of nutrition for people worldwide, and an important ingredient in many animal feed products (Shewry, 2009). The wide acceptability of wheat in the food industry can be attributed to the presence of gluten proteins like glutenin and gliadin which are found in the endosperm, and give the dough viscoelastic properties (Shewry et al., 2003; Sudheesh et al., 2022).

The commonly cultivated and economically significant wheat species are bread wheat, durum, einkorn, and emmer wheat shown in Fig. 1.1. Bread wheat (*Triticum aestivum*) is a very adaptable crop which grows in a wide range of climates and soil conditions due to its genome plasticity and hexaploid nature. Durum wheat (*T. durum*) is tetraploid wheat which is primarily cultivated for the production of pasta due to having more gluten protein (Faris, 2014). Einkorn wheat (*T. monococcum*) is an endemic diploid wheat species found in the Mediterranean. It has higher protein and nutrient content compared to modern wheat varieties and is used for making baked goods, particularly in artisanal products (Xu et al., 2019). Emmer wheat (*T. turgidum subsp*. *diccoccum*) is a tetraploid species and one of the earliest cultivated wheat. It has a tougher husk compared to bread wheat, which helps protect the grain. Emmer wheat is primarily used for making traditional whole-grain bread, pasta, and cereals. These traditional varieties of wheat have had limited exposure to modern breeding methods, which is why they are ancestral seeds (Suchowilska et al., 2012).

Figure 1. 1. Different wheat species. (A) Wild emmer wheat (*T. turgidum ssp. dicoccoides*), (B) domesticated emmer (*T. turgidum ssp. diccoccum*), (C) durum (*T. durum*), and (D) bread wheat (*T. aestivum*). Genome type of the wheat shown with the letters at the lower right corner (Dubcovsky & Dvorak, 2007).

1.1.1. Wheat Distribution

Wheat is the most widely cultivated cereal grain in the world (Yue et al., 2022). The leading producers of wheat in the world include China, India, the United States, Russia, and France (Fig 1.2). Wheat is grown in many other countries as well, and it is an important staple food in many parts of the world.

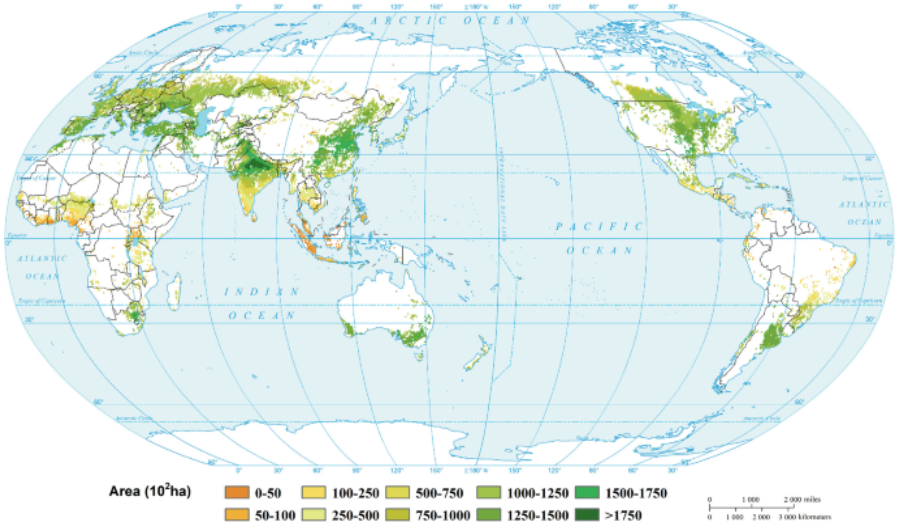

Figure 1. 2. Map of wheat distribution across the world (Yue et al., 2022)

Türkiye is also a major producer of wheat, and the crop is widely grown throughout the country (Fig 1.3). Based on data from the Turkish Statistical Institute (TUIK),

in Türkiye, compared to the 2022, wheat production increased by 11.9% to 19.8 million tons in 2023.

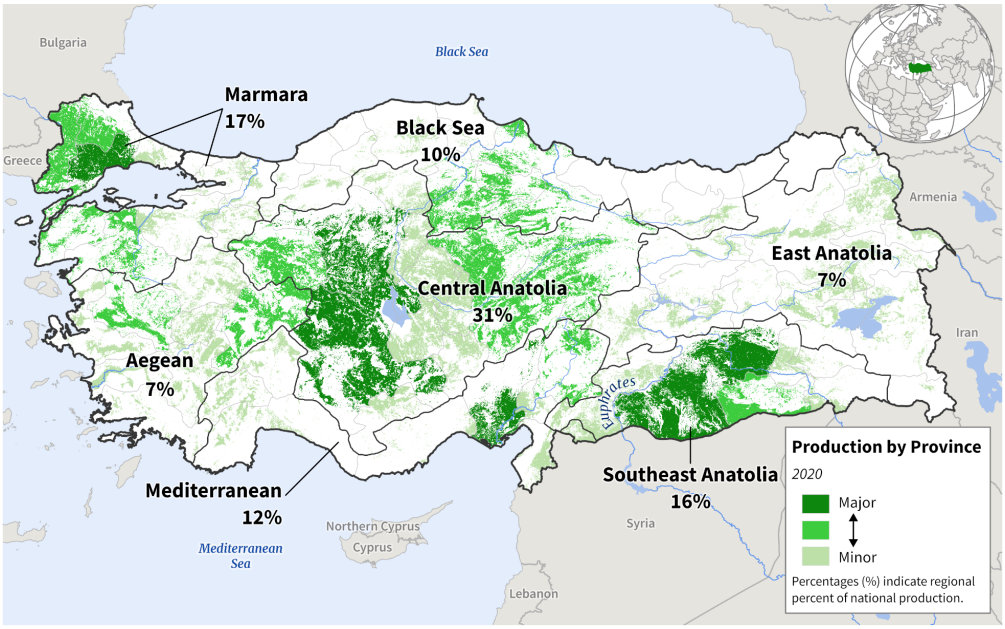

Figure 1. 3. Map of wheat production in Türkiye (Foreign Agriculture Service, 2023)

Wheat is typically adapted in regions with a temperate climate and adequate rainfall (Mediterranean climate), although it can also be grown using irrigation in dryer regions (Shewry, 2009). There are mainly three species of wheat that have been cultivated with six subspecies with a total of ninety-five morphotypes in Türkiye (Morgounov et al., 2016).

Wheat is primarily grown in the Anatolian Plateau, which is in the central and eastern parts of the country. Other significant regions include the Black Sea, the Marmara Region, and the Aegean Region. In Southern coastal regions (from Antalya to İzmir) most grown wheat is *T. durum*. In the Aegean Region farmers mainly sow *T. aestivum.* In Western Black Sea region, these two species grew nearly equal. In a few provinces, such as Bolu and Karabük, einkorn wheat is cultivated. Emmer wheat (Kavılca wheat) and Hulled einkorn (Siyez wheat) are only cultivated in Samsun Province (Morgounov et al., 2016).

1.1.2. Wheat Origin and History

Wheat is a type of grass that belongs to the plant family *Poaceae*. The genus *Triticum* includes wheat, barley, and consists of many species which have different polyploidy levels. Allopolyploidy is a process in which two different species interbreed and produce a new organism with multiple sets of chromosomes, each set inherited from one of the parent species (Feldman & Levy, 2005). Allopolyploidy in wheat occurred when different species of wheat plants hybridized as shown in Fig 1.4.

The progenitors of hexaploid wheat (*Triticum aestivum*) include three species: *T. urartu* (genome A), *Aegilops speltoides* (genome B), and *Ae. tauschii* (genome D) (Dvořák, 2001; Harris et al., 2015; Levy et al., 2022). The first hybridization happened between *Triticum* and *Aegilops* around 500,000-150,000 years ago in the Fertile Crescent and *T. turgidum* evolved, resulting in the progenitor of *T. turgidum subsp. durum* (Charmet, 2011; Pont et al., 2019; Avni et al., 2022). 10,000 years ago, the second hybridization happened between *T. turgidum subsp. dicoccum* and *Ae. tauschii* resulting in bread wheat, *T. aestivum* during the Neolithic period (Dvořák, 2001; Marcussen et al., 2014; Levy et al., 2022).

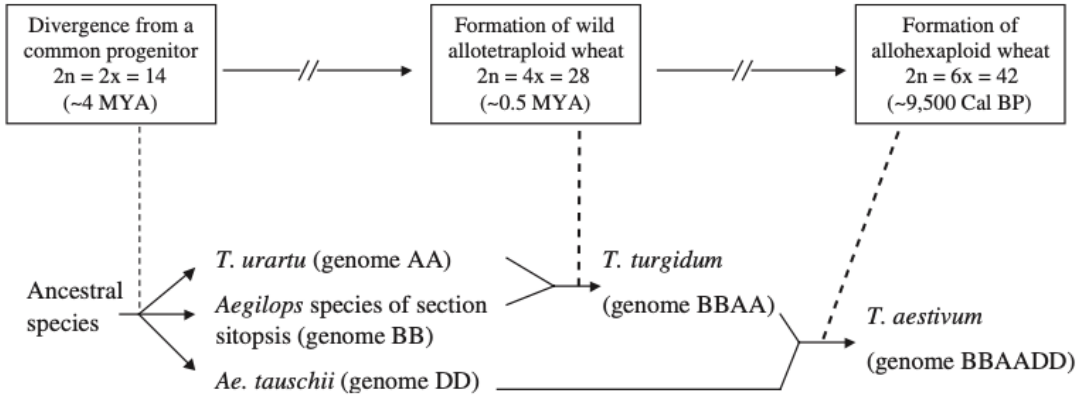

Figure 1. 4. The evolution of allopolyploid in wheat (Feldman et al., 2005)

The hybridization results in the convergence of genomes that were previously adapted to different environments into a new allopolyploid species and creates the

potential to be able to adapt to a wider range of environmental conditions than either of its parent species (*T. turgidum subsp. dicoccum* and *Ae. tauschii*) individually (Faris, 2014). The increased genetic diversity also provides a selective advantage to the *T. aestivum*, making it more resilient to changing conditions and better able to compete with other wheat species and explaining its success in today's most produced crop (Zohary, 2004; Dubcovsky & Dvorak, 2007).

The process of hybridization and polyploidization likely occurred several times in the wild, but it was later domesticated by humans in different regions around the world over time (Wang et al., 2022). There are no wild hexaploid wheat which indicates that hexaploid *T. aestivum* evolved from cultivated tetraploid wheat. The studies suggested the hybridization event occurred at least twice (Giles & Brown, 2016; Pont et al., 2019). Multiple domestication of bread wheat led to the development of many different cultivars with different characteristics, such as hard or soft wheat, spring or winter wheat, and different colors of grain.

1.1.3. Neolithic Revolution

The transition from hunting and gathering to settled agriculture occurred approximately 10,000 years ago, known as the Neolithic Revolution, in the Fertile Crescent region (Fig 1.5) of the Near East which includes the Levant to Mesopotamia and the Zagros Mountains on the border of Iran and Iraq (Brown et al., 2009). The evidence shows that multiple human populations adopted agriculture independently in this region (Gepts, 2004; Broushaki et al., 2016; Lazaridis et al., 2016). Many crop species were first domesticated in the Fertile Crescent region and later spread to surrounding regions; Europe, southern Asia, and North Africa (Gopher et al., 2001; Fuller et al., 2011; Özdoğan, 2011).

Neolithic founder cereal crops were einkorn wheat (*T. monococcum*), emmer wheat (*T. turgidum subsp. dicoccum*), and barley (*Hordeum vulgare*) (Dubcovsky & Dvorak, 2007; Brown et al., 2009; Shewry, 2009; Zohary & Hopt, 2000; Charmet,

2011; Marcussen et al., 2014; Faris, 2014). Einkorn wheat and barley were more commonly cultivated compared to emmer wheat (Fig 1.6). All of these crops shown to have a single core area of plant domestication and limited genetic variability of modern crops compared with their wild progenitors suggesting that these crops were domesticated only once (Zohary & Hopt, 2000; Lev-Yadun et al., 2000; Shewry, 2009). The studies show that domesticated emmer remained as the most cultivated crop until the Bronze Age, where *T. aestivum* replaced the emmer wheat (Dubcovsky, 2007; Marcussen, 2014; Pont et al., 2019). Additionally, genetic studies suggest that emmer wheat might be domesticated in multiple regions whereas einkorn wheat is domesticated in one place and distributed (Brown, 1999; Salamini et al., 2002).

Figure 1. 5. The map of the spread of Neolithic culture over time. Estimated arrival times indicated by numbers in BC (Broushaki et al., 2016).

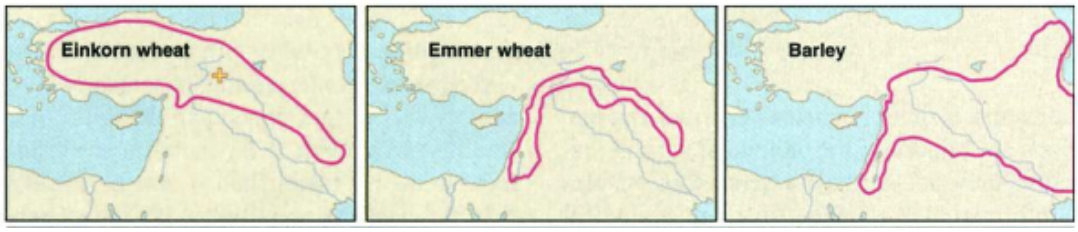

Figure 1. 6. The geographical distribution of the three Neolithic founder cereal crops; einkorn wheat (cross indicates the putative site of its domestication), emmer wheat, and barley in the Fertile Crescent of the Near East (Lev-Yadun et al., 2000).

Additional paleogenetic studies, including the analysis of archaeobotanical samples and archaeological materials from different sites in Anatolia from different time periods, are needed to understand the initiation and spread of agriculture throughout Europe. Western Anatolia is of particular interest because it has the potential to provide insights into the evolution of agriculture and domestication processes about Neolithic migrations from Anatolia to Europe (Horejs, 2019).

1.2. The Kaymakçı Archaeological Site

The Kaymakçı archaeological site is located in the Gediz River valley in Manisa in western Anatolia, Türkiye (Fig. 1.7). The remains found at the archaeological site are well-preserved and the ceramic analyses and radiocarbon dating of remains indicate that the site dates back around 3,500 years BC; the Middle and Late Bronze Age. The site has been the subject of archaeological excavations since 2014, which have revealed several interesting insights into the history and culture of the region during this time period (Luke et al., 2015).

Excavations at Kaymakçı have found houses, alleys, courtyards, and grain silos. Botanical samples show crops were grown for food and animal feed. The charred seeds are various agricultural crop species, including barley (*H. vulgare*), einkorn wheat (*T. monococcum*), free-threshing wheat (*T. aestivum/durum*), emmer wheat (*T. turgidum* subsp. *dicoccum*), bitter vetch (*Vicia ervilia*), chickpea (*Cicer arietinum*) and grape (*Vitis vinifera*) (Fig. 1.8). The ancient wheat seeds were excavated from the area 97.541 at the Kaymakçı site (Fig 1.9) which is situated on the lower, outer terrace of the inner citadel uncovered the remains of seven circular structures and three groups of buildings (Fig 1.10). Wheat seeds excavated from this area have exceptional preservation due to the depth of the stratigraphy of the site (Roosevelt et al., 2018; Çiftçi et al., 2019; Shin et al. 2021). These ancient seeds were compared with modern and wild relatives into identify genetic changes, their origins, and the first time they were cultivated and spread across western Anatolia (Roosevelt et al., 2018; Çiftçi et al., 2019; Shin et al., 2021).

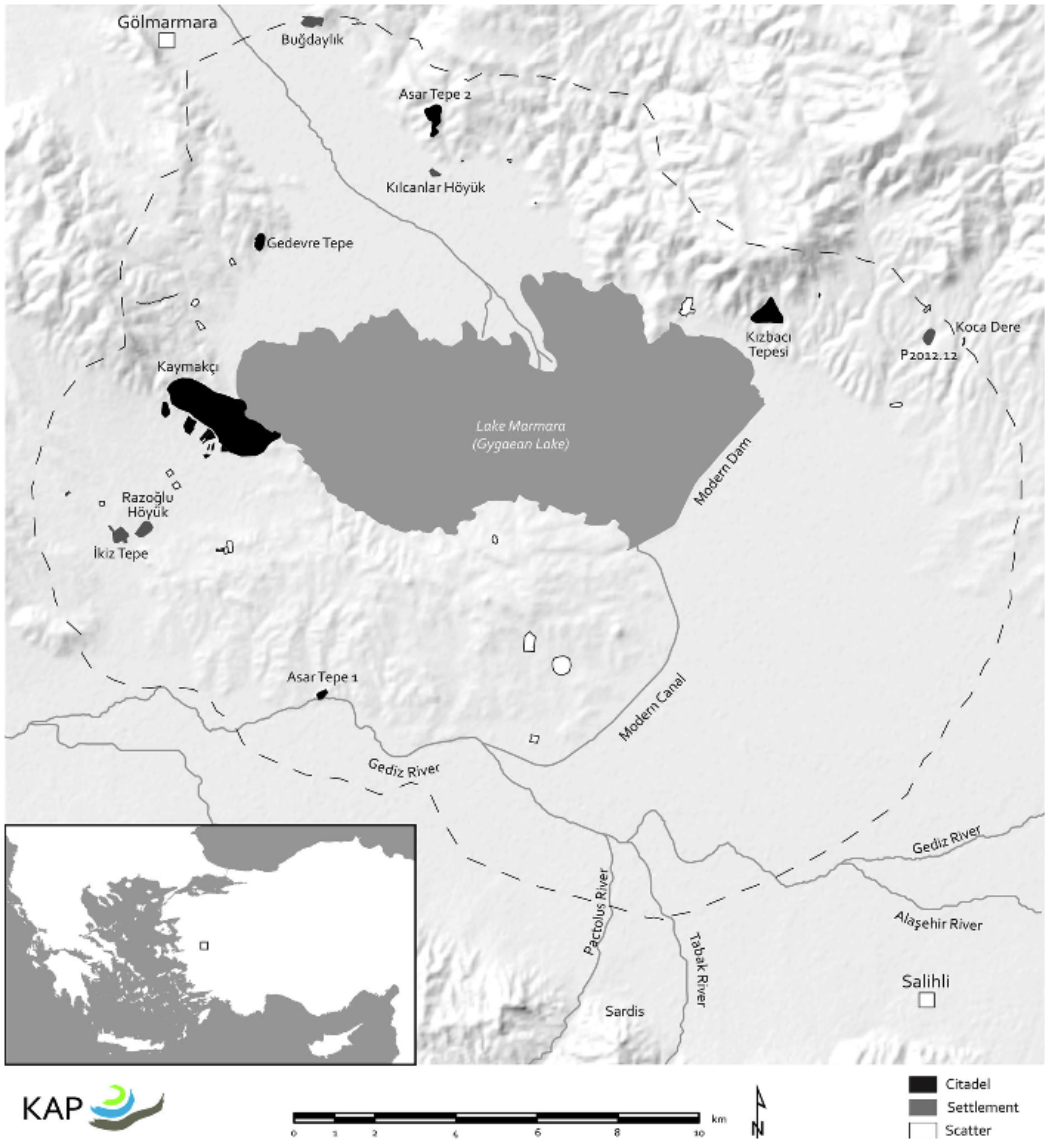

Figure 1. 7. Map showing the location of Kaymakçı and contemporary sites in the Marmara Lake basin of the Gediz River valley. Inset shows location in western Türkiye (Courtesy of the Kaymakçı Archaeological Project) (Çiftçi et al., 2019).

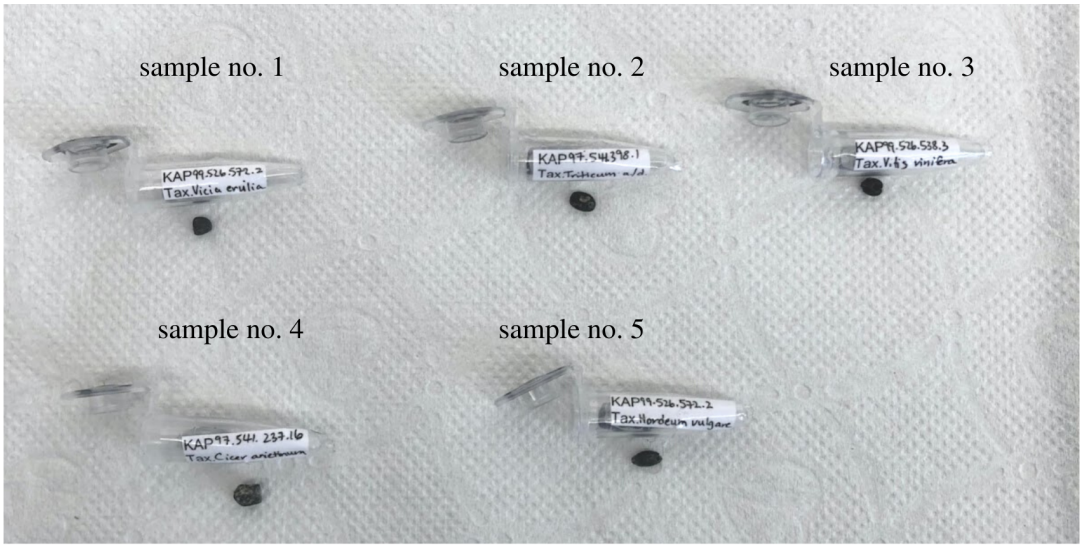

Figure 1. 8. Some charred ancient seeds excavated from Kaymakçı. Sample no. 1: *Vicia ervilia*, sample no. 2: *T. aestivum/durum*, sample no. 3: *Vitis vinifera*, sample no. 4: *Cicer arietinum*, and sample no. 5: *Hordeum vulgare* (Courtesy of the Kaymakçı Archaeological Project).

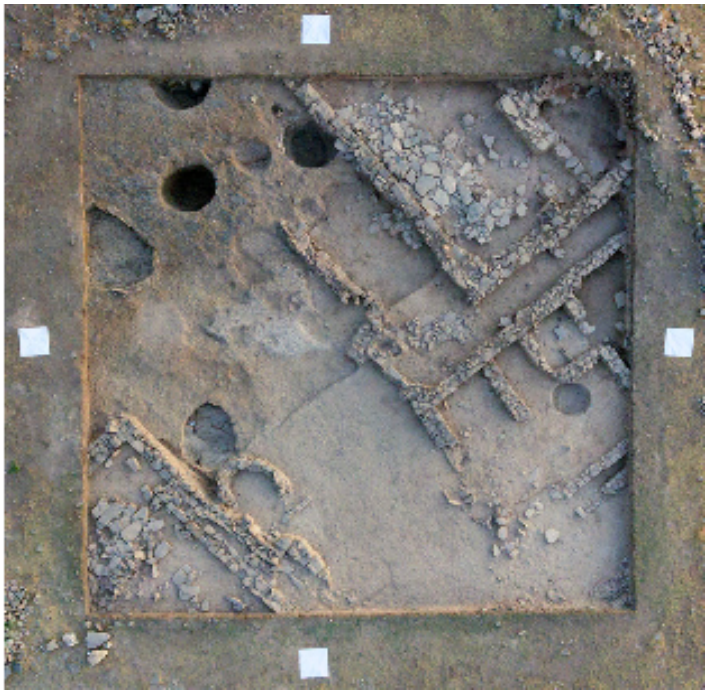

Figure 1. 9. The excavation area 97.541 at Kaymakçı. The white squares (1x1 m) indicate the size of the area. (Courtesy of the Kaymakçı Archaeological Project).

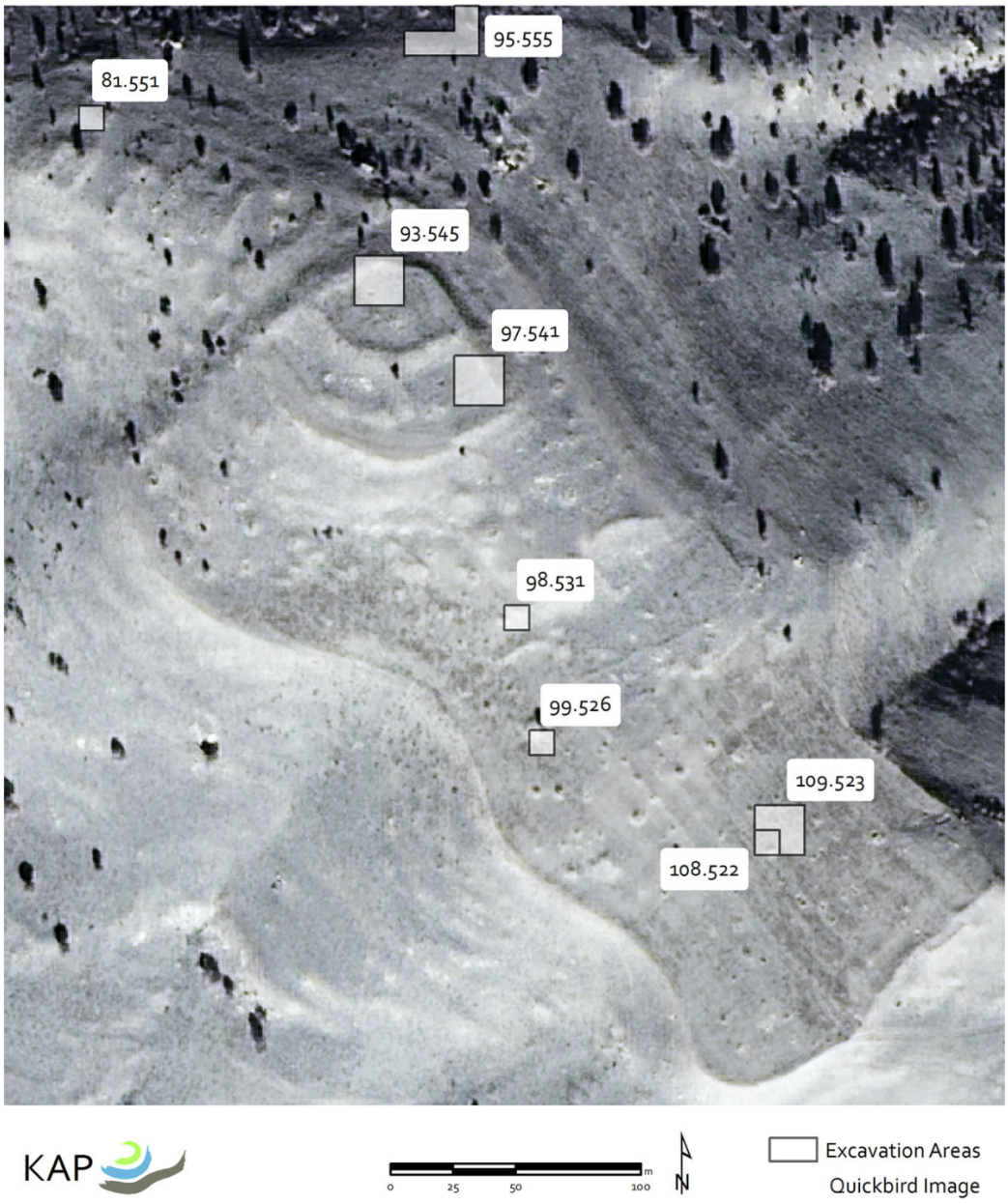

Figure 1. 10. QuickBird satellite image showing the locations of excavation areas at Kaymakçı (Courtesy of the Kaymakçı Archaeological Project) (Çiftçi et al., 2019).

1.3. Ancient DNA Studies

Phylogeny is the study of evolutionary relationships, which involves constructing evolutionary trees of life based on the genetics and ancestry of different species. It helps to understand the evolution and distribution of plants (Scotland et al., 2003). Phylogenetic trees of plants had been constructed according to the morphological features and genetics of the ancient plants in the field of archaeobotany (Allaby, 1997; Schlumbaum et al., 2008). Ancient plant remains provide valuable insights into phylogeny. In addition to that, ancient charred plants give information about ancient civilizations' diets and agricultural practices. The field of paleoethnobotany studies the behavioral and ecological interactions between prehistoric communities and plants through the analysis of pollen grains, charred seeds, and residues (Ford, 1979; Hastorf & Popper, 1988; Warnock, 1998; Pearsall, 2016).

Ancient seeds have been charred over time, often due to being buried in soil or sediment. These seeds are studied using standard dissecting microscopy and other analytical techniques, giving information about their physical characteristics. Comparing archaeological plant remains to modern surface vegetation can be a valuable technique for species identification of ancient seeds (Schlumbaum et al., 2008; Pearsall, 2016). Modern seeds are often larger and have distinct shapes and colors making them easily distinguished from ancient carbonized seeds by their physical characteristics (Pearsall, 2016). Ancient seeds are often excavated with other ancient materials, such as ceramics and artifacts, that can help to determine the period in which the seeds were buried (Kistler, 2018).

However, due to the poor conservation of the charred seeds recovered from archaeological sites, morphological data can be inadequate to classify the plant species accurately (Gugerli et al., 2005; Ucchesu et al., 2016). Besides the poor conservation, most ancient plant remains are found as seeds because the other parts cannot be preserved through charring; hence the species that can be only

differentiated by its leaves or other parts of the plant, may not be identified (Harvey et al., 2005; Schlumbaum et al., 2008). In the case of *Triticum aestivum/durum* discrimination kernel part of the wheat is used, however due to the charring process morphological data is inadequate (Hovsepyan & Willcox, 2008; Vermeulen et al., 2018). Genetic comparison of undetermined archaeological seeds can offer valuable information about their phylogeny (Oliveira et al., 2011).

Modern molecular genetic methods enable the extraction of ancient DNA (*a*DNA) from charred seeds (Allaby et al., 1997; Brown et al., 1998; Gugerli et al., 2005; Kistler et al., 2011; Li et al., 2011; Oliveira et al., 2012; Çiftçi et al., 2019). Analyzing isolated *a*DNA can allow researchers to identify species of the seeds and compare their genetics to modern plant populations (Lindqvist et al., 2019; Wales & Kistler, 2019). Ancient DNA methods have methodological challenges, such as DNA degradation, contamination, low yield, cost, limited temporal, and spatial resolution. DNA degradation makes extracting and analyzing *a*DNA difficult, while contamination can make it difficult to distinguish between ancient and modern DNA (Kistler & Shapiro, 2011; Lindqvist et al., 2019). Contamination can be avoided by setting up a separate *a*DNA laboratory with sterile conditions (Fulton & Shapiro, 2019). Extracted *a*DNA concentration from ancient samples is often low, making it difficult for subsequent analyses. There are methods used in *a*DNA studies to overcome the limitations that come from the low level of isolated *a*DNA. Whole genome amplification (WGA) is used for amplifying the whole genome (Hawkins et al., 2002; Lee & Prys-Jones, 2008; Çiftçi et al., 2019; Değirmenci et al., 2022). The pGEM-T Easy Vector System is utilized to amplify specific *a*DNA fragments and target gene regions (Lister et al., 2008; Fernández et al., 2013).

Despite the challenges involved in their analysis, ancient charred seeds are a valuable source of information for understanding the history and evolution of different plant species, as well as the culture and diet of ancient civilizations.

1.4. Genetic Markers in Ancient Wheat

Genetic markers are used in ancient wheat studies to understand genetic diversity, evolutionary history, and population dynamics. These markers provide valuable insights into ancient wheat varieties' genetic makeup and relationships. They also enable the reconstruction of phylogenetic trees and the analysis of evolutionary processes such as domestication, genetic adaptation, and gene flow (Allaby et al., 1999; Oliveira et al., 2012; Çiftçi et al., 2019).

1.4.1. Nuclear Ribosomal DNA (rDNA) Regions of Wheat

Nuclear rDNA markers are present in multiple copies within a plant genome. This high copy number increases amplification and sequencing success, enhancing the sensitivity and reliability of genetic analyses (Kuzoff et al., 1998). These genetic markers contain both conserved regions, highly similar across species, and variable regions that exhibit variations within and between populations. This combination of conserved and variable regions makes rDNA markers suitable for studying evolutionary relationships and genetic variations. Non-coding nuclear DNA regions give more precise information about recent evolutionary processes, such as the domestication of crop species or recent historical distributions (Lister et al., 2008; Değirmenci et al., 2020).

One of the genetic markers is the 26S rDNA region, which was previously used to successfully identify charred ancient seeds (Carvalho et al., 2011; Çiftçi et al., 2019). This region is particularly suited for ancient plant DNA studies due to its small size and multiple copies in the genome. Also, 26s rDNA has rapid evolution compared to conserved protein-coding regions, providing a good region for phylogenetic analyses (Kuzoff et al., 1998). The ITS2 (the second Internal Transcribed Spacer) region is also used to identify ancient wheat (Alvarez & Wendel, 2003; Li et al., 2011). The ITS2 region is located between the 5.8S and

26S rRNA genes within the ribosomal DNA (Fig 1.11). The ITS2 region is particularly successful in the identification of species in the genus *Triticum* and *Aegilops* due to its high diversity (Baldwin, 1992; Chen et al., 2010; Ganolopous et al., 2017).

Another rDNA barcoding region is the IGS (Intergenic spacer) region used to identify the polyploidy in wheat and to characterize the genome type and species. This region is non-coding and contains repetitive DNA sequences, which show high variation between wheat species (Carvalho et al., 2011).
Accordingly, in the D genome of hexaploid wheat, the IGS region has a 71 base insertion (Brown et al., 1994; Sallares et al., 1995; Li et al., 2011; Oliveira et al., 2012). The inserted region of IGS is highly conserved and used for verifying the presence of the D genome. Therefore, the expected sizes of the PCR products of IGS primers for the D genome is 158 bp, and the A/B genome is 87 bp (Li et al., 2011). In archaeological studies of wheat, finding a whole stem or spike is rare, so naked grains are usually labeled as *"T. aestivum/durum"*. The intergenic spacer (IGS) is particularly important to distinguish morphologically indistinguishable naked wheat species (Değirmenci et al., 2022).

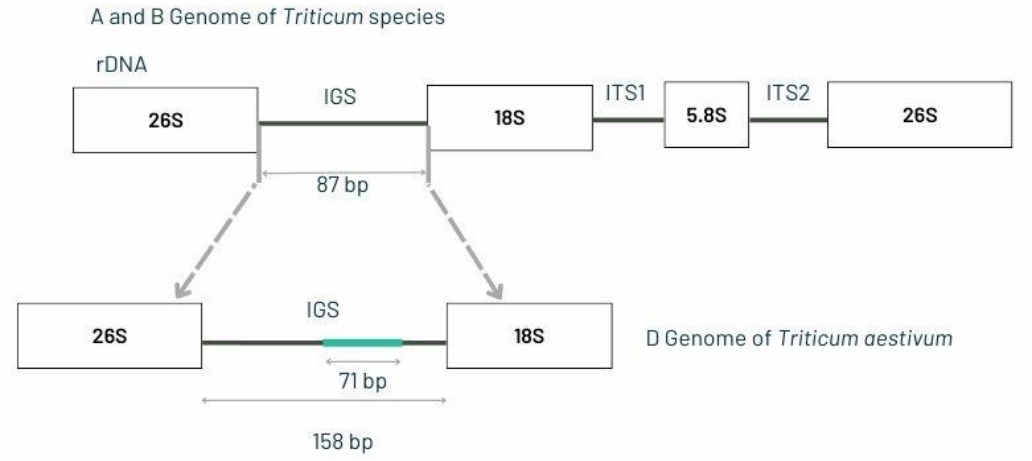

Figure 1. 11. Schematic presentation of the rDNA region structure in wheat. Tandem arrays of the consecutive gene blocks (18S-5.8S-26S). In the D genome of hexaploid wheat, the IGS region has a 71-base pair insertion.

1.4.2. High Molecular Weight (HMW) Glutenin Subunits of Wheat

High molecular weight glutenin subunits (HMW-GS) are a class of proteins found in wheat responsible for the elasticity and strength of wheat dough. These storage proteins comprise long chains of amino acids and are found in the endosperm, the part of the wheat grain milled to make flour (Li et al., 2007). The HMW glutenins are alcohol-insoluble components of gluten with alcohol-soluble gliadin (Shewry et al., 2003).

The HMW glutenins are divided into two subunits encoded by genes located on the chromosome 1 of each genome of the wheat plant. The specific combination and expression of these genes can influence the properties of the HMW glutenins and, therefore, the overall quality of the wheat flour. Some wheat varieties have higher levels of HMW glutenins, which can result in a more elastic dough well suited for bread-making. Other varieties may have lower levels of HMW glutenins, resulting in softer, less elastic dough better suited for pastries and other baked goods (Anjum et al., 2007).

The glutenin locus I (*Glu-1*) is the genetic region in the wheat genome that encodes glutenin proteins and it is located on the long arm of chromosome 1 (Fig 1.12). The *Glu-1* locus is shown to have multiple alleles for each locus, with the highest allelic variation at *Glu-B1* and *Glu-D1*, whereas *Glu-A1* displays lower genetic polymorphism (Li et al., 2020). High variation of D alleles of glutenin genes suggests hexaploid wheat evolved multiple times (Allaby et al., 1999).

Each locus of glutenin contains two genes which encode two different types of HMW; x-type and y-type. *Glu-D1x* gene sequences are more similar to *Glu-B1x* than *Glu-D1y* genes (Thompson et al., 1985). These subunits are also labeled as *Glu-B1-1* (x-type) and *GluB1-2* (y-type) genes (Ravel, 2014).

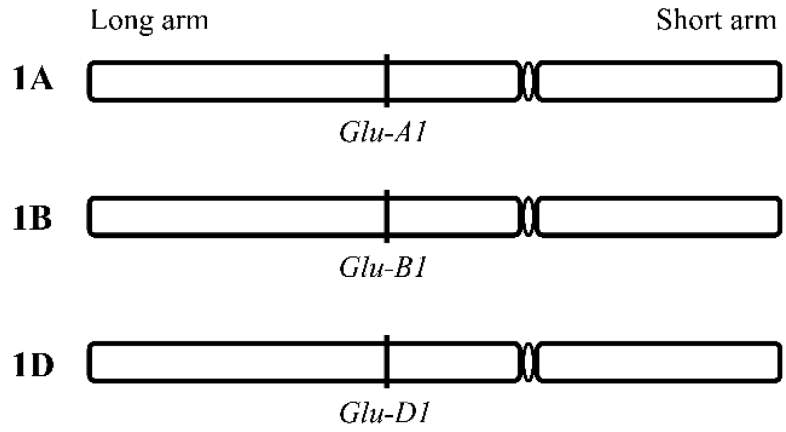

Figure 1. 12. Illustration shows the location of HMW glutenin subunits in the hexaploid wheat chromosome 1 in different genome types; A, B and D (Li et al., 2020).

In *T. durum,* Glu-A1 and Glu-B1 encode HMW glutenin subunits, whereas Glu-A3, Glu B2 and B3 encode LMW glutenin subunits (Roncallo, 2021). Each of these loci encodes for a different type of glutenin protein. The allelic variations of the glutenin locus I are responsible for the quality of wheat dough. Different alleles result in a wide range of gluten quality in wheat, which is important for different end-uses.

The HMW glutenin locus, which comes in several alleles, has been extensively studied in ancient DNA extracted from burned wheat grains excavated from archaeological sites and historical specimens (Allaby et al., 1994; Schlumbaum et al., 1998; Lister et al., 2008; Oliveira et al., 2012). These studies have suggested that *Glu I* locus can provide useful insights into evolutionary relationships. The benefit of this locus is that wheat genomes possess two pairs of HMW glutenin genes (x and y) per nuclear genome in hexaploid wheat, resulting in twelve targets (Allaby et al., 1994). The existence of multiple loci increases the chances of amplification of ancient DNA that is degraded and fragmented, making it a more suitable target for analysis than regions with only a single copy (Lister et al., 2008).

CHAPTER 2

OBJECTIVES OF THE STUDY

The objectives of this study were to make molecular identification of the charred wheat seeds, assess the level of genetic change that occurred over the last 3500 years using molecular markers and investigate evolutionary history.. The primary objective of this thesis was to perform molecular identification of ancient wheat seeds and gain a better understanding of the agricultural practices of early human civilizations and their impact on the evolution and domestication of wheat.

The specific objectives of the study are:

- To successfully extract and amplify ancient DNA from ancient charred wheat seeds and identify the genome type using molecular markers.
- To determine their genetic relationships and evolutionary history, compare the *Glu-1* allelic profile of ancient wheat varieties from the Kaymakçı site with modern wheat cultivars.
- To assess the level of genetic change of ancient wheat varieties compared to their modern relatives using molecular nuclear DNA markers such as 26S rDNA and ITS2 regions.
- To assess the presence of the D genome of ancient wheat samples and accurately determine their respective species identities by IGS region.
- To contribute to understanding the history of wheat cultivation in the region and its relationship to the development of agriculture.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant Material

As part of the TÜBİTAK project (No: 121Z407), ancient seeds of different species were excavated from the Kaymakçı archaeological site in Gölmarmara, Manisa. Amongst these ancient seeds, only two were identified as free-threshing (naked) wheat seeds and used in this study, with their corresponding codes being Ta/d-1 (*T. aestivum/durum*-1) and Ta/d-2 (*T. aestivum/durum*-2) (Table 3.1).

Table 3. 1. Information about the charred wheat seeds excavated from the Kaymakçı archaeological site, Manisa.

Project Inv. No.	Codes	Species	Fraction Size	Weight
KAP97.541.118	Ta/d-1	*Triticum aestivum/durum*	> 2 mm	0.009 g
KAP97.541.398	Ta/d-2	*Triticum aestivum/durum*	> 2 mm	0.016 g

The modern relatives of the ancient wheat seeds were sampled from the Gediz Plain or acquired from the Gema Foundation (Gediz Basin Erosion Control, Afforestation, Environment, and Development Foundation) in April 2020. In addition to ancient seeds and their modern relatives, reference wheat species (*T. aestivum* and *T. durum*) were obtained from the Faculty of Agriculture, Kırşehir Ahi Evran University, in March 2022 (Table 3.2).

Table 3. 2. Information about (a) modern and (b) reference wheat seeds

Codes	Species	Gathering place / Acquired institution
(a) Modern wheat seeds		
Ta1	*T. aestivum*	Gediz Valley
Ta2	*T. aestivum*	Gema Foundation
Ta3	*T. aestivum*	Gema Foundation
Ta4	*T. aestivum*	Gema Foundation
(b) Reference wheat seeds		
Ta	*T. aestivum*	Kırşehir Ahi Evran University
Td	*T. durum*	Kırşehir Ahi Evran University

3.2. DNA Extraction

3.2.1. Sterilization of Isolated Workspace for Ancient DNA Studies

In ancient DNA studies, preventing contamination and obtaining pure ancient DNA from each seed is crucial in terms of the authenticity and reliability of the study (Hebsgaard et al., 2005; Oliveira et al., 2012). To rule out any possible contamination, ancient DNA extraction was carried out in a separate laboratory with new equipment and a sterile cabinet. Before extraction, all surfaces and equipment were wiped with bleach, ethanol, and DNA AWAY™ surface decontaminating solution (Molecular Bioproducts Inc., San Diego, CA). All equipment (tubes, containers, racks and tips) and chemicals were sterilized by autoclave. For non-autoclavable equipment, 70% ethanol and DNA AWAY surface decontaminating solution were used (Çiftçi et al., 2019). 20 mins of ultraviolet (UV) sterilization was applied to all equipment. Necessary precautions were taken during the *a*DNA extraction, such as protective clothing, wearing double gloves and face masks.

3.2.2. DNA Extraction Methods for Ancient Charred Wheat Seeds

Different methods have been used to extract aDNA from ancient charred wheat seeds to assess the accuracy of two *a*DNA extraction methods. Ancient Ta/d-1 seed was isolated using a modified CTAB method developed by Kistler & Saphiro (2011). and the ancient Ta/d-2 seed was isolated using a modified CTAB method developed by Gismondi et al. (2016). The former was used successfully by Çiftçi et al. (2019) and Değirmenci et al. (2022) for DNA extraction from ancient seeds. During all DNA extraction procedures extraction blanks were used as a negative control and *Salix alba* samples were used as a positive control in a separate laboratory.

3.2.2.1. CTAB Based DNA Extraction Modified from Kistler & Saphiro (2011)

Ta/d-1 was placed into the 2.0 mL Eppendorf tube and 500 µl of CTAB extraction buffer (containing 2% (w/v) CTAB; 100 mM Tris/HCl pH 8.0; 20 mM EDTA pH 8.0; 1.4 M NaCl, 0.02 g pvp and 2.5 µl 2-mercaptoethanol) added and shaken for 2 hours at 55 °C. After incubation, the seed in the tube was crushed with a flame-sealed pipette tip. 500 µl of chloroform was added. The tube was centrifuged at 13,000 rpm for 7 minutes and the aqueous phase was transferred to a new sterile Eppendorf tube. The tube was incubated at -20 °C with 0.08 volume of 7.5 M ammonium acetate and 0.54 volume of isopropanol for 45 minutes. Then the mixture was centrifuged at 13,000 rpm for 10 minutes and the supernatant was poured out. The remaining precipitate was washed once with 70% ETOH and centrifuges at 13,000 rpm for 2 minutes and then, added 95% ETOH and incubated in the room tempature for 10 minutes and centrifuged at 13,000 rpm for 2 minutes. After centrifuge, the ETOH is discarded and the Eppendorf tube allowed to air-dry in room temperature. The resulting aDNA sample was dissolved in 25 µl of sterile/nuclease-free water. The extracted aDNA stored in the -20 °C for long term storage.

3.2.2.2. CTAB Based DNA Extraction Modified from Gismondi et al. (2016)

Ta/d-2 was crushed in a mortar with liquid nitrogen and transferred to a 2.0 mL Eppendorf tube with 800 µl extraction buffer (10 mM Tris-HCl pH 8; 100 mM EDTA pH 8; 0.5% SDS; 20 mg/mL RNase A Sigma-Aldrich). The sample was incubated at 37°C for 1 hour and further 3 hours with the addition of 4 µl Proteinase K (100 mg/mL, Sigma-Aldrich). After incubation, 800 µl of cold phenol: chloroform (1:1, pH 8) was added. The tube was gently inverted and centrifuged at 4°C for 5 minutes at 12,000 rpm. Upon transferring the supernatant into a sterile Eppendorf tube, 400 µl of CTAB solution containing 35 mM CTAB and 100 mM NaCl was added, and the lysate was incubated for 1 hour at room temperature.. Then a volume of phenol: chloroform (1:1, pH 8) was added, and the tube was inverted and centrifuged at 4°C for 5 minutes at 12,000 rpm. The supernatant was transferred to a new Eppendorf tube. This step was repeated twice. Following this, a volume of chloroform was added to the tube and centrifuged at 4°C for 5 minutes at 12,000 rpm and the aqueous phase was pipetted into a new sterile 1.5 mL tube containing 100 µL of 2 M NaCl and 1 mL of cold 2-propanol, incubated at –80°C overnight. After incubation, the sample was centrifuged for 30 minutes at 12,000 rpm in 4°C. The supernatant was discarded, the precipitate was air-dried and dissolved in 50 mL of TE buffer (10 mM Tris-HCl pH 8; 1 mM EDTA pH 8). The extracted aDNA stored in the -20 °C for long term storage.

3.2.3. Modern Wheat Seed Extraction

The reference wheat seeds, and the modern wheat seeds sampled from Gediz Valley were planted into pots prior to DNA extraction in order to enhance the amount and quality of the isolated DNA. Each wheat seedling was grown for two weeks, and then the fresh leaves were collected and crushed using mortar and pestle with liquid nitrogen and stored at -80°C until the extraction process.

3.2.3.1. Modified Qiagen DNEasy Plant Mini Kit (Qiagen, Valencia, CA)

The samples weighed 20 mg from previously ground samples. 600 µl of buffer AP1, previously heated at 65°C in a water bath, was added to the samples and homogenized using mortar and pestle. Then the lysate was transferred into a 1.5 mL Eppendorf tube, and 4 µl Proteinase K was added. The tubes were vortexed and incubated at 65°C in a water bath for 20 minutes. 150 µl of cold Buffer P3 was added and the samples were incubated on ice for 30 minutes. The lysate was centrifuged for 15 minutes at 14,000 rpm. The aqueous phase was transferred into a QIAshredder spin column, placed in a collection tube and centrifuged for 10 minutes at 14,000 rpm. The flow-through pipetted into a new 2.0 mL Eppendorf. 675 µl of Buffer AW1 was added into the tubes. 650 µl of the mixture was pipetted into a DNeasy Mini spin column, placed in a collection tube and centrifuged for 5 minutes at 14,000 rpm. The flow-through was discarded and this step repeated for the remaining mixture. The spin column was removed and placed into a new 2 mL collection tube. 600 µl Buffer AW2 was pipetted onto the spin column and centrifuged for 5 minutes at 14,000 rpm. The flow-through was discarded, and this step was repeated. After, the spin column was centrifuged at 8,000 rpm for 2 minutes to ensure the removal of the buffer from the membrane. The spin column was placed into a new 1.5 mL Eppendorf tube. For the elution of the DNA from the membrane, 100 µl of nuclease-free water was added and the samples were centrifuged at 9,000 rpm for 5 minutes.

3.3. DNA Quantification

The amount and quality of the isolated ancient and modern DNA were measured by using Biodrop Duo micro-volume spectrophotometry (Biodrop µLite 7141 V.1.0.4, Department of Biological Sciences, METU). The contamination level of phenolic compounds, protein and secondary metabolites of the sample was estimated by the ratio of A260/A230 nm, and the contamination level of RNA of the sample was estimated by the ratio of A260/A280 nm (O'neill et al., 2011; Yu et al., 2017).

3.4. Whole Genome Amplification

Whole genome amplification of isolated charred ancient seeds was performed following the manufacturer's procedure of the Illustra GenomiPhi HY DNA Amplification Kit (Amersham, GE Healthcare, UK) to obtain better quality and higher concentrations of DNA (Kumar et al., 2007). In a 0.2 mL PCR tube, 1 µl (10 ng) isolated DNA, 9 µl PCR-Grade water and 10 µl denaturation buffer were combined and heated at 95°C for 3 minutes. The samples were incubated at 4 °C for 20 minutes, followed by 90 minutes at 30 °C and heated again to 65 °C for enzyme inactivation in PCR. The samples were cooled to 4 °C and diluted to the working concentration (50X).

3.5. Primers

The nuclear rDNA barcoding primers synthesized by BM Labosis (Çankaya, Ankara) were used for molecular identification of charred seeds. The information of the primer pairs were used in this study is given in Table 3.3.

One of the barcoding primers is specific for the 26S rDNA gene sequence, which was previously used to successfully identify charred ancient seeds (Çiftçi et al., 2019). ITS2 region is also used to identify ancient wheat (Alvarez & Wendel, 2003; Li et al., 2011). The IGS region used to to characterize the genome type and species. (Brown et al., 1994; Sallares et al., 1995; Li et al., 2011; Oliveira et al., 2012).

The HMW glutenin locus was used in this study because it contains important genetic information that can provide insights into the evolutionary history, genetic diversity, and breeding potential of ancient wheat varieties (Allaby et al., 1994; Schlumbaum et al., 1998; Lister et al., 2008; Oliveira et al., 2012). Glu-140 and Glu-240 primers have been designed from upstream of the *Glu - Bx* gene of the *T. turgidum* (Genbank ID: AY621068) (Lister et al., 2008), while Glu-160 primer designed from the *GluB1-1* gene of the *T. aestivum* (Genbank ID: AJ567980).

Table 3. 3. The list of primers

Primer	Primer sequences (F=Forward, R=Reverse, 5'-3')	Expected Size	Reference
26S rDNA	F: TTCCCAAACAACCCGACTC R: GCCGTCCGAATTGTAGTCTG	155 bp	Alvarez & Wendel, 2003
IGS	F: CGCCATGGAAAACTGGGCAA R: ACCTCTCGTACCCGTTCACGT	87-158 bp	Li et al., 2011
ITS2	F: CAAAACACGCTCCCAACCACT R: GCTTCGTTTGTTGCCTCGTTC	145-189 bp	Li et al., 2011
Glu-140	F: CCGATTTTGTTCTTCTCACGC R: TGCTCGGTGTTGTGGGTG	140 bp	Lister et al., 2008
Glu-160	F: CTGCCCTTTTCCAACCGATTTTG R: AGATTGGGGTGCTCGGTGTTG	160 bp	designed
Glu-240	F: GATTACGTGGCTTTAGCAGAC R: TGCTCGGTGTTGTGGGTG	240 bp	Lister et al., 2008

3.6. Optimization of PCR Conditions

Polymerase chain reaction (PCR), 5x HOT FIREPol® Blend master mix (Solis BioDyne, Tartu, Estonia) containing 15mM MgCl2 was used to amplify the selected gene regions. The optimized PCR condition for each gene region and the PCR cycle are provided in Table 3.4. After confirming the presence of the expected size of the product in 3% agarose gel (at 100 V for 45 minutes), stained with ethidium bromide, under UV light (Vilber Lourmat, France), the samples were sent to the BM Labosis (Ankara) for the purification and sequencing procedures.

Table 3. 4. Optimized PCR conditions for gene regions

Primer	Components	Vol. (µL)	PCR Conditions		
Glu140	dH_2O	16.7	Initial Denaturation 5 min		95°C
	Master Mix	3.5	35 cycles	Denaturation 35 sec	95°C
	Primers (10µM)	0.4+0.4		Annealing 40 sec	60°C
	DNA (10ng/µL)	4		Extension 35 sec	72°C
	Total	25	Final Extension 5 min		72°C
Glu160	dH_2O	18.6	Initial Denaturation 5 min		95°C
	Master Mix	3	30 cycles	Denaturation 30 sec	95°C
	Primers (10µM)	0.5+0.5		Annealing 45 sec	56°C
	DNA (10ng/µL)	3		Extension 30 sec	72°C
	Total	25	Final Extension 5 min		72°C
Glu240	dH_2O	17.4	Initial Denaturation 5 min		95°C
	Master Mix	3	35 cycles	Denaturation 35 sec	95°C
	Primers (10µM)	0.3+0.3		Annealing 40 sec	62°C
	DNA (10ng/µL)	4		Extension 35 sec	72°C
	Total	25	Final Extension 5 min		72°C
ITS2	dH_2O	13.6	Initial Denaturation 3 min		95°C
	Master Mix	5	30 cycles	Denaturation 30 sec	95°C
	Primers (10µM)	0.7+ 0.7		Annealing 40 sec	60°C
	DNA (10ng/µL)	5		Extension 1 min	72°C
	Total	25	Final Extension 10 min		72°C

Table 3.4. (continued)

26S	dH_2O	14	Initial Denaturation 5 min		95°C
	Master Mix	5	25 cycles	Denaturation 30 sec	94°C
	Primers (10µM)	0.5+ 0.5		Annealing 30 sec	60°C
	DNA (10ng/µL)	5		Extension 1 min	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	10.2	Initial Denaturation 5 min		95°C
	Master Mix	4	40 cycles	Denaturation 1 min	94°C
	Primers (10µM)	0.4+ 0.4		Annealing 1 min	60°C
	DNA (10ng/µL)	4		Extension 1 min	72°C
	Total	20	Final Extension 10 min		72°C

3.7. Data Analysis

The amplified DNA was sequenced in both forward and reverse directions in BM Labosis by using ABI 310 Genetic Analyzer (PE Applied Biosystem) and an ABI 3730XL 96-capillary automatic sequencer. This approach allowed to overcome any errors or ambiguities in the sequence data, resulting in a more accurate and reliable chromatogram data. Ancient and modern DNA sequences were analyzed using the MEGA 11.0 software for visualization of the chromatogram data, alignment, BLAST research and phylogenetic analysis (Tamura et al., 2021). The NCBI BLAST tool is used for searching databases of sequences to find highly similar sequences (Johnson et al., 2008). Molecular diversity parameters were estimated comparatively between ancient, modern and the BLAST aligned sequences by using the MEGA 11.0 software.

CHAPTER 4

RESULTS

4.1. DNA Extraction Results

Ta/d-1 was isolated using a modified CTAB method developed by Kistler & Saphiro (2011) and Ta/d-2 was isolated using a modified CTAB method developed by Gismondi et al. (2016). The results of the whole genome amplification indicated that the high quality and quantity of aDNA from the ancient seeds were successfully obtained (Table 4.1). The negative controls showed no presence of DNA, indicating the absence of contamination during the extraction process.

Table 4. 1. DNA concentrations of isolated ancient wheat seeds

Code	Species	DNA conc.	230/260 absorbance ratio	260/280 absorbance ratio
Ta/d-1	*Triticum aestivum/durum*	236 µg/ml	0.79	1.32
Ta/d-2	*Triticum aestivum/durum*	3 µg/ml	0.08	2
DNA concentrations after the whole genome amplification of ancient seeds				
Ta/d-1	*Triticum aestivum/durum*	1456 µg/ml	2.15	1.86
Ta/d-2	*Triticum aestivum/durum*	2251 µg/ml	2.22	1.88

Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*).

The DNA concentration, along with the absorbance rates of 230/260 and 260/280, for both the modern and reference wheat seeds are provided in Table 4.2.

Table 4. 2. DNA concentration of isolated modern and reference wheat seeds

Codes	Species	DNA conc.	230/260 absorbance rates	260/280 absorbance rates
Ta1	*T. aestivum*	0.5 µg/ml	1	1.6
Ta2	*T. aestivum*	9 µg/ml	1.12	1.8
Ta3	*T. aestivum*	6 µg/ml	1.5	2
Ta4	*T. aestivum*	9 µg/ml	1.12	1.8
Ta	*T. aestivum*	129.5 µg/ml	2.21	2.04
Td	*T. durum*	17.52 µg/ml	2.06	2.06

Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

4.2. PCR Results

Good quality single bands were observed for the 26s rDNA, ITS2 and Glu primer sets and selected for sequencing (Fig 4.1 and Fig 4.2). No bands were observed in negative controls. For the IGS rDNA region three bands were observed, approximately 87, 157 and 250 bp in length. To obtain chromatogram data free from interference, the 157 bp DNA fragments corresponding to the partial IGS sequence with the 71 bp insertion in the D genome of ancient wheat were purified from the gel by using The Gene Matrix Agarose-Out DNA Purification Kit (Eurx) and sent for sequencing (Fig 4.3).

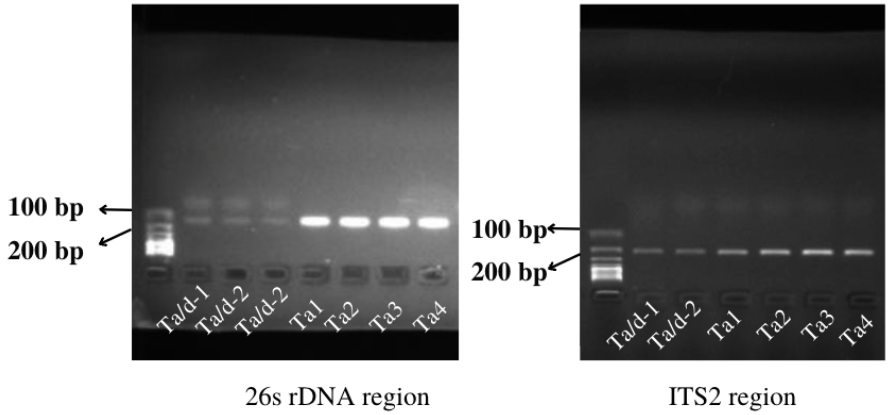

Figure 4. 1. Gel photos of amplified 26s rDNA and ITS2 region of ancient and modern wheat seeds. Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

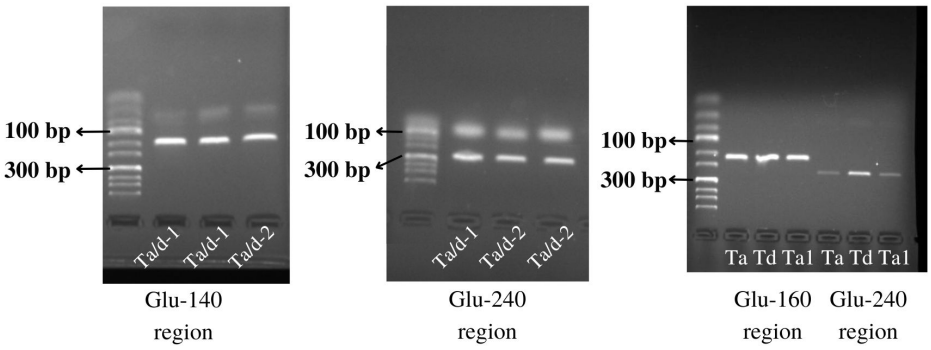

Figure 4. 2. Gel photos of amplified ancient and modern DNA of *Glu* regions. (Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1: modern wheat seed (*T. aestivum*)).

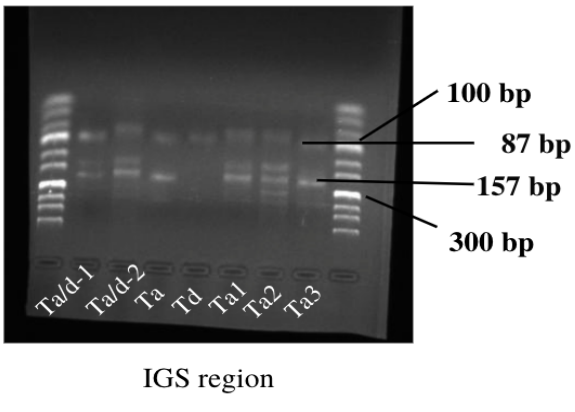

Figure 4. 3. Gel photos of amplified IGS rDNA region of ancient wheats and modern seeds. (Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2 and Ta3: modern seeds (*T. aestivum*)).

4.3. Phylogenetic Analysis

The ancient and modern sequences obtained from the chromatogram data were aligned by using the MEGA 11.0 software and checked with the NCBI BLAST search to determine the genetic similarities between ancient seeds and their contemporaries. The sequences of all gene regions, which are available from the NCBI database for the species in the current study, were also comparatively analyzed.

4.3.1. The 26S rDNA Gene Region

Notably, the ancient 26S rDNA sequences exhibited considerable homology with the sequences derived from their contemporary counterparts. The amplified 26S rDNA sequences of the ancient wheat seed Ta/d-1 were aligned with 95% identity to the sequence of modern *T. aestivum* (MK413186.1) and *T. durum* (MK413187.1) from NCBI. Whereas the ancient charred wheat seed Ta/d-2 was aligned with 96% identity to the sequence of modern *T. aestivum* (MK413186.1) and 97% identity to the sequence of modern *T. durum* (MK413187.1) from NCBI (Table 4.4). Additionally, the sequences of ancient wheat seeds show high homology to the previously studied ancient wheat seed (Çiftçi et al., 2019) from the NCBI databases (MK413185.1) with the identity of 99% and 98%, respectively.

Table 4. 3. The identity comparisons for the 26S rDNA region with the highest aligned sequences retrieved from the NCBI database

	Genebank accession number	Query cover (%)	E value	Identity (%)
(a) Identity comparison of the 26S rDNA sequences of ancient seeds with the sequences from the database				
Ta/d-1 versus *T. aestivum* Ancient	MK413185.1	98	2e-74	99
Ta/d-2 versus *T. aestivum* Ancient	MK413185.1	98	4e-68	98
Ta/d-1 versus *T. aestivum*	MK413186.1	98	2e-58	95

Table 4.3. (continued)

Ta/d-2 versus *T. aestivum*	MK413186.1	98	3e-65	96
Ta/d-1 versus *T. durum*	MK413187.1	98	2e-61	95
Ta/d-2 versus *T. durum*	MK413187.1	98	4e-64	97
(b) Identity comparison of the 26S rDNA sequences of modern seeds (or tissues) with the sequences from the database				
Ta1 versus *T. aestivum* Ancient seed	MK413185.1	98	4e-68	98
Ta2 versus *T. aestivum* Ancient seed	MK413185.1	99	3e-57	93
Ta3 versus *T. aestivum* Ancient seed	MK413185.1	98	9e-64	93
Ta4 versus *T. aestivum* Ancient seed	MK413185.1	98	9e-64	93
Ta versus *T. aestivum* Ancient seed	MK413185.1	98	9e-64	93
Td versus *T. aestivum* Ancient seed	MK413185.1	98	9e-64	93
Ta1 versus *T. aestivum*	MK413186.1	98	4e- 57	93
Ta2 versus *T. aestivum*	MK413186.1	99	1e- 66	96
Ta3 versus *T. aestivum*	MK413186.1	98	1e- 66	96
Ta4 versus *T. aestivum*	MK413186.1	98	1e- 66	96
Ta versus *T. aestivum*	MK413186.1	98	1e- 66	96
Td versus *T. aestivum*	MK413186.1	98	1e- 66	96
Ta1 versus *T. durum*	MK413187.1	98	4e-57	93
Ta2 versus *T. durum*	MK413187.1	98	4e-68	98
Ta3 versus *T. durum*	MK413187.1	98	9e-75	98
Ta4 versus *T. durum*	MK413187.1	98	9e-75	98
Ta versus *T. durum*	MK413187.1	98	9e-75	98
Td versus *T. durum*	MK413187.1	98	9e-75	98

Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

Substitutions were observed between the Ta/d-2 and aligned Blast sequence of *T. aestivum* (MK413186.1) at base positions of 39, 56 and 57. Additionally substitutions were observed for the ancient Ta/d-1 are at the base positions of 23, 76 and 78. All of the reference and modern wheat seeds have the same sequences with each other and are compared to the aligned Blast sequence of *T. aestivum,* the substitutions were observed at base positions of 22, 27, 76, 87, 113 and 126. The ancient wheat seeds Ta/d-1 and Ta/d-2 aligned with each other with only three nucleotide changes at base positions of 23, 76 and 78 (Table 4.4). Moreover, ancient sequences of Ta/d-1 26s rDNA show eleven base substitutions in 151 base pair long sequence when aligned with the blast sequence of *T. aestivum* (MK413186.1) from the NCBI databases with a 96% matching score (Table 4.3 and Table 4.4).

Table 4. 4. 26S Sequence alignments of the ancient seeds and their contemporaries

Triticum Spp.	22	23	27	39	56	57	76	78	87	113	126
Modern wheat seeds											
MK413186.1 *T.aestivum*	C	T	A	G	C	G	T	A	C	C	T
MK413185.1 *T.aestivum*	.	A	.	T	A	C	.	C	-	.	.
MK413187.1 *T.durum*	T	.	.	.	.	.	C	.	.	T	.
Ta	T	.	G	.	.	.	C	.	T	T	C
Td	T	.	G	.	.	.	C	.	T	T	C
Ta1	T	.	G	.	.	.	C	.	T	T	C
Ta2	T	.	G	.	.	.	C	.	T	T	C
Ta3	T	.	G	.	.	.	C	.	T	T	C
Ta4	T	.	G	.	.	.	C	.	T	T	C
Ancient wheat seeds											
Ta/d-1	.	A	.	T	A	C	C	C	.	.	.
Ta/d-1.1	.	A	.	T	A	C	C	C	.	.	.
Ta/d-2	.	.	.	T	A	C	.	.	-	.	.
Ta/d-2.1	.	.	.	T	A	C	.	.	-	.	.

Identical bases were marked by dots. The numbers above sequences indicate the base positions. Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

4.3.2. The ITS2 rDNA Gene Region

The alignments of the sequences are made with the IT2 region of both *T. aestivum* and *T. durum* available in NCBI (MF480399.1, MK612075.1). The ancient *Triticum* seeds and the modern seeds Ta1 and Ta4 have the same sequences of the ITS2 region with the sequence of modern *T. aestivum* (MF480399.1) and *T. durum* (MK612075.1) from the NCBI databases (identity 100%) (Table 4.5). Whereas the ancient sequences are aligned *Aegilops tauschii* (MK387303.1) with identity %95 showing Kaymakçı ancient wheat samples very likely belong to the *Triticum* genus (not shown).

Table 4. 5. The identity comparisons for the ITS2 region with the highest aligned sequences retrieved from the NCBI database

	Genbank accession number	Query cover (%)	E value	Identity (%)
(a) Identity comparison of the ITS2 rDNA sequences of ancient seeds with the sequences from the database				
Ta/d-1 versus *T. aestivum*	MF480399.1	99	9e-103	100
Ta/d-2 versus *T. aestivum*	MF480399.1	99	9e-103	100
Ta/d-1 versus *T. durum*	MK612075.1	99	9e-103	100
Ta/d-2 versus *T. durum*	MK612075.1	99	9e-103	100
(b) Identity comparison of the ITS2 rDNA sequences of modern seeds (or tissues) with the sequences from the database				
Ta1 versus *T. aestivum*	MF480399.1	99	9e-103	100
Ta2 versus *T. aestivum*	MF480399.1	99	4e-95	99
Ta3 versus *T. aestivum*	MF480399.1	99	9e-97	99
Ta4 versus *T. aestivum*	MF480399.1	99	9e-103	100
Ta1 versus *T. durum*	MK612075.1	99	9e-103	100
Ta2 versus *T. durum*	MK612075.1	99	4e-95	99
Ta3 versus *T. durum*	MK612075.1	99	9e-97	99
Ta4 versus *T. durum*	MK612075.1	99	9e-103	100

Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

Additionally, in the modern wheat Ta3, one nucleotide change is observed at the base position of 175 and in modern wheat Ta2 two base substitutions are observed at 164th and 175th base positions (Table 4.5). From these nucleotide changes, only the one at the base position at 175 is parsimony informative (Table 4.13).

Table 4. 6. ITS2 region sequence alignments of the ancient and modern seeds

Sequence ID	164	175
Modern wheat seeds		
MF480399.1 *T. aestivum*	T	A
MK612075.1 *T. durum*	.	.
Ta1	.	.
Ta2	A	G
Ta3	.	G
Ta4	.	.
Ancient wheat seeds		
Ta/d-1	.	.
Ta/d-1.1	.	.
Ta/d-2	.	.
Ta/d-2.1	.	.

Identical bases were marked by dots. The numbers above sequences indicate the base positions. Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

4.3.3. The *Glu* Gene Regions

Glu-140 and Glu-240 primers were designed from upstream of the *Glu - Bx* gene of the *T. turgidum* (Lister et al., 2008). Glu-240 primers amplify the same region as the Glu-140 primers and have an additional 100 base pairs upstream. These primers share a reverse primer, and they were previously shown to successfully amplify

multiple alleles of the glutenin locus, which also increases the amplification success in ancient wheat seeds (Lister et al., 2008; Oliveira et al., 2012). However, for modern samples, it causes multiple PCR products in each amplification and therefore, problems in sequencing. To overcome this, a new primer was designed (Glu160), which has a specific reverse primer that only amplifies the *GluB1* allele. The sequences amplified by the Glu160 primer are shown in Table 4.7 with an asterisk (*). However, the primer failed to amplify the ancient wheat samples because it targets only one allele of the glutenin locus.

4.3.3.1. Glu-140/160 Region

The sequence of Glu-140 for ancient wheat Ta/d-1 and the blast samples of the *GluB1* allele of the glutenin gene of *T. aestivum* (KM116485.1) and *T. durum* (JQ689004.1) aligned completely with identity of %100 (Table 4.7). Whereas ancient wheat Ta/d-2 had six nucleotide substitutions at the base positions of 36, 58, 114, 115, 120 and 123 (Table 4.8). There were some nucleotide changes observed in modern wheat samples compared to the Blast samples of the *GluB1* allele of the glutenin gene of *T. aestivum* (KM116485.1) and *T. durum* (JQ689004.1).

Table 4. 7. The identity comparisons for the Glu140 region with the highest aligned sequences retrieved from the NCBI database

	Genebank accession number	Query cover (%)	E value	Identity (%)
(a) Identity comparison of the Glu140 DNA sequences				
Ta/d-1 versus *T. aestivum*	KM116485.1	96	2e- 70	100
Ta/d-2 versus *T. aestivum*	KM116485.1	98	7e- 51	94
Ta/d-1 versus *T. durum*	JQ689004.1	98	1e- 72	100
Ta/d-2 versus *T. durum*	JQ689004.1	98	6e- 53	94

Table 4.7. (continued)

(b) Identity comparison of the Glu140 DNA sequences				
Ta1 versus *T. aestivum*	KM116485.1	96	2e- 51	92
Ta2 versus *T. aestivum*	KM116485.1	98	4e- 52	100
Ta3 versus *T. aestivum*	KM116485.1	97	4e- 71	100
Ta4 versus *T. aestivum*	KM116485.1	100	3e- 65	96
Ta versus *T. aestivum*	KM116485.1	99	6e- 59	94
Td versus *T. aestivum*	KM116485.1	100	8e- 73	98
Ta1 versus *T. durum*	JQ689004.1	96	2e- 51	92
Ta2 versus *T. durum*	JQ689004.1	98	4e- 52	100
Ta3 versus *T. durum*	JQ689004.1	97	4e- 71	100
Ta4 versus *T. durum*	JQ689004.1	100	3e- 65	97
Ta versus *T. durum*	JQ689004.1	99	6e- 59	94
Td versus *T. durum*	JQ689004.1	100	1e- 67	98

Table 4. 8. Glu-140 region sequence alignments of the ancient and modern seeds

Triticum Spp.	1	2	20	21	23	32	36	42	47	48	49	52
KM116485.1 *T. aestivum GluB1-2*	A	T	A	C	C	T	C	T	T	C	G	G
JQ689004.1 *T. durum GluB1-2*	.	.	.	.	.	.	.	.	.	.	.	.
Ancient wheat seeds												
Ta/d-1	.	.	.	.	.	.	.	.	.	.	.	.
Ta/d-1.1	.	.	.	.	.	.	.	.	.	.	.	.
Ta/d-2	.	.	.	.	.	.	T	.	.	.	.	.
Ta/d-2.1	.	.	.	.	.	.	T	.	.	.	.	.
Modern wheat seeds												
Ta1*	C	A	.	.	.	.	T	A	C	.	C	.
Ta2	C	.	.	.	.	.	.	.	.	.	.	.
Ta3	G	.		A	T	.	.	.	.	.	.	.

Table 4.8. (continued)

Ta4*	.	A	.	.	.	.	T	.	.	A	.	.
Ta*	C	A	G	.	.	.	T	A	.	.	.	.
Td*	.	A	.	.	.	C	.	.	.	.	A	A
Triticum Spp.	53	57	58	114	115	118	120	123	124	140	142	143
KM116485.1	T	C	A	A	T	C	T	T	C	C	A	G
JQ689004.1	.	.	.	.	.	.	.	.	.	.	.	.
Ancient wheat seeds												
Ta/d-1	.	.	.	.	.	.	.	.	.	.	.	.
Ta/d-1.1	.	.	.	.	.	.	.	.	.	.	.	.
Ta/d-2	.	.	G	C	C	.	C	C	.	-	-	-
Ta/d-2.1	.	.	G	C	C	.	C	C	.	-	-	-
Modern wheat seeds												
Ta1*	G	A	G	T	.	T	.	.	T	A	C	C
Ta2	.	.	.	-	-	-	-	-	-	-	-	-
Ta3	.	.	.	.	.	.	.	.	.	.	.	A
Ta4*	G	.	.	.	.	.	.	.	.	.	C	C
Ta*	G	.	G	.	.	.	.	.	T	A	C	C
Td*	.	.	.	.	.	.	.	.	.	.	C	C

* labels the samples amplified by Glu-160 primer.

Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

4.3.3.2. Glu-240 Region

For the Glu-240 region, all sequences are aligned with the Blast sequences of the *GluB1* gene with one exception (Table 4.9). Amplified sequences of ancient wheat Ta/d-1 had aligned to sequences of the *GluA1.1* gene with identity of 98.1% to *T. aestivum* (KM116478.1) and 97.56% to *T. durum* (JQ689002.1). Amplified

sequences of ancient wheat Ta/d-2 and their contemporaries are aligned to the sequences of the *GluB1*. Ancient wheat Ta/d-2 aligned with identity of 100% to the *T. aestivum* (KM116486.1) and 95.18% to the *T. durum* (JQ689005.1) (Table 4.9).

Table 4. 9. The identity comparisons for the Glu240 region with the highest aligned sequences retrieved from the NCBI database

	Genebank accession number	Query cover (%)	E value	Identity (%)
(a) Identity comparison of the Glu240 sequences				
Ta/d-1 versus *T. aestivum Glu A1-1*	KM116478.1	89	2e- 79	98
Ta/d-2 versus *T. aestivum Glu B1-2*	KM116486.1	98	7e- 134	100
Ta/d-1 versus *T. durum Glu A1-1*	JQ689002.1	89	9e- 78	98
Ta/d-2 versus *T. durum Glu B1-2*	JQ689005.1	98	1e- 100	95
(b) Identity comparison of the Glu240 sequences				
Ta1 versus *T. aestivum Glu B1-2*	KM116486.1	92	4e- 102	94
Ta2 versus *T. aestivum Glu B1-2*	KM116486.1	93	1e- 94	96
Ta3 versus *T. aestivum Glu B1-2*	KM116486.1	93	1e- 98	93
Ta4 versus *T. aestivum Glu B1-2*	KM116486.1	92	4e- 102	94
Ta versus *T. aestivum Glu B1-2*	KM116486.1	92	2e- 119	98
Td versus *T. aestivum Glu B1-2*	KM116486.1	92	7e- 108	96
Ta1 versus *T. durum Glu B1-2*	JQ689005.1	92	3e- 88	91
Ta2 versus *T. durum Glu B1-2*	JQ689005.1	92	2e- 112	98
Ta3 versus *T. durum Glu B1-2*	JQ689005.1	93	3e- 114	97
Ta4 versus *T. durum Glu B1-2*	JQ689005.1	93	2e- 112	97
Ta versus *T. durum Glu B1-2*	JQ689005.1	92	1e- 98	93
Td versus *T. durum Glu B1-2*	JQ689005.1	92	1e- 95	93

Four base substitutions were observed between sequences of ancient wheat Ta/d-1 and *T. aestivum* at the base positions 49, 143, 145 and 151, whereas an additional nucleotide change between *T. durum* at the base position 235 (Table 4.10). Ancient wheat Ta/d-2 had eight nucleotide substitutions at the base positions of 16, 88, 134, 143, 145, 146, 167 and 213 when aligned to the *T. durum* (JQ689005.1) (Table 4.11).

Table 4. 10. Glu-240 region sequence alignments of the ancient wheat Ta/d-1

Sequence ID	49	143	145	151	235
KM116478.1 *T.aestivum GluA1-1*	A	T	T	T	T
JQ689002.1 *T. durum GluA1-1*	.	.	.	.	C
Ancient wheat seeds					
Ta/d-1	T	C	C	C	.
Ta/d-1.1	T	C	C	C	.

Identical bases were marked by dots. The numbers above sequences indicate the base positions. Ta/d-1: ancient wheat seed 1 (*T. aestivum/durum*)

Table 4. 11. Glu-240 region sequence alignments of the ancient wheat Ta/d-2 and modern seeds

Sequence ID	14	16	17	29	33	76	88	134	143	145	146
KM116486 *T.aestivum GluB1-2*	A	T	G	G	T	T	G	A	A	A	C
JQ689005.1 *T. durum GluB1-2*	.	C	.	.	.	.	T	G	T	G	T
Ancient wheat seed											
Ta/d-2	.	.	.	.	.	.	.	.	.	.	.
Ta/d-2.1	.	.	.	.	.	.	.	.	.	.	.
Modern wheat											
Ta1	.	G	T	.	A	G	C	.	T	.	.
Ta2	.	G	T	.	.	G	.	.	T	.	.
Ta3	C	G	T	.	A	.	.	.	T	.	.
Ta4	C	G	.	.	A	.	C	.	.	.	.
Ta	C	.	.	.	.	.	.	.	.	.	.
Td	C	G	T	T	.	.	.	G	T	G	T

Table 4.11. (continued)

Sequence ID	167	179	196	197	198	203	205	206	213	216
KM116486	C	A	C	A	A	T	T	C	C	A
JQ689005.1	T	.	.	.	.	.	.	.	T	.
Ancient wheat seed										
Ta/d-2	.	.	.	.	.	.	.	.	.	.
Ta/d-2.1	.	.	.	.	.	.	.	.	.	.
Modern wheat										
Ta1	.	T	T	.	G	C	.	T	.	T
Ta2	.	T	.	.	.	C	.	T	.	.
Ta3	.	.	.	T	.	.	.	.	.	T
Ta4	.	.	.	T	G	C	.	.	.	.
Ta	.	.	.	.	.	C	.	.	.	T
Td	.	.	.	.	.	.	C	.	T	.

Identical bases were marked by dots. The numbers above sequences indicate the base positions. Ta/d-2: ancient wheat seed (*T. aestivum/durum*); Ta and Td: reference seeds (*T. aestivum* and *T. durum*); Ta1, Ta2, Ta3 and Ta4: modern wheat seed (*T. aestivum*).

4.3.4. The IGS Gene Region

For the IGS rDNA region, only the bands of ancient wheat were purified from the gel and sent for sequencing. Only ancient wheat seed Ta/d-1 can be successfully sequenced. Amplified sequence has been aligned with identity of 100% to different species' sequences from the NCBI database; *Aegilops tauschii* (KF482112.1), *T. spelta* (AF147500.1) and *T. aestivum* (M372699) (Table 4.11 and Table 4.12).

Table 4. 12. The identity comparisons for the IGS region with the highest aligned sequences retrieved from the NCBI database

	Genebank accession number	Query cover (%)	E value	Identity (%)
(a) Identity comparison of the IGS rDNA sequences of ancient seeds with the sequences from the database				
Ta/d-1 versus *Triticum aestivum*	M37269.1	97	1e- 42	100
Ta/d-1 versus *Aegilops tauschii*	KF482112.1	97	4e- 42	100
Ta/d-1 versus *Triticum spelta*	AF147500.1	97	1e- 42	100

Table 4. 13. IGS region partial sequence alignments of the ancient wheat Ta/d-1

Sequence ID	1	15	78	89	98
KF482112.1 *Aegilops tauschii*- External Transcribed Spacer	G	C	C	A	A
AF147500.1 *Triticum spelta* L. 26S-18S Intergenic Region	.	.	.	.	.
M372699 *Triticum aestivum* NOR D3 locus	.	.	.	.	.
Ta/d-1	.	.	.	.	.
Ta/d-1.1	.	.	.	.	.

Identical bases were marked by dots. The numbers above sequences indicate the base positions. Ta/d-1: ancient wheat seed (*T. aestivum/durum*)

According to phylogenetic analysis performed among ancient and modern wheat species, the length of the 26S rDNA was found to be 151 bases long with 68.66% GC content. Nucleotide diversity between 11 sequences were 0.035. The length of the ITS2 rDNA sequences were 196 bases long with 60.2% GC content. Nucleotide diversity between ancient and modern wheat sequences are found to be 0.035. The length of the Glu-140 region is 144 bases long with 47.92% GC content. Nucleotide diversity for the Glu-140 region is 0.045, and only 14 of the total 23 variable sites are parsimony informative. The amplified sequence of the Glu-240

Glu-A1 region had a length of 262 bases and a GC content of 48.47%. The nucleotide diversity within this region is 0.012 among 4 sequences. The length of the Glu-240 *Glu-B1* region was 238 bases long with 45.3% GC content. Nucleotide diversity for the Glu-240 region is 0.195 between the 12 sequences, and only 16 of the total 31 variable sites are parsimony informative sites. For the IGS partial sequences, the GC content is 58.12% and nucleotide diversity is zero (Table 4.13).

Table 4. 14. Estimated molecular diversity parameters based on the sequences of ancient wheat seeds from Kaymakçı and their contemporary

	26s rDNA	ITS2	Glu-140 *Glu-B1*	Glu-240 *Glu-B1*	Glu-240 *Glu-A1*	IGS
Number of sequences*	11	10	12	10	3	4
Sequence length (bp)	151	196	144	238	262	117
GC content (%)	68.66	60.2	47.92	45.3	48.47	58.12
Conserved sites	140	194	121	207	257	117
Variable sites	11	2	23	31	5	0
Parsimony informative sites	11	1	14	16	0	0
Number of indels	0	0	0	0	0	0
Nucleotide diversity	0.035	0.035	0.045	0.195	0.012	0

*The ‘‘number of sequences’’ include ancient and contemporary samples of the same species and the sequences of the same species or close relatives of the studied species available from the NCBI database

CHAPTER 5

DISCUSSION

The analysis of ancient DNA (*a*DNA) has emerged as a crucial and valuable resource for studying the genetic modifications undergone by cultivated species throughout the course of agricultural history. *a*DNA studies can provide insights into the genetic diversity of crop species in the past, including ancestral varieties and wild relatives that may have possessed traits that are no longer present in modern cultivated varieties (Allaby et al., 1997; Kistler, 2018).

Extracting DNA from ancient plant material can be challenging. Over time DNA degrades and fragments in harsh environmental conditions such as fluctuating temperatures and exposure to humidity (Allaby et al., 1997; Hovsepyan & Willcox, 2008). Researchers have developed specialized methods to extract good-quality DNA from archaeobotanical remains (Kistler & Saphiro, 2011; Gismondi et al., 2016; Çiftçi et al., 2019). Among all plant tissues, seeds and grains are particularly advantageous in crop species due to their relatively high DNA content. The inherent characteristics of seeds contribute to their long-term preservation and successful retrieval, making them favorable for genetic analysis (Lister et al., 2008; Değirmenci et al., 2022).

The wheat seeds excavated from Kaymakçı archeological site have been successfully isolated with relatively good quality. One of the reasons is the graphical location of the archeological site. Due to the altitude of Kaymakçı and its distance from the Marmara Lake, ancient seeds remained far from the wetland, and the conditions in which they were buried prevented them from being exposed to humidity and annual temperature changes. As a result, although the wheat seeds were charred, they were well preserved. Another reason, after the Late Bronze Age,

no permanent community settled in this area, meaning that subsequent civilizations did not disturb or build upon the site. As a result, the original layers and artifacts have been preserved well, allowing archaeologists to study and interpret them effectively (Roosevelt et al., 2015; Çiftçi et al., 2019).

Out of the two ancient DNA extraction methods that were used in this study, the modified CTAB method developed by Kistler & Saphiro (2011) resulted in higher DNA concentrations compared to the modified CTAB method developed by Gismondi et al. (2016). The reason for high concentration of DNA may be the DNA contamination from external sources. However, the negative controls showed no presence of DNA, showing the absence of contamination during the extraction process. However, extracted *a*DNA is very fragmented; hence the selection of DNA markers are made according to their high copy number, length of the region and highly polymorphic genetic loci. Also, these regions have been selected due to their effectiveness of identifying species and used in previous studies (Allaby et al., 1997; Brown et al., 1998; Gugerli et al., 2005; Lister et al., 2008; Li et al., 2011; Oliveira et al., 2012; Fernández et al., 2013; Çiftçi et al., 2019; Değirmenci et al., 2022).

In this study, all selected gene regions are in the nuclear genome. Although chloroplast markers have been traditionally favored due to the presence of multiple copies of the chloroplast genome per cell, nuclear DNA provides much more information on a plant species developmental history and allows for the exploration of qualitative traits and phenotypes (Zeder et al., 2006; Koch et al., 2006; Calonje, 2009). Non-coding nuclear DNA regions give more precise information about recent evolutionary processes, such as the domestication of crop species or recent historical distributions (Lister et al., 2008).

Ribosomal RNA (rRNA) genes and spacer regions are generally used for phylogenetic analysis. They are in tandem arrays with consecutive gene blocks (18S, 5.8S, and 26S) in eukaryotic cells. ITS1 and ITS2 separate the rDNA genes

and intergenic spacers (IGS) separate these gene blocks and play regulatory roles in rRNA transcription and processing (Poczai & Hyvönen, 2010). These regions used to study evolutionary relationships, classify species, and understand genetic diversity.

The 26s nuclear rDNA region proves to be particularly suited for ancient plant DNA studies due to its small size and multiple copies in the genome (Kuzoff et al., 1998). The sequences of 26s rDNA of ancient wheat seeds Ta/d-1 and Ta/d-2 aligned with each other with only three nucleotide changes showing very high similarity. Moreover, ancient sequences show 11 base substitutions in 151 base pair sequences when aligned with the sequence of *T. aestivum* (MK413186.1) from the NCBI database with a 96% match. These base substitutions may be the result of single nucleotide mutations. The GC content of 26s rDNA is 68.66% indicating a more conserved region. Observed high levels of homology between ancient and modern samples are expected. Even though 26s rDNA has rapid evolution compared to conserved protein-coding regions, many differences are not anticipated within the *Triticum* genus. As a result, this region remains a reliable and informative marker for differentiating the taxa in familia *Poaceae*.

The alignments of the sequences are made with the ITS2 region of both *T. aestivum* and *T. durum* available in NCBI (MF480399.1, MK612075.1). The ancient sequences are aligned perfectly (identity 100%) with both *T. aestivum* and *T. durum.* Since the sequences of ITS2 region of both species are identical, it is not possible to identify the species of the ancient wheat. Whereas the ancient sequences are aligned *Aegilops tauschii* (MK387303.1) with identity %95 showing the Kaymakçı ancient wheat samples very likely belong to the *Triticum* genus. This result also shows success of ITS2 region in the identification of species in the genus *Triticum* and *Aegilops* (Chen et al., 2010; Ganolopous et al., 2017). The ITS2 region has 60.2% GC content, similar to the 26s rDNA region, although slightly lower, indicating a highly conserved region. Observed variety makes this region a valuable marker for distinguishing between closely related plant species

by showing high diversity between species of *Triticum* and *Aegilops* (Baldwin, 1992).

The study conducted by Oliveira et al. (2012) found that ancient wheat samples from pre-Hispanic times on the island of Gran Canaria, Spain, showed 100% homology with ITS2 marker of *T. durum* and *T. aestivum* confirming our results and indicating this marker is inadequate for identification of naked (free-threshing) archeological wheats (*T. aestivum/durum)*.

The IGS region is particularly important for the species that cannot be identified morphologically, such as ancient charred naked wheat seeds. These ancient wheats are undetermined and labeled as *T. aestivum/durum*. In the D genome of hexaploid wheat, the IGS region has a 71-base pair insertion (Brown et al., 1994; Sallares et al., 1999; Li et al., 2011; Oliveira et al., 2012). The inserted region of IGS is highly conserved and used for verifying the presence of the D genome. Therefore, the expected sizes of the PCR products of IGS primers for the D genome is 158 bp and the A/B genome is 87 bp (Li et al., 2011).

The absence of the D genome-specific band (158 bp) on the gel may be due to DNA degradation or preferential amplification of the smaller 87 bp product, not due to the absence of the D genome in ancient wheat (Oliveira et al., 2012; Değirmenci et al., 2022). Hence, ancient wheat samples were subjected to different PCR conditions to ensure the presence or absence of the D genome-specific band (Appendix B).

For the IGS rDNA region, only the bands of ancient wheat were purified from the gel and sent for sequencing. This purification step was essential to minimize any potential contamination or noise that could affect the accuracy of the resulting sequence data. However, only ancient wheat seed Ta/d-1 was successfully sequenced. For the ancient wheat seed Ta/d-2, the chromatogram data cannot be

analyzed. This could be due to the insufficient quantity or degradation of the PCR product or problems during the purification step.

Amplified sequence of Ta/d-1 is 117 base pair in length and has been aligned perfectly (identity of 100%) with different species' IGS sequences available from the NCBI database; *Aegilops tauschii* (KF482112.1), *T. spelta* (AF147500.1) and *T. aestivum* (M372699). According to the alignment, ancient wheat seed Ta/d-1 is successfully identified as *T. aestivum.* For the ancient wheat seed Ta/d-2, although the sequencing has not been done successfully, the presence of approx. 87 and 158 bands on the gel photos (Appendix B) indicates that it is also *T. aestivum.*

Glu-140 and Glu-240 primers have been designed from upstream of the *Glu - Bx (GluB1)* HMW glutenin gene of the *T. turgidum* (Lister et al., 2008). Glu-240 primers amplify the same region as the Glu-140 primers and have an additional 100 base pairs upstream. These primers share a reverse primer, and they are previously shown to successfully amplify multiple alleles of the glutenin locus, which also increases the amplification success in ancient wheat seeds (Lister et al., 2008). However, for modern wheat samples, it causes multiple similar size PCR products in each amplification and therefore problems in sequencing.

Even with the bad quality of the modern seed sequences, many of the Glu-240 sequences successfully aligned to the NCBI database sequences. However, some Glu-140 sequences from modern and reference wheat seeds have a very poor quality. To overcome this problem, a new primer (Glu-160) was designed from the *GluB1-1* gene of the *T. aestivum* (AJ567980). Because it has a specific reverse primer, the sequences of modern seeds had a good quality and aligned nearly perfectly with the sequences from the NCBI database.

Previous ancient DNA studies show that due to degradation, there are specific types of nucleotide changes in the ancient DNA. The most commonly observed ones are C-to-T and G-to-A transitions due to post-mortem DNA deamination

(Stiller et al., 2006; Briggs et al., 2010; Fernandez et al., 2013; Lan & Lindqvist, 2019). From six base substitutions of ancient wheat 5 for the Glu140 primer, only one may be a C-to-T transition could be due to deamination, showing that the other single nucleotide changes are most likely not from DNA degradation but due to evolutionary change and allelic variation.

The ancient wheat seed Ta/d-1 has the identical sequence to the aligned blast samples for *T. aestivum* and *T.durum*. The study conducted by Oliveira et al. (2012) found that these ancient wheat samples from Spain, showed 100% homology with the 140 bp fragment of the *Glu-Bx* gene of *T. durum* and *T. aestivum*. Another study also found 100% identity between ancient wheat samples and Blast sequences of *GluBx* gene in *T. turgidum* (Lister et al., 2008). These results indicate this marker is inadequate for differentiation of *T. aestivum/durum*.
For the Glu-240 region, all sequences are aligned with the sequences of the *GluB1* gene with one exception of the ancient wheat seed Ta/d-1 which is aligned to sequences of the *GluA1.1* gene. Alignment of different loci of the HMW genes is expected because the primers can recognize each locus, but have a preference for *Glu-Bx,* where it had been designed from the study of Lister et al. (2008). This study showed that the *Glu* primers successfully amplified both *GluA1* and *GluB1* upstream regions of ancient wheat samples. Degradation of the primer binding region in *GluB1.2* can be the reason why only one of the ancient wheat sequences is aligned to a different *Glu* gene.

Amplified sequences of ancient wheat seed Ta/d-1 had aligned to sequences of the *GluA1.1* gene. There are four base substitutions between sequences of ancient wheat 1 and *T. aestivum*, whereas five nucleotide changes between *T. durum.* None of the substitutions are due to post-mortem DNA deamination. However, none of the variable sites are also parsimony informative sites.

Amplified sequences of ancient wheat seed Ta/d-2 and their contemporary relatives were aligned to the sequences of the *GluB1.* The ancient wheat seed Ta/d-2 aligned

with identity of 100% to the *T. aestivum* (KM116486) and 95.18% to the *T. durum* (JQ689005.1). This result indicates that ancient wheat seed Ta/d-2 is most likely to be *T. aestivum.* Nucleotide differences that were observed in the contemporary relatives' sequences due to high variety shown in different subspecies of *T. aestivum*.

The HMW glutenin locus, which comes in several alleles, has been extensively studied in aDNA isolated from charred wheat grains excavated from archaeological sites and historical specimens. The diversity of HMW glutenin sequences plays a pivotal role in wheat breeding, dough quality improvement, and understanding the evolutionary history of wheat. Wheat aDNA studies (Allaby et al., 1994; Schlumbaum et al., 1998; Lister et al., 2008; Oliveira et al., 2012) underscore the significance of understanding and utilizing the HMW glutenin locus to advance wheat-related research and applications.

CHAPTER 6

CONCLUSION

Present study investigated the genetic polymorphism and relationships among ancient wheat and their contemporaries using molecular markers 26S rDNA, ITS2, IGS and HMW glutenin gene regions. The study included two ancient wheat seeds of *Triticum aestivum/durum* excavated from Kaymakçı archaeological site, four modern *T. aestivum* varieties that were gathered from the Gediz Valley or acquired from Gema Foundation and two reference wheat seeds *T. aestivum* and *T. durum*. The results showed that the genetic markers effectively distinguished the different species of ancient wheat. The amplified 26S rDNA region showed the highest level of genetic polymorphism among the samples, while the ITS2 marker showed the lowest level of polymorphism.

In addition to the molecular markers mentioned above, the intergenic spacer (IGS) region has also been used to investigate the presence or absence of the D genome in ancient wheat. Although sequencing of the IGS region resulted in the successful alignment of the D genome in only one of the two ancient wheat samples, the gel photos revealed the presence of the D genome in both specimens. The study results revealed that two ancient wheat seeds, previously designated as *T. aestivum/durum* based on morphological characteristics, were molecularly identified as *T. aestivum*. This approach allowed for a more thorough assessment of the genetic relationships and differentiation patterns among the ancient wheat species, enabling a deeper understanding of their domestication history.

The discovery of well-preserved ancient charred wheat grains in the Kaymakçı archaeological site, along with the successful extraction and analysis of their ancient DNA, has provided valuable insights into the cultivation and domestication

of wheat. The use of molecular techniques, such as nDNA markers, has enabled us to identify the hexaploid wheat species present in the archaeological samples.

Overall, our study underscores the significance of molecular marker analysis in uncovering the genetic diversity and relationships within ancient wheat populations. The success of this study also suggests a great potential for further research on ancient plant DNA, which could help shed light on the history and domestication of wheat in Türkiye. Further investigations, employing additional markers and larger sample sizes, could provide a more comprehensive understanding of the genetic landscape of ancient wheat and its implications for wheat breeding, agricultural productivity, and resilience in the face of changing environments.

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APPENDICES

A. CHROMATOGRAM DATA EXAMPLES OF ANCIENT SAMPLES

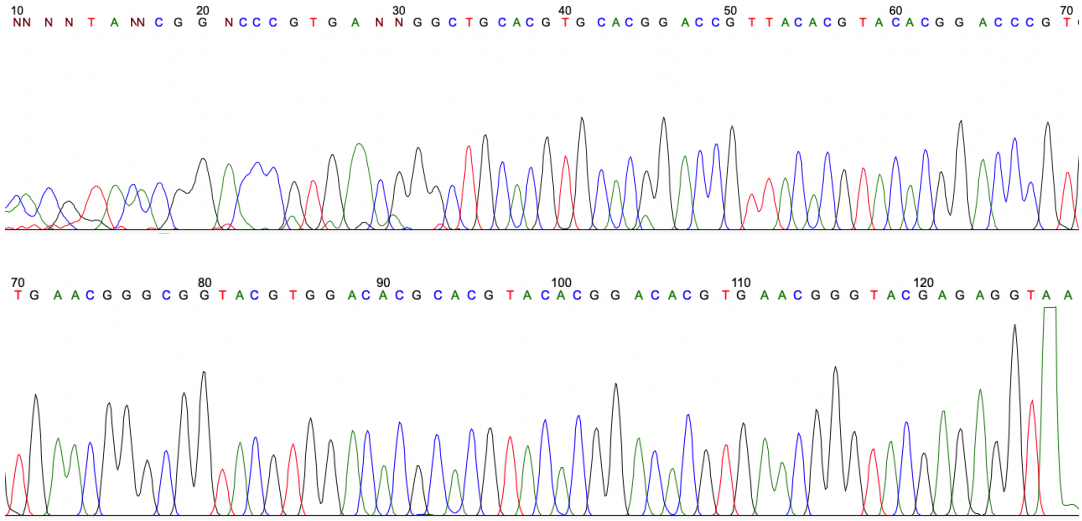

Figure A.1 Chromatogram of IGS region of ancient wheat Ta/d-1

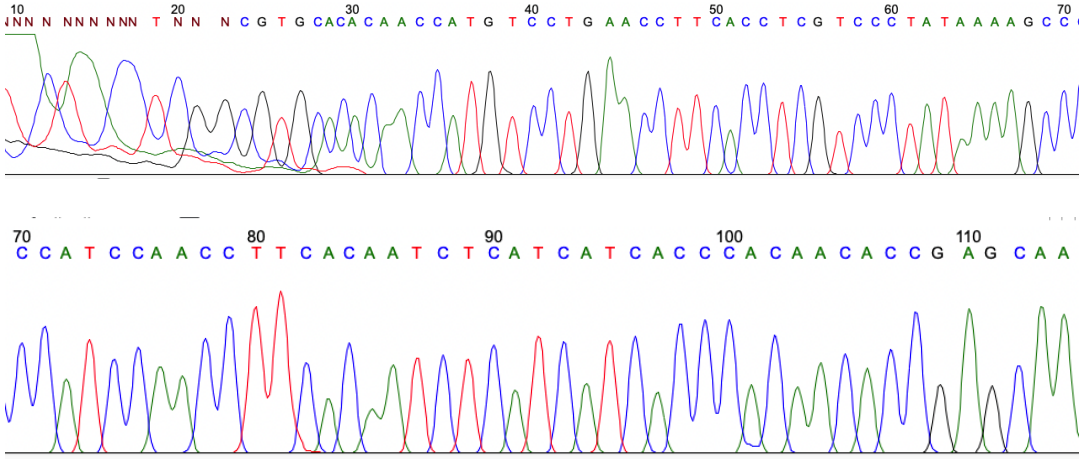

Figure A.2 Chromatogram of Glu-140 region of ancient wheat Ta/d-1

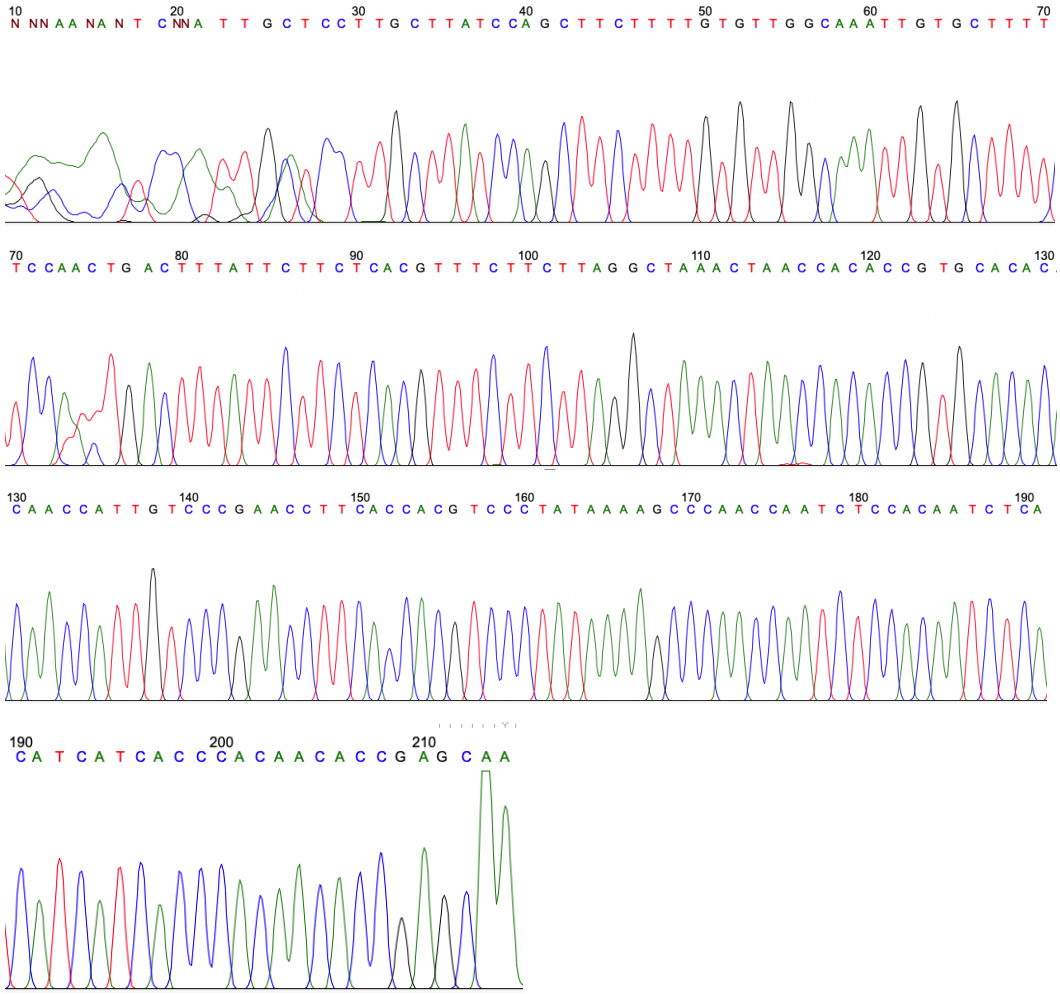

Figure A.3 Chromatogram of Glu-240 region of ancient wheat Ta/d-2

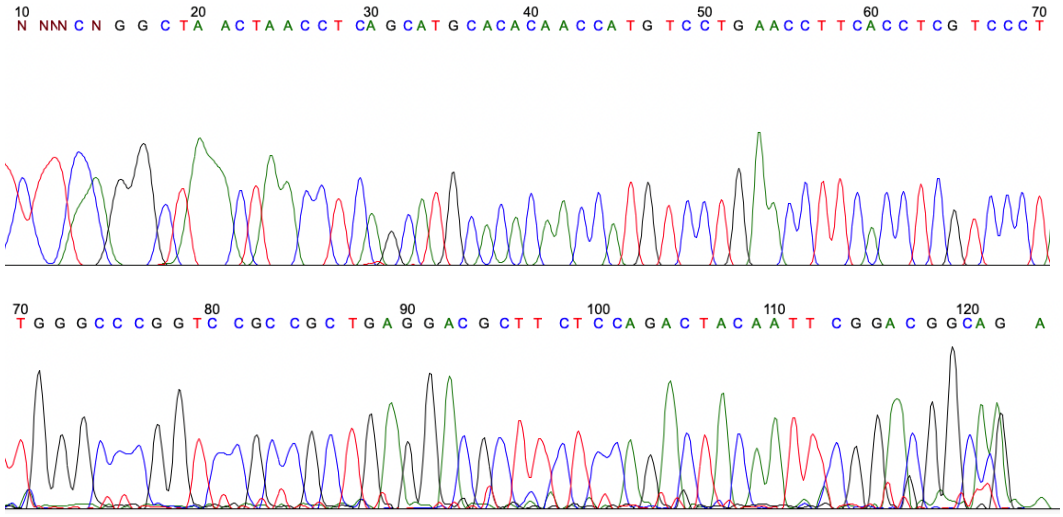

Figure A.4 Chromatogram of 26S region of ancient wheat Ta/d-1

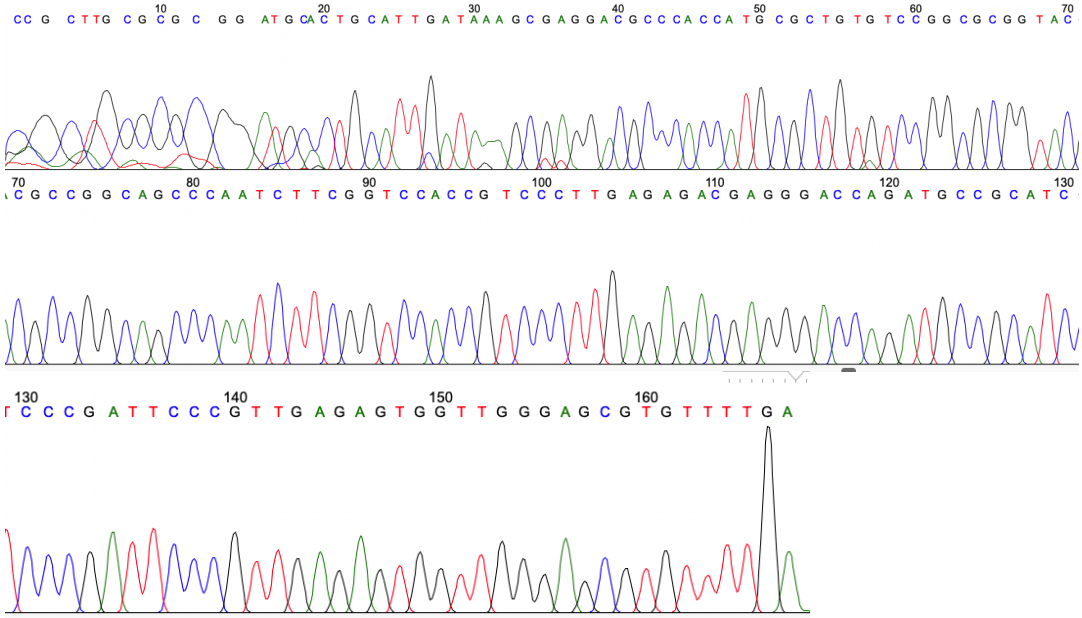

Figure A.4 Chromatogram of ITS2 region of ancient wheat Ta/d-2

B. PCR CONDITIONS AND CYCLINGS OF IGS REGION

Table B. Different PCR conditions and cycling parameters for charred wheat seeds

Primer	Components	Volume (µL)	PCR Conditions		
IGS	dH_2O	13	Initial Denaturation 5 min		95°C
1. Cond.	Master Mix	5	35 cycles	Denaturation 45 sec	95°C
	Primers (10µM)	0.5+0.5		Annealing 45 sec	60°C
	DNA (10ng/µL)	6		Extension 45 sec	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	14	Initial Denaturation 5 min		95°C
2. Cond.	Master Mix	4	35 cycles	Denaturation 30 sec	95°C
	Primers (10µM)	0.5+0.5		Annealing 30 sec	62°C
	DNA (10ng/µL)	6		Extension 45 sec	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	12.7	Initial Denaturation 5 min		95°C
3. Cond.	Master Mix	3.5	35 cycles	Denaturation 1 min	94°C
	Primers (10µM)	0.4+0.4		Annealing 1 min	60°C
	DNA (10ng/µL)	5		Extension 1 min	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	9.4	Initial Denaturation 5 min		95°C
4. Cond.	Master Mix	4	40 cycles	Denaturation 45 sec	95°C
	Primers (10µM)	0.4+0.4		Annealing 45 sec	61°C
	DNA (10ng/µL)	5		Extension 45 sec	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	12	Initial Denaturation 5 min		95°C

5. Cond.	Master Mix	4	45 cycles	Denaturation 1 min	95°C
	Primers (10µM)	0.5+0.5		Annealing 1 min	60°C
	DNA (10ng/µL)	5		Extension 1 min	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	12.8	Initial Denaturation 5 min		95°C
6. Cond.	Master Mix	5	35 cycles	Denaturation 2 min	95°C
	Primers (10µM)	0.6+0.6		Annealing 1 min	60°C
	DNA (10ng/µL)	6		Extension 1 min 45 sec	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	10.7	Initial Denaturation 5 min		95°C
7. Cond.	Master Mix	3.5	35 cycles	Denaturation 2 min	95°C
	Primers (10µM)	0.4+0.4		Annealing 1 min	60°C
	DNA (10ng/µL)	5		Extension 1 min 45 sec	72°C
	Total	25	Final Extension 10 min		72°C
IGS	dH_2O	13	Initial Denaturation 5 min		95°C
8. Cond.	Master Mix	5	45 cycles	Denaturation 1 min	95°C
	Primers (10µM)	0.5+0.5		Annealing 1 min	56°C
	DNA (10ng/µL)	6		Extension 1 min	72°C
	Total	25	Final Extension 10 min		72°C

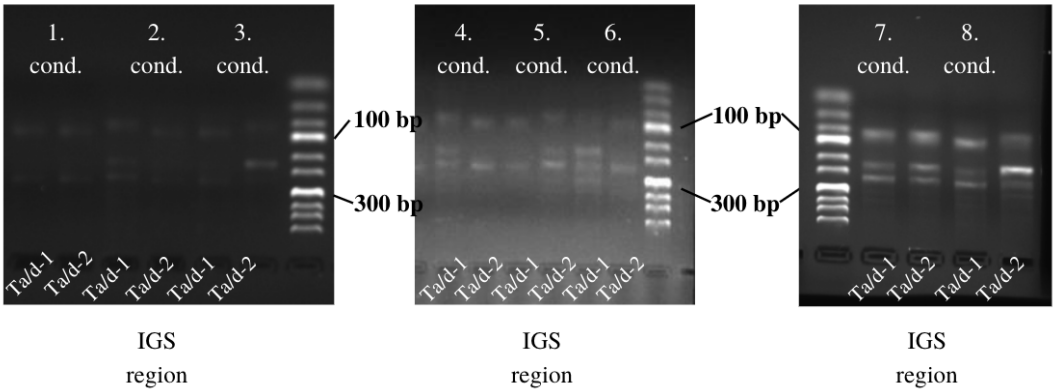

Fig. B1 Amplification results from different conditions for the IGS gene region (Ta/d-1 and Ta/d-2: ancient wheat seeds (*T. aestivum/durum*)).