ASSESSMENT OF PLANT BIODIVERSITY FROM 3500-YEAR-OLD CERAMICS RECOVERED FROM THE ARCHAEOLOGICAL SITE OF KAYMAKÇI USING A METAGENOMICS ANALYTICAL APPROACH

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ABSTRACT

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This research delves into the connection between communities residing in the Kaymakçı citadel and their natural environment. By studying the ceramics found at the site using metagenomics, the goal was to assess the diversity of plants at genus level. Through analysis focusing on the *rbc*L (Ribulose-1,5-bisphosphate carboxylase/oxygenase Large Subunit) gene, a variety of plant species were identified, with *Populus* and *Quercus* being prominent among 95 plant genera.

The *rbc*L gene sequence based phylogenetic tree derived from the ceramic samples suggests that the 33 labeled ceramic pieces (KAP2- KAP37) fall into four groups which are Cluster 1.a, 1.b, 1.c and 2. These groups are categorized based on the types of plants found and how often they appear; The first group is mainly *Papaver*, the second is mostly *Populus*, the third is dominated by *Quercus* and the fourth is predominantly *Apium*. This classification prompts archaeologists to investigate further to understand the connections, in terms of location and time periods. This study highlights the importance of investigation in archaeology offering insights into plant diversity and environmental intricacies.

Keywords: Metagenomics Analysis, Ceramics, Ancient Plant Biodiversity, *rbc*L Gene, Kaymakçı Archaeological Site

ÖZ

KAYMAKÇI ARKEOLOJİK ALANINDAN ELDE EDİLEN 3500 YILLIK SERAMİKLERDEN METAGENOMİK ANALİZ YAKLAŞIMI İLE BİTKİ ÇEŞİTLİLİĞİNİN DEĞERLENDİRİLMESİ

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Bu araştırma, Kaymakçı kalesinde yaşayan toplumların doğal çevreleri ile olan bağlantısını inceliyor. Çalışma, yerleşimde bulunan seramiklerin metagenomik kullanarak incelenmesi yoluyla, bitkilerin çeşitliliğinin, cins seviyesinde değerlendirilmesi amaçlanmaktadır. *rbc*L genine odaklanan analizler sonucunda, çeşitli bitki türlerinin belirlendiği görüldü; *Populus* ve *Quercus* başta olmak üzere 95'ten fazla diğer cinsin yer aldığı tespit edildi. Seramiklerden oluşturulan antik *rbc*L gen dizilerine dayanan filogenetik ağaç, 33 etiketli seramiğin (KAP2-KAP37) Küme 1.a, 1.b, 1.c ve 2 olarak dört kategoriye ayrıldığını göstermektedir. Bu kategoriler tür kompozisyonlarına ve sıklıklarına göre oluşturulmuştur: Küme 1'de *Papaver*, Küme 2'de *Populus*, Küme 3'te *Quercus* ve Küme 4'te *Apium* hakimdir. Bu gruplandırma, arkeologların, bu kümelerin mekansal ve zamansal değerlendirmesi için araştırma yapmalarını teşvik eder. Bu çalışma, bitki çeşitliliği ve çevresel karmaşıklıkların ortaya çıkarılmasında arkeolojik çalışmaların önemini gösterir.

Anahtar Kelimeler: Metagenom analizi, seramik antik bitki biyoçeşitliliği, *rbc*L geni, Kaymakçı arkeolojik alanı

to my family and all women in science

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ABBREVIATIONS

aDNA	: ancient DNA
BLAST	: Basic Local Alignment Search Tool
BOLD	: Barcode of Life Data System
Buffer SL	: Lysis buffer SL
CD-Hit	: Clustering by Fast Search and Hierarchy Construction using k-Mer Hits
CTAB	: Cetyltrimethylammonium Bromide
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylene Diamine Tetra Acetic Acid Disodium Salt
ITS	: Internal Transcribed Spacer
IUPAC	: International Union of Pure and Applied Chemistry
KAP	: the Kaymakçı Archaeological Project
LBA	: Late Bronze Age
Lyse SL	: Lysis buffer
*mat*K	: Maturase K
NCBI	: National Center for Biotechnology Information
NGS	: Next- Generation Sequencing
OTU	: Operational Taxonomic Units
PCR	: Polymerase Chain Reaction
PNG	: Portable Network Graphics
eDNA	: environmental DNA
PR buffer	: Proteinase K reaction buffer
QIIME2	: Quantitative Insights Into Microbial Ecology 2
*rbc*L	: Ribulose-1,5-bisphosphate carboxylase/oxygenase Large Subunit
RLB	: Reverse Line Blot
RNA	: Ribonucleic Acid
rpm	: Revolutions per minute

SDS	: Sodium Dodecyl Sulphate
sedaDNA	: sedimentary ancient DNA
sedDNA	: sedimentary DNA
SILVA	: Systematic Identification, aLignment and Visualization of
Sol SL buffer	: Solubilization buffer
*trn*H-*psb*A	: Transfer RNA Histidine - Photosystem II D1 protein
UV	: Ultraviolet
Wash SLX1	: First wash buffer
Wash SLX2	: Second wash buffer

CHAPTER 1

INTRODUCTION

1.1. The Neolithic Transition: A global phenomenon of sedentary settlement and agricultural expansion

The Neolithic Revolution, which led to settled living and focused on agriculture began independently across times and places worldwide before spreading to all continents over time (Diamond & Bellwood, 2003; Gepts, 2004). Based on anthropological and archaeological findings, it is believed that the Neolithic Transition took place approximately 14,000 years ago in regions such as the southern Levant, northern Mesopotamia, southern and central Anatolia, the Taurus Zagros borders and northern Mesopotamia (Özdoğan, 2011; Riehl *et al*., 2013; Broushaki *et al.*, 2016). During this time, agriculture spread from its Mediterranean birthplace to Iran and Central Asia, bringing with it new crops, livestock, and farming implements. This agricultural method spread presumably as a result of herders migrating in search of pastures and population growth brought on by increased food production. Some movements were also influenced by migrations of herders looking for grazing lands (Bar-Yosef & Bar-Yosef Mayer, 2002, p.340). The Neolithic period began after the cold and dry Younger Dryas era ended, ushering in wetter climates that allowed trees and cereal grasses to propagate naturally. This recovery initially took place in the Mediterranean before spreading across the Near East over time. It took over a millennium for conditions, in Iran to become conducive for agriculture with a delay observed between Iran and Central Asia (Hole, 2004). The discoveries made at villages and farming methods provide a deep insight, into how agriculture evolved in the early stages the shift towards permanent settlements.

These observations play a role, in unraveling the beginnings of society and the influences that shaped its growth (Thompson, 2015).

The Neolithic Revolution from roaming hunter gatherer lifestyles to settled communities was influenced by a mix of changes such as development of farming, domestication of animals, technological progress and shifts in society. About 14,000 years ago, as the Earth warmed at the tail end of the Ice Age, wheat and barley thrived in the Fertile Crescent region sparking the beginnings of agriculture (Morrell & Clegg, 2007). This era also witnessed the taming of crops and animals like emmer wheat, einkorn wheat, barley, lentils, chickpeas, peas, flax along with pigs, goats, sheep and cattle. It led to the establishment of settlements supported by farming methods. These advancements played a role in transitioning from groups of wandering hunter gatherers to larger settlements based on agriculture and early forms of civilization (Martin-Merino, 2021). The Neolithic Revolution brought about surplus resources that facilitated trade networks and exchanges of goods and ideas among cultures while also leading to hierarchies taking root. This period marked a moment in history that laid the groundwork for future innovations seen during the Bronze Age and Iron Age as well, as paving way for early civilizations (Putterman, 2006).

Contrary to the belief that agriculture and animal domestication originated in the Fertile Crescent, evidence from archaeology, anthropology, and genetics suggests that these practices were carried out by societies across various regions of the Middle East. This indicates that agricultural development occurred in multiple areas rather than at a singular location in time. (Lazaridis et al., 2016). Among the eleven communities unearthed in Central Anatolia region, five show traces of techniques such as stone tool craftmanship, ceramic pot production in their remnants (Baird, 2012). It has been observed that there were both cross regional socials as well as economic exchanges, between these societies and other Neolithic communities located in the Fertile Crescent region (Gerard & Thissen, 2002; Özbaşaran, 2012). The Hittites nurtured trade ties across these regions to oversee

territories with trade routes, in Western Anatolia and establish links (Yakubovich, 2010).

1.2. Kaymakçı: A Middle Bronze Age hub and its agricultural significance

During the Middle Bronze Age, the Marmara Lake basin in the middle Gediz Valley was thought to have been a transport hub with numerous small settlements. Among these settlements, the Kaymakçı stood out as the largest (Figure 1.1). It had a castle, a residential area and scattered ruins. Given its size and significance, the Kaymakçı is thought to have served as the capital during this period (Shin *et al.*, 2021).

In order to investigate the archaeological site of the Kaymakçı, the Kaymakçı Archaeological Project (KAP) was initiated in 2013. During the excavations of KAP, a sequence of Late Bronze Age (LBA) fills, or layers of material and waste deposited over time, was discovered (Roosevelt *et al.*, 2018).

Excavations at the Kaymakçı since 2014 have uncovered semi-circular structures, streets, courtyards, and houses built of rock and earth. As in many other regions of central and western Anatolia, these semi-circular structures were thought to have served as grain silos. The semi-circular structures as grain silos are especially significant as they indicate a Bronze Age society that was highly adept at managing and storing surplus food (Roosevelt *et al.*, 2018).

The study of 263 soil samples from the Kaymakçı archeological site revealed plant species, which indicate that the people living there grew crops for both their animals and everyday needs. Barley (*Hordeum vulgare L.*), wheat (*Triticum aestivum/durum*), emmer wheat (*Triticum turgidum spp. dicoccum* (Schrank) Thell.), and einkorn (or spelt) wheat (*Triticum monococcum L.*) were among the grains grown at the time. Legumes were also found, including peas (*Lathyrus sativus L.),* chickpeas (*Cicer arietinum*), lentils (*Lens culinaris Medik.*), and bitter vetch (*Vicia ervillia*). Grape seeds were the only evidence for fruit cultivation. Based on radiocarbon analysis, the approximate age of these seeds was 3500 years (Roosevelt *et al.*, 2018).

An in-depth investigation of soil fertility, fertilization methods, and irrigation systems is made possible by analyzing soil samples from archaeological sites such as the Kaymakçı. These insights are essential for comprehending the development of agriculture. Plant development and yield are significantly influenced by soil fertility, underscoring the significance of appropriate soil management techniques for long-term productivity and sustainability (Yadav *et al.*, 2023).

Synthetic or organic fertilization both greatly increase soil fertility by giving plants the micro and macronutrients they need, because they release nutrients gradually, organic fertilizers—which are made from natural materials like compost and manure— help restore soil fertility over time, although at a slower pace. Conversely, synthetic fertilizers give instant benefits and quick absorption, but treating them carefully is necessary to prevent environmental pollution (Wolf, 2023).

Another important aspect influencing agricultural productivity and soil fertility is irrigation. It guarantees water availability, especially in areas with little natural rainfall, promoting soil health and agricultural growth. But the quality of irrigation water is crucial because too much salinity, acidity, or alkalinity can harm plant development and soil fertility (Wolf, 2023).

These farming methods have a significant impact on the local economy and social fabric. A community's wealth distribution may change because of greater commerce and economic activity brought on by higher agricultural output. In addition, the use of irrigation systems and sophisticated farming techniques point to the sophistication of ancient cultures' organizational structures and technological developments (Van Der Crabben & Rebler, 2023).

It is possible to gain insights into the living circumstances and everyday life organization of communities such as the Kaymakçı by comprehending its architectural elements, infrastructure systems, and water supply processes. The way that people live, work, and interact with the environment is reflected in the design of their residences, public spaces, and irrigation systems.

Studying how ancient societies stored their goods is crucial, for understanding their way of life. The way they built and used storage facilities like granaries, ceramic pots and other containers can tell us a lot about what they ate and how they lived. For instance, the designs of pots give us insights into the types of food and products they stored and used along with highlighting the technological advancements of that era. By examining the size, shape and distribution of these storage vessels we can uncover information about the produce grown their trade practices and even the societal hierarchy, in place (Peña-Chocarro *et al.*, 2015).

Moreover, the existence and complexity of storage systems can provide insights into the behaviors of the community such as how excess goods were managed and how assets were distributed and safeguarded in times of shortage (Bintliff, 2012b). By examining these storage methods in conjunction with infrastructure features, we may gain an understanding of the everyday routines, financial tactics and environmental adjustments made by the residents of the Kaymakçı and comparable societies.

In conclusion, a comprehensive understanding of past agricultural practices, and their effects on society evolution can be gained through the examination of soil samples from archaeological sites. We learn about the lives of our predecessors, how they interacted with their surroundings, and how human civilization developed via painstaking excavation and investigation (Van Der Crabben & Rebler, 2023).

Soil analysis findings are vital for uncovering the techniques used in societies (Janni, 2002) like the Kaymakçı. By studying soil quality, fertilizer application and irrigation methods archaeologists can deduce how civilizations farmed (Weiss & Bradley, 2001). These evaluations provide insights into the factors impacting soil fertility and agricultural efficiency in periods. For instance, comprehending the availability of fertilizers and the effectiveness of mineral supplements helps in understanding how ancient farmers chose fertilization methods (Marston *et al.*, 2015).

Similarly, investigating irrigation systems and their effects on crop yields and water usage offers knowledge on water management practices (McMahon, 2019). Analyzing how these systems influenced farming outcomes in previous civilizations allows for a grasp of the economy's role within the community (Lim, 2020). Soil analysis can unveil details about cultivated crops the intensity of farming activities and the sustainability of practices, over time (Helwing, 2003).

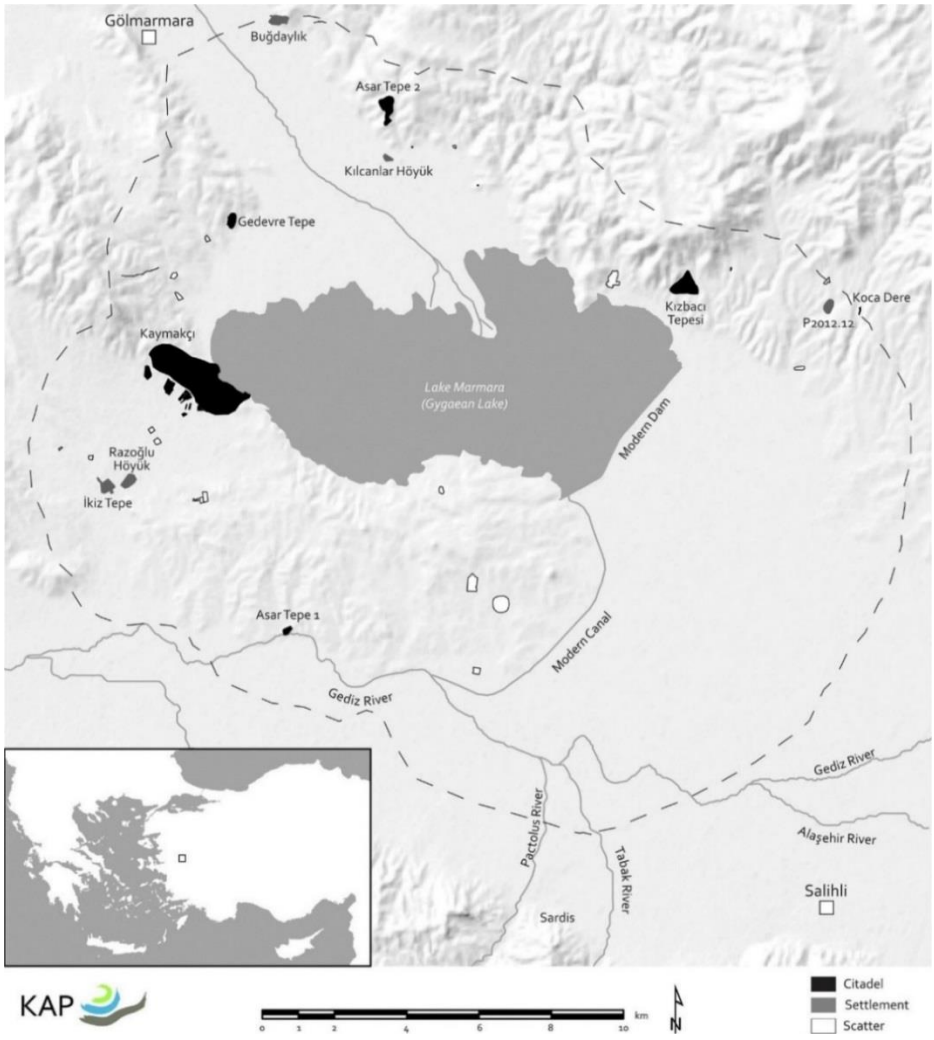

Figure 1. 1. Map showing the location of modern sites and the Kaymakçı in the Marmara Lake basin of the Gediz River valley (courtesy of the Kaymakçı Archaeological Project) (Ciftci et al., 2019).

Exploring how agriculture impacted trade networks, sources of income methods of product distribution and other economic aspects illuminates the dynamics of

societies (Lim, 2020). Understanding these aspects assists, in grasping how farming activities influenced trade networks, social hierarchies and economic exchanges within and outside the settlement (Helwing, 2003).

Moreover, exploring the components of infrastructure systems and water supply gives a perspective on living conditions and daily life organization (McMahon, 2019). The availability and caliber of water resources were crucial for operations factors significantly impacted by soil quality and management techniques (Janni, 2002). The extension of water distribution systems in eras benefited stakeholders, including suppliers, processors and agricultural enterprises (Weiss & Bradley, 2001). These advancements likely played a role in the expansion and sustainability of regions focused on agriculture by revitalizing farmlands and increasing the number of producers within the society (Marston *et al.*, 2015).

By merging soil analysis discoveries with contexts, we can reconstruct a precise and detailed portrayal of the agricultural and economic livelihoods of ancient societies (Janni, 2002; Helwing, 2003) such as Kaymakçı.

1.3. Analyzing ceramics for historical insights

Ceramics can be recognized by their chemical composition, which is also key to determining their age, origin and how well they have withstood damage from storage conditions (Chari, 2023). In archaeology, there has been a growing interest in the study of ceramics and the biological remains found within them due to their porosity and absorbency. Lipids and fatty acid ratios are among the studied substances in ceramics as well as the concentrations of these components in the samples being analyzed. In addition, the identification of food-specific chemicals is valuable for the study of ancient pottery (Kałużna-Czaplińska *et al.*, 2017).

The ancient pottery discovered at the site of the Kaymakçı, which dates back 3500 years offers insights into the technological advancements, craftsmanship and cultural traditions of that era. Although there are no specific patterns on the samples, symbols and designs on the ceramics unearthed at the Kaymakçı ceramic

analysis is a utilized method in studies to comprehend the material culture of past civilizations (Luke *et al.*, 2015). This kind of analysis can unveil details about the expertise of that time including the pottery making techniques employed types of clay utilized and methods of firing.

The classification of clay into coarse and fine categories reflects the multifunctional nature of the clay used in the ceramic by indicating its particle size. Aside from the fact that different jar, bowl, jug, and handled jar sizes and forms suited a variety of domestic demands, site characteristics may offer contextual hints on the function and importance of the pottery (Gingras & Sneed, 2019). Samples from the inside of the citadel may imply formal or ceremonial contexts, whilst samples from the exterior may show they were part of daily activity outside the castle walls. Archaeologists can reconstruct the social, cultural, and economic facets of the community by classifying ceramic samples, which helps them to acquire a thorough grasp of the material culture and everyday activities of this ancient civilization (Fusaro, 2021).

When it comes to determining where these ceramics were in relation to trade routes and how they spread to regions is taking an approach that involves ceramic analysis. The ceramics found at the Kaymakçı alongside artifacts can offer insights into the social and economic frameworks of the communities in that area. For example, discovering ceramics from areas could indicate trading connections while observing a variety of styles and techniques within the site might indicate a diverse range of cultural influences and interactions.

The link between ceramics and the social and economic setups in the areas where they were discovered can be investigated using approaches. Studying styles of pottery observing where ceramics are found in settings such as households, ceremonies or trade and exploring how they were distributed offers archaeologists valuable information about their usage in everyday life, social structures, and economic activities within communities (Ashkanani & Kovár, 2021) like the site at the Kaymakçı. It is essential for archaeologists to understand the origins of clay

used in pottery to reconstruct production methods and trade routes because distinct mineral compositions in clay from sources can be linked back to specific geographic regions (Finlay *et al.*, 2012). By analyzing the geochemistry of ceramics, researchers can determine whether they were made locally or brought from afar providing insights into trade paths and cultural interactions.

Moreover, clays often retain traces of plants and microorganisms transported by liquids that could be trapped in the pottery during its making or use. Sophisticated techniques like DNA analysis enable the detection and identification of these residues offering perspectives on past environments and human behaviors (Pérez, 2022).

Studying DNA found in layers is a new and exciting area that provides valuable insights into the past. By identifying the presence of plant and animal species in the area, researchers can learn more about ancient communities' economic and social structures. This includes discovering practices, trade items as well as insights into the diet and health of people from that period. When combined with methods like stylistic analysis and distribution studies, a clearer picture of life in settlements like the Kaymakçı emerges. The study of ceramics, including their style, materials, and biological remnants, enhances our understanding of previous societies by revealing details about how they made and used ceramics (Sołtysiak, 2021; Frankel & Webb, 2012).

Beneath the 2-meter-wide walls, archaeologists found a layer of pottery between 0.05 and 0.10 meter thick, which was uncovered during the Kaymakçı Archeological Project (KAP). The discovery provides insights into the lifestyle, culture and economy of the citizens living in Anatolia during the Middle and Late Bronze Ages (2000- 1200 BC). The ceramic samples (Figure 1. 2) recovered from the Kaymakçı archaeological site contain various objects, including bowls and jars, each with distinct characteristics. The samples identified by their catalogue numbers (e.g., KAP 02, KAP 03), were excavated from various locations within a

citadel, including places with varying uses, internal sections, and external spaces with distinct soil compositions.

The samples analyzed were excavated from sites coded 93.545, 95.555, 97.541, and 109.523. Of the total 33 samples, 12 were from 95.555, 2 were from 93.545, 11 were from 97.541, and 8 were from 109.523 (Figure 1.3 and Figure 1.4). The differences in sample size, composition, and inclusion presence suggest a variety of materials and environments from which they were made. The diversity of the samples allows for a wide range of data to be analyzed, which can shed light on the dietary preferences, cultural practices, and agricultural activities of those who lived and worked in the citadel (Roosevelt *et al.*, 2018).

Figure 1. 2. Ceramic vessels excavated at the Kaymakçı between 2014-2016. They include a rear flask (99.526.58.1) and a small jug (95.555.66.1) from left to right, and a front lamp (99.526.550.1) as well as cups (99.526.324.1, 99.526.452.1, 109.523.113.1) from left to right (Roosevelt *et al.*, 2018).

The methods used to produce ceramics have changed considerably over time, reflecting improvements in both craftsmanship and technology. The ceramic pieces and fuel were combined in one of the first techniques, called open firing, and fired in an atmosphere high in oxygen. This ancient but expert technique did not call for building structures, but it did call for careful management of the fire process, which

included controlling the temperature and making sure the ceramics were sufficiently dried to avoid explosion. In open firing, the temperature, length, and pre-firing drying time had an impact on the ceramics' distinctive reddish coloring. Utilizing kilns, which could either combine the fuel and vessel or separate them, was another technique. Due to the kilns ability to retain heat, it was challenging to achieve the temperatures typically reached in firing methods. Nonetheless, kilns offered the advantage of controlling the atmosphere during firing, which played a role in ensuring top notch ceramic production (Safina *et al.*, 2020). Despite the heat involved recent research indicates that ancient DNA (aDNA) can sometimes be preserved within ceramics. The temperature drying process in kilns can impact the capture and preservation of DNA within ceramic (Gong *et al.*, 2022). Throughout firing the interior of the pottery may reach temperatures that could potentially degrade or eliminate biological materials such as DNA. However, certain DNA fragments might endure, especially if they are shielded by clay matrix or nestled within pores of the material (Jordán *et al.*, 2020). These preserved DNA fragments offer insights into environments by revealing remains of plants, animals and microorganisms that interacted with the ceramics. Advanced techniques, like aDNA (ancient DNA) analysis enable researchers to extract and identify these remains shedding light on diets, farming practices and environmental circumstances (Orlando *et al.*, 2021).

For example, finding plant remains in pottery containers can give clues about the kinds of crops that were stored or processed while identifying animal DNA can provide information on domesticated animals or food supplies. Despite the difficulties caused by temperatures, successfully extracting DNA from ceramics may offer new opportunities to learn about the daily lives and economic activities of past communities. By examining styles, distribution patterns and studying preserved DNA, archaeologists can create a more detailed and nuanced understanding of historical societies.

The designs, symbols, and decorations on ceramics are examples of artistic expressions that provide insight into the social mores and cultural norms of the eras

in which they were produced (Safina *et al.*, 2020). These expressions could be anything from intricate representations of vegetation, animals, and mythological characters to straightforward geometric patterns. The societies that made and used these ceramics have storytelling customs and aesthetic sensibilities that are reflected in the motif selection and degree of detail in the artwork (Gong *et al.*, 2022). Such artistic components point to a vigorous and inventive side of ancient societies and imply a profound involvement with visual culture as well as a comprehensive comprehension of design principles (Gingras & Sneed, 2019; Campbell, 2010).

Ceramics have expanded over many locations, demonstrating their importance in trade networks as commodities and cultural exchange vehicles. The distribution patterns may reveal information about how interconnected various societies are, how much they interact, and how ideas and technologies are shared across national boundaries. Rebuilding the historical economic and cultural environments requires an understanding of these trade routes (Lim, 2020).

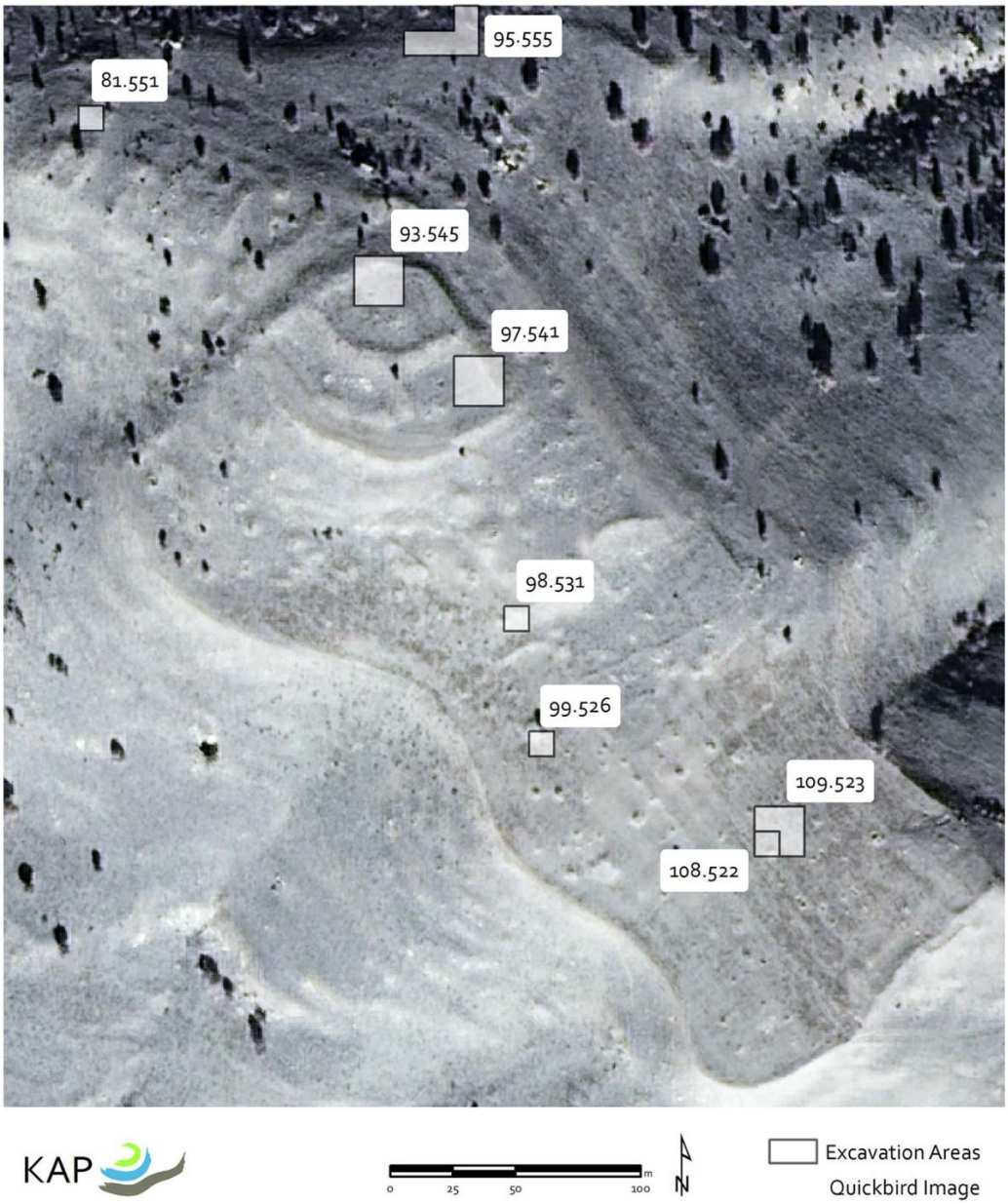

Figure 1. 3. QuickBird satellite image of the Kaymakçı showing excavation areas between 2014 and 2016 (Roosevelt *et al.*, 2018).

Figure 1. 4. This area, coded as 95.555, consists of a rectangular shape measuring 9 x 29 meters and an additional area measuring 9 x 9 meters, forming an "L" shape, located along the northern walls of the castle. Various phases of construction and activity have been observed in this area, including the original construction of the 2-meters LBA wall and several buildings (Roosevelt *et al.*, 2018).

1.4. Utilizing metagenomic analysis to identify plant species traced in ancient ceramics

Metagenomic analysis focuses on studying the population in a sample rather than individual organisms. This approach is valuable for investigating the diversity and complexity of life in environments such as soil, water, and the human body. Metagenomic analysis has applications in fields like healthcare, engineering, agriculture, environmental conservation and ecology (Lema *et al.*, 2023). The analytical process includes activities like sample management, data organization and storage statistical interpretation, experimental design sequencing technology utilization for assembly and annotation. These steps are essential to ensure that the

data generated through sequencing leads, to meaningful outcomes (Thomas *et al.*, 2012).

In analysis, the initial and crucial step is a proper sample collection. The sample is collected to extract DNA and may need to be collected several times depending on the scope of the DNA isolation process required for studies. This stage is pivotal as it sets the foundation for analyses. Next step is to prepare the sequencing library and perform the sequencing procedures.

After generating reads, researchers apply sequence data analysis (Thomas *et al.*, 2012). The primary goal of the study is this analysis which involves refining the sequenced data to extract nucleotide sequences for information extraction. The sequencing data consists of samples containing billions of sequences reads. Various bioinformatics pipelines and platforms, like the Galaxy platform are utilized to handle the amount of data. These tools are specifically designed to evaluate the genetic and the genomic data (Ghosh *et al.*, 2019 p. 184). One approach to analyzing metagenomes is to compare them using a taxonomic perspective to known sequences in databases or sequences associated with specific biological functions. Depending on the objectives of the studies, one can prefer either of taxonomic analysis which compares metagenomes with known sequences in databases, and functional analysis, which focuses on the biological activities of the sequence. Understanding techniques and having processing and storage capacity are essential for analyzing and interpreting metagenomic data (Navgire *et al.*, 2022).

Previous research on data from samples has focused on ancient DNA (aDNA) and metagenomic analysis to gain insights into past ecosystems, lifestyles, and diseases. by utilizing NGS technology. Examined aDNA, offering valuable information, about ancient lifestyles and population migrations (Sarkissian *et al.*, 2021). The field of paleogenetic has seen a growth due to the progress in studying DNA found in deposits offering valuable insights into the evolution of animals and hominins without relying on physical biological remnants. However, challenges persist in

identifying, quantifying and verifying communities and addressing potential modern contamination issues (Gilbert *et al.*, 2005 p. 542).

While most metagenomic investigations concentrate on microorganisms (Navgire *et al.*, 2022), this thesis aimed to determine the plant species existing during that era. Drawing from research in the area the goal was to comprehend diversity from earlier times to present by integrating archaeological, geological and molecular biological methodologies. These interdisciplinary approaches enabled researchers to extract DNA from artifacts discovered at the Kaymakçı archaeological site for metagenomic analyses. Metagenomic analysis is advantageous to identify plant species in different component. For instance, detecting plant DNA in samples aids in recognizing consumed plant species linked to practices and dietary habits (Raime *et al.*, 2020). Understanding the interaction between agricultural methods and ecosystems is also largely dependent on metagenomic analysis, especially when it comes to sustainability. Through the investigation of microbial communities in soil and plant settings, this analytical method provides lots of insights into the intricate relationships that exist between microbes and their hosts. The integrity of the soil ecosystem, the cycling of nutrients, and the general health of the plants rely on these interactions (Nwachukwu & Babalola, 2022).

One important area of focus in metagenomic investigations is soil microbial diversity which is influenced by land-use practices, agricultural management, and environmental factors. The soil surrounding the rhizosphere, contains microbes that can improve plant growth. They also contribute to the sustainability of conventional and urban agriculture by helping to transform harmful pollutants into non-toxic forms. The dynamic character of soil habitats and their influence on plant health and productivity is highlighted by the fact that the concentration of metabolites generated by these bacteria vary depending on factors including temperature, pH, and nutrient content (Masenya *et al.*, 2024).

Furthermore, metagenomic analysis has demonstrated the importance of microbial communities in soilless growth systems. Microbial activity plays a major role in

these systems in breaking down organic materials into forms that plants can use. The identification of microbial biomarkers and symbiotic interconnectivities, among other insights from metagenomic research, might promote sustainable urban agriculture (Marco, 2017).

Moreover, a promising method for deciphering the intricate relationships between microbial communities and their host species is the combination of metagenomics with machine learning and epigenetics. The identification of important microbial taxa, physiological processes, and epigenetic markers that influence host-microbe interactions is made possible by this integrated approach, which may result in new discoveries and agricultural applications (Vecherskii *et al.*, 2021).

DNA barcoding is a method used for categorizing species based on their DNA sequences. Various fields such as ecology, aerobiology, biosecurity and forensics utilize this technique (Cristescu, 2014; Bell *et al.*, 2016).

When studying samples like soil or water, it is helpful to compare their DNA sequences with a database of known plant species for identification purposes (Bell *et al.*, 2017). The *rbc*L (Ribulose-1,5-bisphosphate carboxylase/oxygenase Large Subunit) gene is a choice for DNA barcoding because of its length making it easy to overlap reverse and forward reads in sequencing. This overlapping feature is crucial for making comparisons. Since the *rbc*L gene is found in the chloroplast, it is commonly used as an identifier for plant species (Kang *et al.*, 2017). When comparing the accuracy of the *rbc*L gene with DNA barcoding methods, significant variations in plant species identification become apparent (Ho *et al.*, 2021).

Studying the ceramic samples at the Kaymakçı archaeological site can reveal valuable insights into the diet, farming techniques and environmental circumstances of Bronze Age inhabitants. By examining the extracted DNA, the plant species that once thrived in the area establishing a connection between the archaeological artifacts and the local ecosystem can be pinpointed. This method allows researchers to understand the techniques and environmental conditions of that time. It also helps trace plant residues found in ceramics back to ancient trade

routes and exchange networks. For example, the discovery of wheat DNA would imply that the ceramic was intended for grain storage, demonstrating the area's importance on cereal farming. Moreover, investigating how these plant remains in ceramics relate to trade routes and exchanges is an avenue for exploration. Exploring the benefits of the innovative technologies employed in studies and outlining future research paths underscores the transformative power of metagenomic analyses in reshaping our comprehension of ancient civilizations and their interactions, with nature. By enabling the investigation of microbial diversity, population dynamics, and functional activities within ecosystems, these technologies help to clarify the intricate relationships that exist between microbes and their surroundings. This method has revealed previously unidentified bioactive compounds and microbial roles with consequences for the domains of agriculture and health. Furthermore, metagenomics has been used to safeguard the environment by locating possible biocatalysts and methods for cleaning up contaminated areas. Future research promises to further broaden the knowledge base as researchers continue to develop and improve these technologies. These opportunities present previously unheard-of chances to transform our understanding of the past and its lasting linkages to the present and future (Pavlopoulos *et al.*, 2023).

CHAPTER 2

OBJECTIVES OF THE STUDY

The research involves investigating the array of plant species discovered in the ceramic samples, from the Kaymakçı site and to reveal the possible factors that influence this plant diversity. Through the application of tools in research, it strives to advance archaeology and provide new perspectives on how ancient societies engaged with their environment. The main objective of this study was to identify plant diversity at the Kaymakçı site.

The specific aims of this study included:

- To examine the plant species composition in the ceramics from the Kaymakçı site.
- To identify the possible factors that impact plant biodiversity in these samples.
- To contribute to the wider field of environmental archaeology studies.
- To explore how metagenomics analysis techniques can be effectively utilized for archaeological research.

CHAPTER 3

MATERIALS AND METHODS

3.1. Ceramic samples

Koç University Department of Archeology and Art History, within the scope of the Gygaia Project (Gygaia Projects - Culture - Society - Nature, 2023), 33 of the 37 samples excavated from the Kaymakçı archaeological site were provided by Prof. Chris Roosevelt and Assoc. Prof. Christina Luke research team. The information in Table 3.1 provides a thorough summary of the archaeological excavations carried out at a particular site, highlighting different facets of the discoveries while Figure 3.1 shows two of the ceramic samples from the site. The systematic approach to recording the archaeological environment is facilitated by the inclusion of sample identification number, weight, method of analysis, classification, and site characteristics in each entry. The variety of methods such as RLB (Reverse Line Blot), gold wash, and coarse were used to evaluate the materials from the Kaymakçı site. The categories, which include handled objects, bowls, and jars, further emphasize the variety of the artifacts findings.

Ceramic pot manufacturing, including the clay used, the cooking technique, and the kiln for firing, hold equal significance to the samples preserved inside. Due to ceramics' porous design, it may allow the exchange of chemicals such as moisture. This may have an impact on the preservation and retrieval of DNA (Smenderovac *et al.*, 2024).

Table 3. 1. Comprehensive Overview of Archaeological Excavations from Kaymakçı Site (Kaymakçı Archaeological Project-Ebru Kaner)

Excavation Locations	Sample No.	Weight (g)	Analysis Method	Classification	Site Characteristics
95.555.346.322	KAP 02	1.0048 g	Coarse	Jar	Exteriror of citadel. Mixed.
95.555.359.69	KAP 03	1.0070 g	Coarse	Jar	Interior of citadel.
95.555.361.212	KAP 04	1.0146 g	Gold wash	Bowl	On the slope. Pebbly area
95.555.361.214	KAP 05	1.0221 g	Coarse	Jar	On the slope. Pebbly area
95.555.361.216	KAP 06	1.0425 g	Coarse	Jar (handled)	On the slope. Pebbly area
95.555.361.218	KAP 07	1.0794 g	RLB	Bowl	On the slope. Pebbly area
95.555.361.222	KAP 08	1.0104 g	Coarse	Jar	On the slope. Pebbly area
95.555.366.143	KAP 10	1.0133 g	RLB	Bowl	On the slope. Same level of rubble of the W. 113
95.555.367.17	KAP 11	1.0432 g	RLB	Bowl	Interior of citadel.
95.555.370.81	KAP 13	1.0571 g	RLB	Bowl	Seems like interior of space with heart/oven
95.555.370.83	KAP 14	1.0672 g	Gold wash	Bowl (handled)	Seems like interior of space with oven
95.555.370.85	KAP 15	1.0595 g	Coarse	Jar	Seems like interior of space with heart/oven
93.545.354.20	KAP 16	1.0502 g	Gray ware	Bowl	Inner citadel.
93.545.385.12	KAP 17	1.0739 g	RLB	Bowl (flat base)	Inside the pit.
97.541.875.8	KAP 18	1.0923 g	RLB	Bowl	Room. Does not have a number
97.541.875.10	KAP 19	1.0205 g	RLB	Jug (neck)	Room. Does not have a number
97.541.917.1	KAP 20	1.0130 g	RLB	Jug	Outside of the spaces. Open area.

Table 3.1. (continued)

97.541.942.6	KAP 21	1.0362 g	Coarse	Uncertain	Wall in the middle of the context.
97.541.971.7	KAP 22	1.0671 g	RLB	Jug (spouted)	Seems like interior of space with oven
97.541.971.8	KAP 23	1.0246 g	Coarse	Bowl	Seems like interior of space with oven
97.541.971.9	KAP 24	1.0115 g	Coarse	Jar (handled)	Seems like interior of space with oven
97.541.1027.7	KAP 25	1.0991 g	RLB	Jug (handled)	Exterior of citadel.
97.541.1058.10	KAP 26	1.0049 g	Gold wash	Bowl	Interior of space.
97.541.1064.3	KAP 27	1.0244 g	Coarse	Jug (handled)	Outside of the spaces. Open area.
97.541.1093.5	KAP 28	1.0686 g	Coarse	Bowl	Outside of the spaces. Open area.
109.523.668.6	KAP 30	1.0927 g	Coarse	Jug	On the alley.
109.523.681.6	KAP 31	1.0887 g	Coarse	Bowl (burnt)	Interior of space.
109.523.681.12	KAP 32	1.0111 g	Coarse	Jar/bowl	Interior of space.
109.523.698.7	KAP 33	1.0212 g	Gold wash	Bowl	Interior of space.
109.523.705.8	KAP 34	1.0837 g	Coarse	Jar	Interior of space.
109.523.713.17	KAP 35	1.0318 g	RLB	Jar	Interior of space.
109.523.733.10	KAP 36	1.0180 g	RLB	Jug	Room. Does not have a number.
109.523.748.9	KAP 37	1.0346 g	RLB	Jar	Interior of space.

Figure 3. 1. KAP 07 and KAP 17 from the Kaymakçı archaeological site, respectively (the Kaymakçı Archaeological Project-Ebru Kaner).

3.2. aDNA Extraction Methods

The ceramic samples excavated from the Kaymakçı site have not been washed prior to the ancient DNA extraction because the washing process might harm the source of aDNA that found surface of the ceramics and cause cross- contamination.

To enhance the quantity and quality of DNA extracted from samples different methods were utilized to extract the ancient DNA (aDNA) from ceramics. Techniques such as the Modified Qiagen DNEasy Plant Mini Kit (Qiagen, Valencia, CA), the EURx GeneMATRIX Soil DNA Purification Kit (the EURx, Gdańsk, Poland) and the modified CTAB based DNA extraction (Gismondi *et al.*, 2016) method were employed. Since the EURx GeneMATRIX Soil DNA Purification Kit gave the best results, it was used for all the samples.

3.2.1. EURx GeneMATRIX Soil DNA Purification Kit (EURx Ltd., 2023)

30 µl of activation Buffer SL (lysis buffer) were gently applied onto the DNA binding spin column without any spinning motion. It was then left at room temperature until the lysate was poured onto the spin column for a minimum of 10 minutes. This step plays a role, in ensuring that the membranes were properly wetted, and that optimal DNA binding occurred. It was recommended to carry out this step before commencing the isolation process.

For soil samples, up to 250 milligrams were added to the Bead Tube, which contained glass beads and a buffer that assisted in dispersing soil particles and breaking down cells. While the kit was designed for 0.25 grams of soil, it was suggested to reduce the sample weight to between 0.1 and 0.15 grams to obtain more DNA. In cases of water samples, after filtration, the membrane was placed into the Bead Tube. The tube was gently shaken to mix the sample thoroughly, then 60 µl of Lyse SL buffer (lysis buffer) of the kit were added and shaken for 1 minute. If cloudiness was noticed in the buffer at temperatures below 20°C, it was warmed up in a water bath at 37°C until it became clear. The Bead Tubes were positioned horizontally and vortexed for 10 minutes, or a cell disrupter was utilized for improved yield. After blending, the tube was placed in the centrifuge for a duration of 2 minutes. With caution, 400 µl of the liquid was carefully transferred to a 2 ml tube. Following this, 400 µl of PR buffer (Proteinase K reaction buffer) was mixed gently, given a shake. Then it was allowed to rest on ice for 5 minutes. The tube was then spun more and 600 µl of the liquid was transferred to another tube, combined with 600 µl of Sol SL buffer (solubilization buffer), and blended with 200 µl of ethanol. Subsequently, 600 µl of this mixture was applied onto the DNA column and spun at 11,000 × g (force of gravity) for half a minute. This step was repeated prior to moving the remaining solution to the column and spinning it for a minute. Next, 500 µl of Wash SLX1 buffer (first wash buffer) was spun for a minute. The column was then removed, and any residual content passing through it was discarded before returning it to its tube. Another round involved adding 500 µl Wash SLX2 buffer (second wash buffer) followed by spinning for another minute to ensure the removal of any lingering traces of the wash buffer. The column underwent one spin before being placed in a tube where approximately 50–100 µl of Elution buffer was added to release any trapped DNA molecules within it. After letting it settle at room temperature for 2 minutes, it underwent another spin cycle lasting 1 minute. The column was then set aside, and the collection tube was removed. The activation step referred to the preparation phase where reagents and samples were mixed according to the protocol, while the lysis step disrupted cells

to release DNA into solution, which was especially crucial for extracting nucleic acids.

The washing step removed proteins and other contaminants from the purification matrix, ensuring the purity of the extracted DNA. Finally, the elution step released the DNA from the purification matrix into a low-ionic-strength solution, preparing it for use in downstream applications (Ali *et al.*, 2017). The DNA was then prepared for examination or could be kept for storage at temperatures ranging from 2–8°C or −20°C, respectively.

3.3. DNA Quantification

The Biodrop Duo instrument was utilized to analyze the amount and quality of the DNA extracted from samples (Biodrop µLite 7141 V.1.0.4, Department of Biological Sciences, METU). The A260/A280 ratio is commonly employed to assess the purity and integrity of DNA calculated based on absorption at 260 nm (nanometer) (A260) and 280 nm (A280). A typical ratio ranging from 1.78 to 1.84 indicates no contamination from RNA, proteins, or impurities in the sample. Furthermore, the A260/A230 ratio is determined by evaluating absorption at 260 nm (A260) and 230 nm (A230) to estimate contamination levels from various compounds, proteins, and secondary metabolites. An A260/A230 ratio ranging between 2.0 and 2.2 typically implies no contamination levels in the sample aiding in identifying substances that absorb light at wavelengths not commonly absorbed by DNA indicating contamination levels with these compounds (Yu *et al.*, 2017).

3.4. *rbc*L primer for metagenome analysis

The *rbc*L gene, a component of the chloroplast DNA, is essential for categorizing plants based on taxonomy. It plays a role in forming a DNA barcode for terrestrial plants by combining with the *trn*H- *psb*A spacer region. The *rbc*L gene acts as a marker aiding in classifying a specimen into its respective family, genus and generally even species. On the other hand, the *trn*H- *psb*A spacer region, known for its variability helps pinpoint the species identification when the *rbc*L gene alone

may not provide enough information especially in diverse plant genera (Pang *et al.*, 2012). Both genetic markers can be easily amplified using primers making them versatile for types of land plants. This dual locus plant barcode is currently being utilized to create a database encompassing, over 700 significant medicinal plants of the world. (Ho *et al.*, 2021).

In Kress & Ericson's research in 2007, scientists have successfully analyzed both *mat*K (Maturase K) and *rbc*L sequences and the BLAST findings revealed matching results, for both genes. The *rbc*L gene has proven to be more reliable than the *mat*K gene region of chloroplast genome in identifying sequences in plants considering sequences with a minimum of 80% identity for identification. The BLAST outcomes consistently found matches for both *mat*K and *rbc*L genes whereas the BOLD (Barcode of Life Data System) results displayed variations, due to limitations, in their databases size and completeness. The *rbc*L gene is known for its effectiveness in identifying species since it is well studied, and highly conserved. (Kress & Erickson, 2007).

The *rbc*L gene primer sequences used in this study were as follows; the forward primer sequence was 'CTTACCAG***Y***CTTGATCGTTACAAAGG' while the reverse primer sequence was 'GTAAAATCAAGTCCACC***R***CG'. It has been confirmed that the reverse primer sequence aligns with the *rbc*L DNA barcode region (Kress& Erickson, 2007). This primer sequence is commonly used as the standard in plant barcoding. Using this pair of primers results in an amplicon of a size of 379 base pairs, across the alignment (Hollingsworth *et al.*, 2009).

The *rbc*L primer above contains both "Y" and "R" which makes it a degenerative primer. Degenerative primers are designed to attach to target sequences with variations, which is vital, for amplifying a set of related DNA sequences. These primers incorporate bases at positions using IUPAC codes like "R" for A or G and "Y" for C or T enabling them to bind with sequences containing either of the specified bases at those positions (Linhart & Shamir, 2005).

3.5. Optimization of PCR conditions

The selected gene region was amplified using the Polymerase Chain Reaction (PCR), with 2x KAPA HiFi HotStart Master Mix (Roche Diagnostics, Basel, Switzerland) containing 2.5mM MgCl2. Following confirmation of the expected product size on a 3% agarose gel (run at 100 Volt for 60 minutes), it was stained with ethidium bromide (10 mg/mL) and viewed under UV light (Vilber Lourmat, France). Then, the samples were sent to a sequencing company (BM Labosis, Ankara, Türkiye) for purification and sequencing procedures. Detailed PCR optimization conditions for all ceramic samples using the *rbc*L primer can be found in Table 3.2.

Table 3. 2. Optimized PCR conditions of aDNA amplifications from ceramic samples

Components	**Volume (µL)**	**PCR Conditions**			
dH_2O	12	Initial Denaturation 3 min			95°C
Master Mix	6		Denaturation	30 sec	95°C
Primers (10µM)	0.5+0.5	45 cycles	Annealing	30 sec	51°C
DNA (10ng/µL)	6		Extension	30 sec	72°C
Total	25	Final Extension		10 min	72°C

3.6. Data analysis

Qiime2, a tool tailored for studying communities heavily depends on the SILVA database. This database is specifically fine-tuned to recognize bacteria and archaea, by analyzing their 16S ribosomal DNA sequences (Hall & Beiko, 2018). However, when it comes to identify plant genomes, Qiime2 2024.5 version faces limitations as plants possess markers, like the Internal Transcribed Spacer (ITS) and the Ribulose-1,5-bisphosphate carboxylase/oxygenase Large Subunit (*rbc*L) making the analysis of plant genomes more intricate (Dubois *et al.*, 2022).

In this study, to analyze the metagenome data from aDNA of ceramics, a customized program was written that involved in writing scripts for Linux version 5.10.16.3 (*The Linux Kernel Archives*, 2024), RStudio version 3.6.0+ 2024.04.2+764 (*RStudio Desktop - Posit*, 2024) and Python (*Welcome to Python.org*, 2024) version 2024.6.0. The process started with downloading the *rbc*L gene database, from the National Center for Biotechnology Information (NCBI). Next, the initial and subsequent read pairs were combined by converting the resulting file from FastQ to Fasta format. Changes to the header of the Fasta file were made to decrease its size. Using this modified dataset, Operational Taxonomic Units (OTUs) were created by applying the CD-Hit (Clustering by Fast Search and Hierarchy Construction using k-Mer Hits) algorithm to identify sequences with a 90% or higher similarity level. Selection of 90% or higher similarity is primarily due to the need to have accurate identification and differentiation of species. A higher similarity threshold ensures that only closely related organisms which are considered matches. This will reduce the likelihood of false positives caused by distant evolutionary relationships. An abundance table was then generated based on these OTU (Operational Taxonomic Units) matches aiding in retrieving taxonomic identification IDs from the NCBI database using accession numbers. A filtering process, finding only plant species among the taxon IDs, was applied to characterize plant species found in the sample. Lastly, a stack bar plot was produced to display the distribution of plant species present, in each sample.

The *pandas*, *matplotlib*, and *numpy* libraries were used by the Python code to analyze and visualize data. The Python script read an Excel file into a DataFrame and imported the required libraries first. After that, the Python scripts created a list of different colors for plotting by filtering out columns where all values were less than or equal to 0. Then, it arranged the remaining data according to its columns in ascending order. After this preparation, the Python scripts used *matplotlib* to build a stacked bar plot in which each bar represented a distinct genus and the height of each segment inside a bar represented the genera's percentage value. The x-axis

tick labels were rotated for improved reading, and the plot was personalized with labels, a legend, and a title. The plot was then shown after being saved as a PNG image file. The data was efficiently analyzed and visualized by this procedure, giving a clear summary of the percentage distribution across several samples in an eye-catching way.

To understand plant diversity and dominance in study samples, several analytical metrics such as frequencies, averages, medians, and modes were also used. Frequencies showed the most and least common plants by counting how often each plant genus appeared. Averages (means) indicated overall prominence by showing the general distribution of plant types. Medians provided the middle value of the dataset, less influenced by extremes, highlighting central tendencies. Modes identified the most prevalent plant, being the most frequently occurring value in the dataset. These descriptive statistics (median, mode, and average) were crucial for understanding plant diversity and dominance, as well as the unique botanical and ecological characteristics of a region (Kalusová *et al.*, 2016).

The steps during the experiments and data analysis of this thesis are given as a flow chart in the Figure 3.2 below.

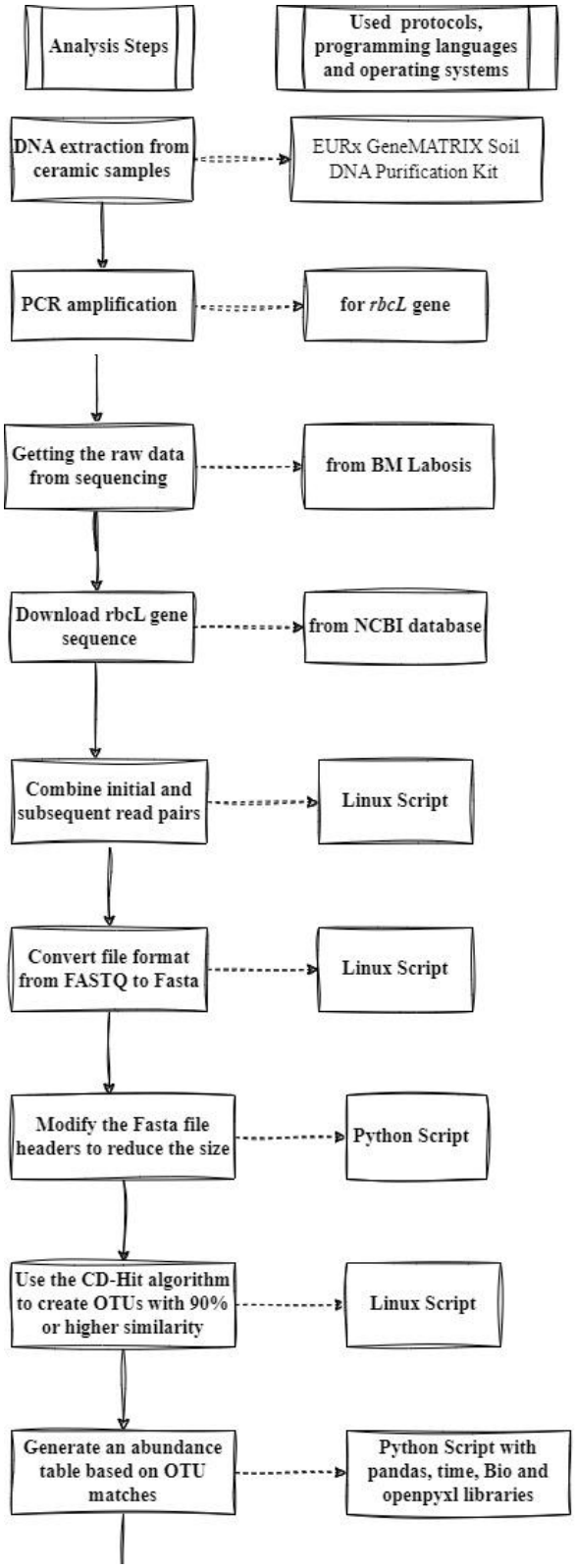

Figure 3. 2. Steps during the experiments and data analysis of this thesis

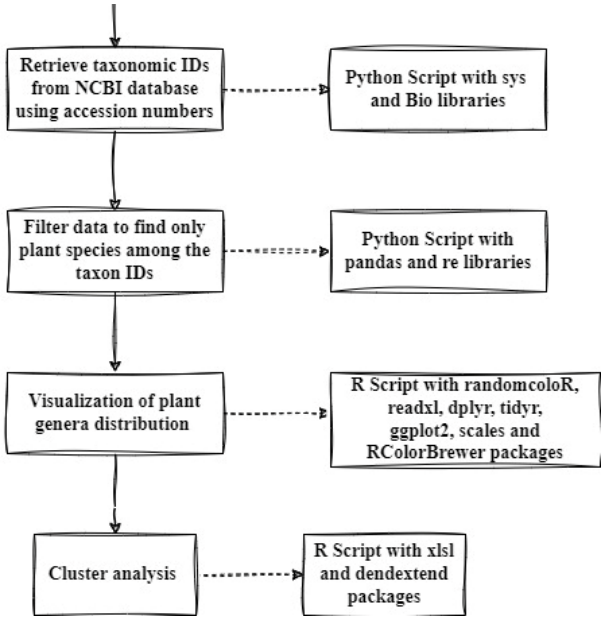

Figure3.2 (continued)

3.7. Cluster analysis

Understanding the Distance Metric in Cluster Analysis involves several concepts. The Euclidean Distance is a measure used to calculate the distance between points in a multi-dimensional space, where each point represents a sample characterized by its features (e.g., KAP plant genera and their frequency values). This metric helps quantify how similar or dissimilar samples are. In the analysis of this study, the distance matrix calculated these distances for all pairs of samples, with values ranging from 0 (indicating identical samples) to higher numbers indicating greater differences (Barnova *et al.*, 2023).

The distance scale on the dendrogram x-axis interpreted these distances from 0 to 120. A value of 0 signified no distinction between samples, while 120 indicated the maximum dissimilarity observed in the dataset. The length of each branch in the dendrogram corresponded to these distances, with branches suggesting differences between clusters or samples. These distances played a role in cluster formation; samples that are alike (smaller distances) typically cluster together early on, forming tight clusters. The hierarchical nature of clustering, especially when using the linkage method (method = "complete"), ensured that clusters were created

based on the distance between any members of different clusters, fostering a structure where members within each cluster were closely related (Bakker, 2024).

When using it practically, examining the dendrogram helped in making decisions about how many clusters best depicted the selected data (Costa & Weese, 2019). For instance, choosing a cut-off point at 30 could result in a different cluster arrangement compared to selecting one at 60. This method linked the measured distances to the likeness between samples, which was essential for understanding clusters in data analysis (Costa & Weese, 2019).

In this study, hierarchical clustering and data visualization were accomplished with an R script that focused on examining the relationships between samples rather than their individual attributes. The first step was to install the required packages (*dendextend* for manipulating and extending dendrograms, and *xlsx* for reading Excel files). The script imported these packages, then read information from an Excel file. The data was to carry out then transposed to facilitate sample clustering rather than using the original categories (such as "genus"). The original column names were changed from numeric indices to row names, which served as unique identifiers for every sample.

The next step was to carry out hierarchical clustering, which groups similar samples together by calculating the Euclidean distance matrix between samples and by using the "complete" linking approach. The result was used to produce the dendrogram, which visually illustrates the connections among data points often generated from clustering analyses. To enhance the dendrogram's readability, branch colors were assigned according to the clusters found during the process. The used four distinct colors correspond to several clustering groups.

After that, samples were positioned on the right side of the dendrogram to make it simpler to compare samples that were part of the same cluster. The map had annotations for the average distance between clusters, which was determined by averaging the merge heights from the hierarchical clustering process. Branches

were colored based on the previously established scheme. This final stage offered a thorough visual representation of the data, emphasizing the links between samples according to their attributes as well as the data's structure. Each tier of the dendrogram represented the level of similarity or dissimilarity between the data points, like genera in a family tree. The top tier indicated broader groupings, while lower tiers refined these groupings further. The merging height of two data points reflected their proximity or distance from one another.

However, interpreting a dendrogram necessitates careful analysis since it condenses data into a simplified form, potentially missing subtle nuances found in the original dataset. Despite this challenge, dendrograms remain a valuable tool for grasping the structure of data and pinpointing natural clusters within it (Forina *et al.*, 2002).

CHAPTER 4

RESULTS

4.1. DNA extraction results

DNA extraction from all samples was performed using the GeneMATRIX Soil DNA Purification Kit provided by EURx Ltd. The results of these extractions are detailed in the Appendix 1.a. Some of the best and the worst results can be found in Table 4.1 below.

Table 4. 1. DNA concentrations of some isolated ceramic samples

Sample No.	Weight(g)	DNA Conc. (µg/ml)	A260/230	A260/280
KAP03	0.25	6.721	0.627	1.806
KAP04	0.25	7.004	0.159	2.331
KAP08	0.25	3.706	0.236	2.339
KAP20	0.25	10.620	0.841	1.605
KAP30	0.25	0.406	0.113	1.000
KAP32	0.25	30.66	0.605	1.643

Based on the information shown in Table 4.1, the DNA extraction outcomes from ceramic samples exhibited notable differences in both concentration and purity levels. For instance, KAP32 had the highest DNA concentration at 30.66 µg/ml while KAP30 had the lowest DNA concentration at 0.406 µg/ml. The purity assessment (A260/230 and A260/280 ratios) suggested contaminations such as organic compounds or salts in the samples. These results indicated that while certain samples like KAP32 were more suitable for further analysis, additional

purification steps may be necessary for other samples such as KAP04, KAP08 and KAP30.

4.2. PCR results

Good-quality single bands approximately 350 bp in length were observed in all 33 samples onto 3% agarose gel (Figure 4.1).

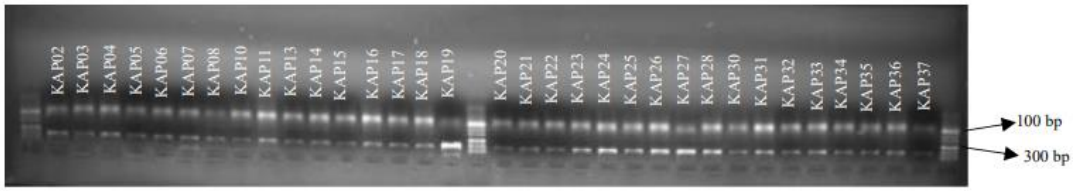

Figure 4. 1. The gel photo showing the amplified PCR products of *rbc*L gene for 33 KAP ceramic samples from the Kaymakçı.

The sequencing results of KAP20 sample were not sufficient for further analysis, so it was not used in data and cluster analysis.

4.3. Metagenome analysis results

The analyses of metagenomic data from 32 ceramic samples resulted in presence of 95 plant genera as follows: *Heracleum, Apium, Quercus, Vincetoxicum, Salix, Papaver, Laurus, Populus, Pinus, Solanum, Cynodon, Salvia, Phaseolus, Lathyrus, Forsythia, Euphorbia, Vicia, Launaea, Carthamus, Astragalus, Triticum, Daucus, Lamium, Melissa, Helianthus, Chamira, Rosa, Ranunculus, Fritillaria, Atractylodes, Lactuca, Musa, Acer, Cucumis, Tragopogon, Linaria, Hypericum, Onosma, Cephalaria, Arabidopsis, Prunus, Artemisia, Petrorhagia, Noaea, Medicago, Cynanchum, Convolvulus, Crepis, Glycine, Silybum, Allium, Hordeum, Secale, Lapsana, Oxybasis, Dianthus, Smyrnium, Styphnolobium, Juniperus, Citrus, Anethum, Ailanthus, Cicer, Pericallis, Polygonum, Scrophularia, Sonchus, Anthemis, Brassica, Gypsophila, Pyracantha, Cedrus, Corylus, Anemone, Centaurea, Robinia, Fraxinus, Lithocarpus, Machilus, Eruca, Elymus, Chenopodium, Echinops, Corydalis, Pilosella, Urtica, Ziziphora, Cyclospermum, Monarda, Heteromorpha, Nuttallanthus, Vigna, Lauraceae, Onobrychis.*

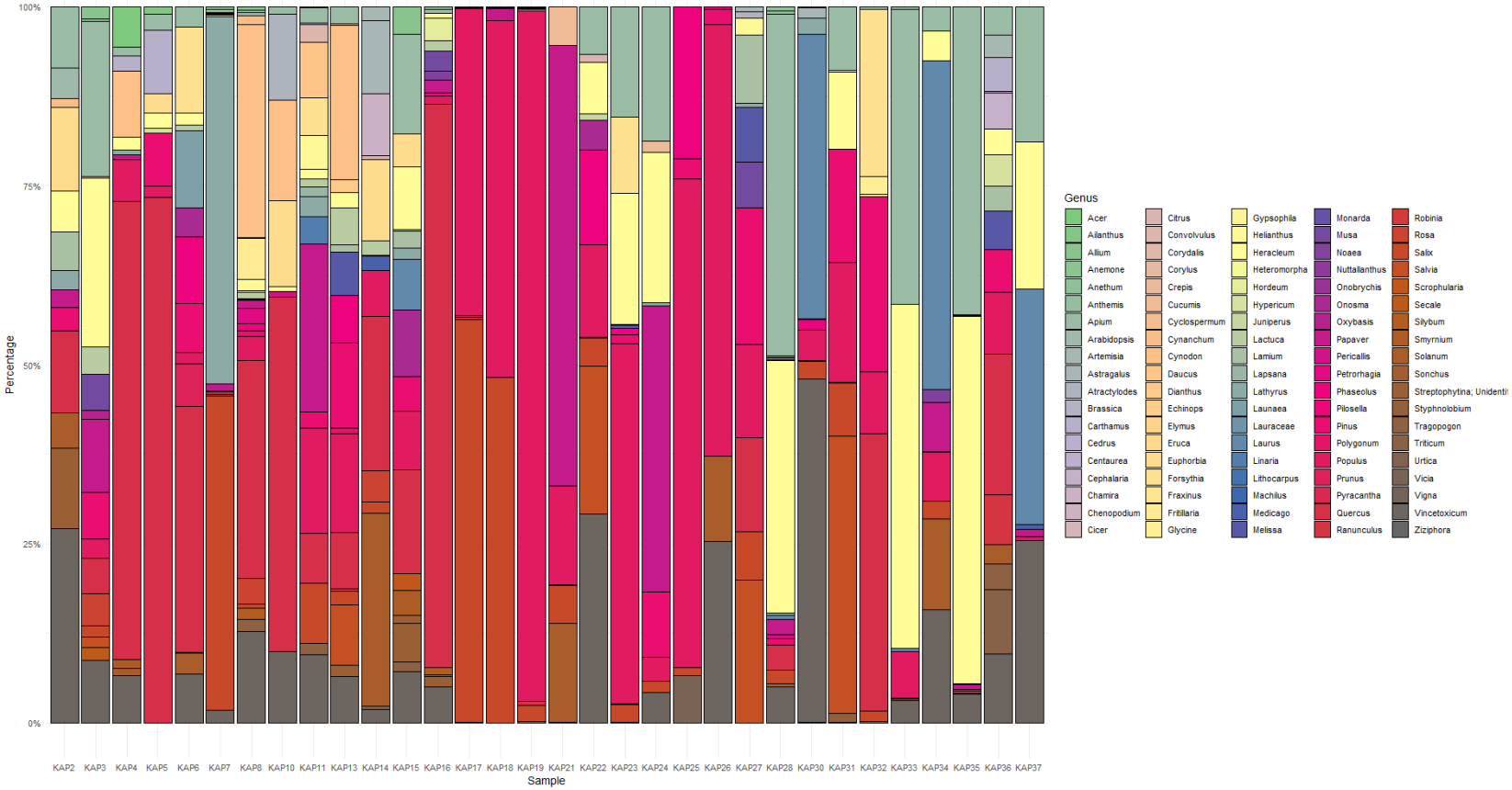

Figure 4. 2. The highest frequency of species found in the samples belongs to the *Quercus* and *Populus* genera. For enlarged form of the Figure 4.2, please refer Appendix 2.a and Appendix 2. b

4.4. Clustering of ceramic samples based on the plant genera and their frequencies

The tree diagram generated by analyzing the KAP samples from the Kaymakçı site (Figure 4.3) shows interesting clustering patterns while Table 4.2 shows clusters, which samples belong to which cluster, excavation locations for clusters, most and least common plant genus. The analysis yielded two primary clusters, with one of these further subdividing into three subclusters.

The sampled plant species have different patterns and traits that the hierarchical clustering analysis has revealed, putting them into clusters that are comparable in terms of genetic makeup and prevalence. Every cluster has distinctive characteristics, such as dominating plant species and genetic characteristics that set them apart from other groups.

In Cluster 1.a, *Papaver* stood out as the prevalent genera while *Ziziphora* was noted as the least common one. Moving onto Cluster 1.b, a similar trend was observed with *Ziziphora*, being the least common genus, but *Apium* taking the lead as the most common one. Cluster 1.c, on the other hand, promotes *Quercus* as the most common genus and *Fraxinus* as the least prevalent one. Cluster 2 displayed a profile with *Populus* being highly abundant and *Musa* being less prevalent.

In Table 4.3, Table 4.4, Table 4.5, and Table 4.6, the genera names and the order of genera from most common to least common for the Cluster1.a, 1.b, 1.c and 2 were provided, respectively.

Table 4. 2. Table showing clusters, which samples belong to which cluster, excavation locations for clusters, most and least common plant genera

Cluster No.	**Sample No.**	**Excavation Locations of the Ceramic Samples**	**Most Common Plant Genus**	**Least Common Plant Genus**
1.a	KAP7, KAP17, KAP18, KAP21, KAP30, KAP34, KAP37	3 of the samples are from 109.523, 2 of them are from 97.541, 1 of them is from 93.545 and 1 of them is from 95.555.	*Papaver*	*Ziziphora*
1.b	KAP2, KAP3, KAP11, KAP13, KAP15, KAP22, KAP24, KAP27, KAP28, KAP31, KAP33, KAP35, KAP36	5 of the samples are from 95.555, 4 of them are from 97.541, 3 of them are from 109.523.	*Apium*	*Ziziphora*
1.c	KAP4, KAP5, KAP6, KAP8, KAP10, KAP14, KAP16, KAP32	6 of the samples are mainly from 95.555, 1 of them is from 93.545 and 1 of them is from 109.523.	*Quercus*	*Fraxinus*
2	KAP19, KAP23, KAP25, KAP26	All the samples are from 97.541.	*Populus*	*Musa*

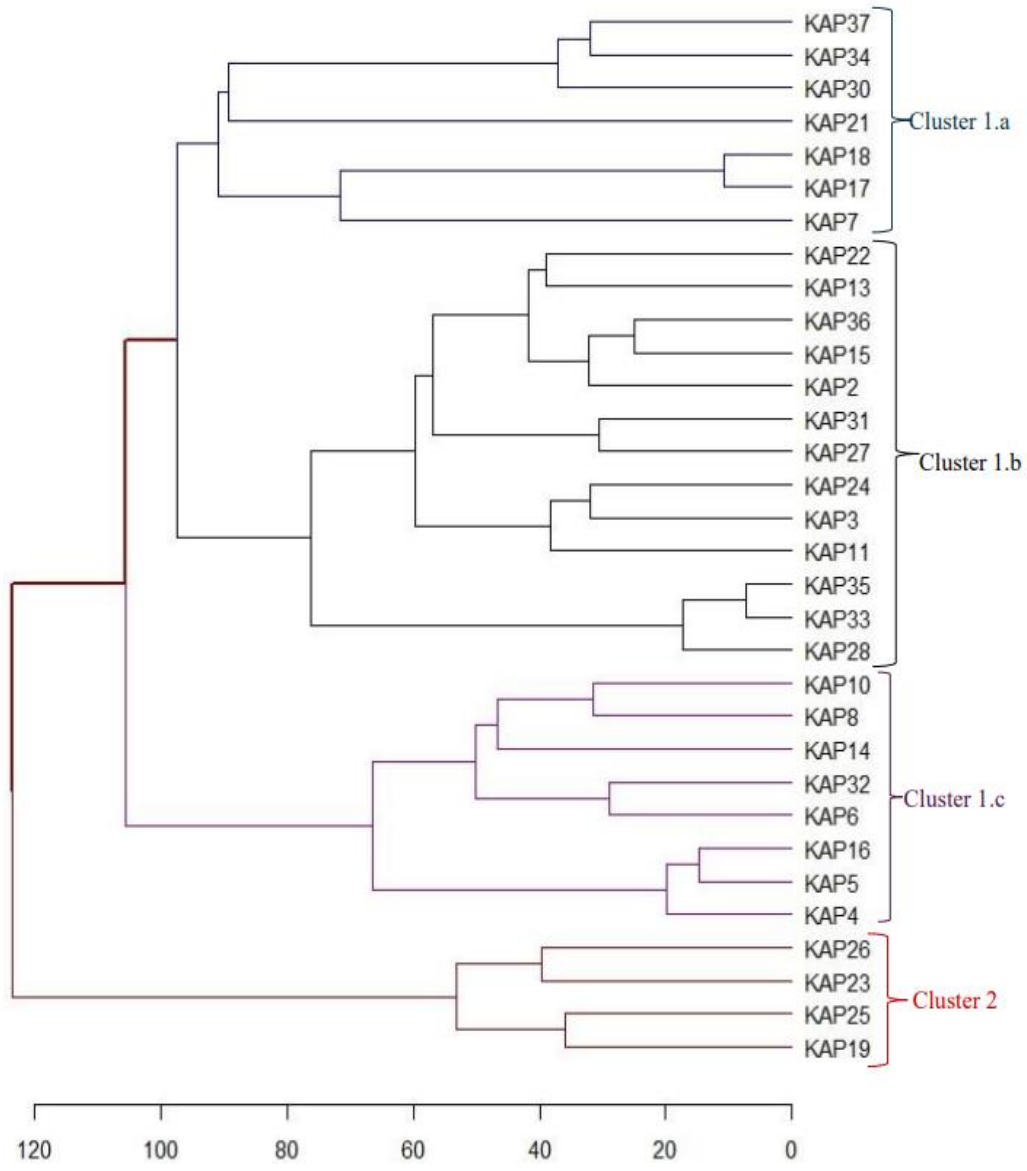

Figure 4. 3. Hierarchical cluster tree for KAP samples.

Table 4. 3. Presence of plant genera and their percentages in ceramic samples of Cluster1.a

Plant Genera	KAP7	KAP17	KAP18	KAP21	KAP30	KAP34	KAP37
Papaver	0.97	0	1.67	61.47	0	6.83	0.93
Lathyrus	51.29	0.04	0.03	0.01	2.24	0	0
Solanum	0	0	0	13.87	0	12.68	0.06
Heracleum	0	0	0.08	0.02	0	4.2	20.53
Apium	0.5	0	0	0	0	3.4	18.9
Cucumis	0.17	0	0	5.42	0	0	0
Noaea	0	0	0	0	0	1.8	0
Phaseolus	0.04	0	0	0.01	1.47	0	0
Astragalus	0	0	0	0	1.41	0	0
Quercus	0.38	0.35	0	0.08	0.04	0	0
Pinus	0.06	0	0.05	0	0	0.12	0.56
Medicago	0	0	0	0	0	0	0.77
Allium	0.32	0	0	0.01	0	0	0
Rosa	0.23	0	0	0	0	0	0
Fraxinus	0.21	0	0	0	0	0	0
Gypsophila	0	0.17	0	0	0	0	0
Pyracantha	0	0.17	0	0	0	0	0
Anemone	0	0	0	0	0.16	0	0
Chenopodium	0.1	0	0	0	0	0	0
Musa	0	0	0.09	0	0	0	0
Machilus	0	0	0	0	0.08	0	0
Corydalis	0.08	0	0	0	0	0	0
Eruca	0	0	0.07	0	0	0	0
Elymus	0	0	0	0	0.06	0	0
Ziziphora	0	0	0	0	0.02	0	0

Table 4. 4. Presence of plant genera and their percentages in ceramic samples of Cluster1.b

Plant Genera	KAP2	KAP3	KAP11	KAP13	KAP15	KAP22	KAP24	KAP27	KAP28	KAP31	KAP33	KAP35	KAP36
Apium	8.51	21.65	2.11	2.31	13.98	6.69	18.76	0	47.64	8.85	41.28	42.92	4.00
Heracleum	5.75	23.55	1.33	2.16	8.69	7.17	21.07	0	35.30	10.81	48.02	51.26	3.50
Vincetoxicum	27.11	8.69	9.51	6.47	7.09	29.16	4.24	0	4.99	0.06	3.10	3.97	9.62
Populus	0	2.73	14.79	13.84	8.20	12.98	3.37	12.98	0	16.82	6.42	0.16	8.64
Salvia	0	1.54	0	8.47	0	20.68	0	19.88	0	38.62	0	0.02	0
Quercus	11.53	5.00	6.90	7.91	14.49	0.04	0	13.13	3.45	0.06	0	0.34	19.66
Pinus	3.18	6.45	2.25	11.93	4.88	0	9.06	19.07	0.88	15.77	0.01	0	5.98
Papaver	2.50	10.20	23.39	0	0	0	39.96	0	2.13	0	0	0.76	0.02
Salix	0	1.52	8.41	1.91	0	3.93	1.57	6.81	1.96	7.47	0.25	0	0
Lamium	5.41	0	0	1.05	2.38	0	0	9.50	0	0	0	0	3.48
Cynodon	0	0	0	21.50	0	0	0	0	0	0	0	0	0
Phaseolus	0	0	0	6.61	0	13.16	0	0	0	0	0	0	0
Melissa	0	0	0	6.02	0.03	0	0	7.61	0	0	0	0	5.38
Streptophytina	11.19	0.03	0	1.55	5.41	0	0	0	0	0	0	0	0
Euphorbia	11.67	0	0	0	4.59	0	0.01	0	0	0	0	0	0
Onosma	0	0	0	0	9.30	4.14	0	0	0	0	0	0	0
Musa	0	5.02	0	0	0	0	0	6.40	0	0	0	0	0.02
Lactuca	0	3.91	1.16	5.15	0.27	0	0	0	0	0	0	0	0
Triticum	0	0	0	0	0	0	0	0	0	0	0	0	8.99
Lathyrus	2.70	0	2.77	0	1.53	0	0.44	0.63	0.31	0	0.01	0	0

Table 4.4 (continued)

Solanum	4.95	0	0	0	0	0	0	0	0.46	0.02	0	0.12	2.70
Laurus	0	0	0	0	7.01	0	0	0	0.57	0	0.44	0.08	0.02
Daucus	0	0	7.69	0	0	0	0	0	0	0	0	0	0
Helianthus	0	0	4.69	0	0	0	0	2.38	0	0.25	0	0	0
Ranunculus	0	0	0	0	0	0	0	0	0	0	0	0	6.87
Tragopogon	0	0	1.58	0	1.37	0	0	0	0	0	0	0	3.61
Allium	0	1.67	0	0	3.83	0	0	0	0	0	0	0.08	0
Forsythia	0	0	5.25	0	0	0	0	0	0	0	0	0	0
Cephalaria	0	0	0	0	0	0	0	0	0	0	0	0	5.11
Carthamus	0	0	0	0	0	0	0	0	0	0	0	0	4.76
Rosa	0	4.44	0	0	0	0	0	0	0	0	0.13	0	0
Hypericum	0	0	0	0	0	0	0	0	0	0	0	0	4.37
Arabidopsis	4.32	0.04	0	0	0	0	0	0	0	0	0	0	0
Linaria	0	0	3.83	0	0	0	0	0	0	0	0	0	0
Smyrnium	0	0	0	0	3.42	0	0	0	0	0	0	0	0
Artemisia	0	0	0	0	0	0	0	0	0.22	0	0	0	3.09
Convolvulus	0	0	2.47	0	0	0	0	0	0.23	0	0	0	0
Scrophularia	0	0	0	0	2.34	0	0	0	0	0	0	0	0
Crepis	0	0	0	0.28	0	0	1.54	0	0.02	0	0	0.12	0
Dianthus	0	0	0	1.81	0	0	0	0	0	0	0	0	0
Secale	0	1.75	0	0	0	0	0	0	0	0	0	0	0
Lapsana	0	0	1.36	0	0	0	0	0	0	0	0	0	0
Cynanchum	1.18	0	0	0	0	0	0	0	0	0	0	0.13	0

Table 4.4 (continued)

Styphnolobium	0	0	0	0	0	0	0	0	0	1.25	0	0	0
Oxybasis	0	1.24	0	0	0	0	0	0	0	0	0	0	0
Anethum	0	0.35	0	0.02	0	0	0	0	0.54	0	0.32	0	0
Sonchus	0	0	0	0	1.11	0	0	0	0	0	0	0	0
Citrus	0	0	0	0	0	1.11	0	0	0	0	0	0	0
Juniperus	0	0	0	0	0	0.93	0	0	0	0	0	0	0
Astragalus	0	0	0	0	0	0	0	0.71	0.21	0	0	0	0
Brassica	0	0	0	0	0	0	0	0.85	0	0	0	0	0
Polygonum	0	0	0	0.76	0	0	0	0	0	0	0	0	0
Petrorhagia	0	0	0	0	0	0	0	0	0.57	0	0	0	0
Anthemis	0	0	0	0	0	0	0	0	0.53	0	0	0	0
Cedrus	0	0	0.30	0	0	0	0	0	0	0	0	0	0
Robinia	0	0	0	0.23	0	0	0	0	0	0	0	0	0
Corylus	0	0.22	0	0	0	0	0	0	0	0	0	0	0
Centaurea	0	0	0	0	0	0	0	0	0	0	0	0	0.17
Ailanthus	0	0	0.14	0	0	0	0	0	0	0	0	0	0
Echinops	0	0	0.05	0	0	0	0	0	0	0	0	0	0
Urtica	0	0	0	0	0.05	0	0	0	0	0	0	0	0
Monarda	0	0	0	0	0	0	0	0.03	0	0	0	0	0
Lauraceae	0	0	0	0	0.03	0	0	0	0	0	0	0	0
Vigna	0	0	0	0.02	0	0	0	0	0	0	0	0	0
Onobrychis	0	0	0	0	0	0	0	0	0	0	0	0.02	0
Heteromorpha	0	0	0	0	0	0	0	0	0	0	0	0	0.02

Table 4.4 (continued)

Cyclospermum	0	0	0.01	0	0	0	0	0	0	0	0.01	0	0
Nuttallanthus	0	0	0.01	0	0	0	0	0	0	0	0.01	0	0
Ziziphora	0	0	0	0	0	0	0	0	0	0.01	0	0	0

Table 4. 5. Presence of plant genera and their percentages in ceramic samples of Cluster1.c

Plant Genera	KAP4	KAP5	KAP6	KAP8	KAP10	KAP14	KAP16	KAP32
Quercus	63.91	73.38	34.37	30.39	49.53	21.47	78.76	38.83
Cynodon	9.25	0	0	29.77	14.04	0	0	0
Vincetoxicum	6.58	0	6.80	12.71	9.87	1.88	4.96	0.14
Pinus	0.04	7.33	6.91	0.83	0	0.05	0.47	24.48
Forsythia	0	0	11.94	0	0	0	0	23.33
Solanum	1.05	0	2.92	0	0	26.87	0	0
Populus	5.85	1.61	1.52	3.29	0	6.38	1.06	8.54
Euphorbia	0	2.70	0	0	12.05	11.28	0	0
Atractylodes	0	0	0	0	12.05	0	0	0
Launaea	0	0	10.84	0.19	0	0	0	0
Carthamus	2.13	8.80	0	0	0	0	0	0
Phaseolus	0	0	9.23	0.99	0	0	0	0
Astragalus	0	0	0	0	0	10.17	0	0
Chamira	0	0	0	0	0	8.68	0	0
Apium	1.19	2.29	2.89	0.34	1.00	0	0.54	0.41
Rosa	0	0	0	3.61	0	4.49	0	0
Heracleum	1.74	2.18	1.74	0.25	0.64	0	0.72	0.30
Prunus	0	0	5.93	0.02	0	0	0	0
Fritillaria	0	0	0	5.72	0	0	0	0
Acer	5.66	0	0	0	0	0	0	0
Lactuca	0	0	0.72	0.92	0	2.04	1.49	0

Table 4.5 (continued)

Onosma	0	0	4.05	0	0	0	0	0
Salix	0	0	0.13	0.56	0	1.55	0	1.43
Papaver	0.69	0	0	1.12	0	0	1.83	0.02
Hordeum	0	0	0	0	0	0	3.12	0
Musa	0	0	0	0	0	0	2.75	0
Silybum	0	0	0	1.64	0	0	0.91	0
Glycine	0	0	0	0	0	0	0	2.49
Artemisia	0	0	0	0.47	0	1.95	0	0
Tragopogon	0	0	0	1.68	0	0.46	0	0
Petrorhagia	0	0	0	2.12	0	0	0	0
Medicago	0	0	0	0	0	1.99	0	0
Helianthus	0	0	0	1.58	0	0	0	0
Smyrnium	1.23	0	0	0	0	0	0.32	0
Streptophytina	0	0	0	0	0	0	1.45	0
Noaea	0	0	0	0	0	0	1.24	0
Cynanchum	0	0	0	1.24	0	0	0	0
Ailanthus	0	1.02	0	0	0	0	0	0
Allium	0	0	0	0.49	0	0	0.36	0
Pericallis	0	0	0	0	0.81	0	0	0
Lathyrus	0.68	0	0	0.05	0	0	0	0
Hypericum	0	0.70	0	0	0	0	0	0
Cicer	0	0	0	0	0	0.60	0	0
Lithocarpus	0	0	0	0	0	0.14	0	0

Table 4.5 (continued)

Echinops	0	0	0	0.04	0	0	0	0
Pilosella	0	0	0	0.02	0	0	0	0
Laurus	0	0	0	0	0.01	0	0	0.01
Vicia	0	0	0.01	0	0	0	0	0.01
Fraxinus	0	0	0.01	0	0	0	0	0

Table 4. 6. Presence of plant genera and their percentages in ceramic samples of Cluster 2

Plant Genera	KAP19	KAP23	KAP25	KAP26
Populus	96.42	50.22	68.29	60.21
Vincetoxicum	0.21	0.03	0	25.33
Phaseolus	0	0.88	21.20	0.27
Heracleum	0.14	18.36	0	0.05
Apium	0	15.38	0	0
Solanum	0	0	0	11.84
Forsythia	0	10.60	0	0
Vicia	0	0	6.62	0
Pinus	0.07	1.25	2.81	2.20
Salix	2.23	2.48	1.07	0
Quercus	0.52	0.18	0.01	0.05
Medicago	0	0.50	0	0
Lactuca	0.17	0	0	0
Astragalus	0.13	0	0	0
Cicer	0.07	0	0	0
Euphorbia	0.06	0	0	0
Salvia	0	0	0	0.05
Allium	0	0.04	0	0
Laurus	0	0.03	0	0
Papaver	0	0.03	0	0
Lamium	0	0.03	0	0
Musa	0	0.01	0	0

CHAPTER 5

DISCUSSION

This study delves into utilizing the *rbc*L gene to identify present plant genera of ancient ceramic samples from the Kaymakçı archaeological site via metagenomics analysis. Good extraction results were obtained by performing soil DNA isolation kit due to the similarity between clay, which the ceramic is made, and soil itself. This may be explained why other methods yielded poor results.

The integration of DNA-based methods for plant identification from soil samples has become increasingly feasible through molecular techniques. DNA metabarcoding allows for the rapid assessment of plant biodiversity using short DNA fragments extracted from soil, reflecting both current and previous vegetation. This method, as demonstrated by Yoccoz *et al.* (2012), has shown that short DNA fragments from soil samples can efficiently assess plant taxonomic diversity, with results consistent with conventional above-ground surveys. This technique opens up new opportunities for large-scale DNA-based biodiversity studies using standardized metabarcoding approaches.

In this study, 95 different genera were found, and those genera were created 2 main clusters with one of them having 3 subclusters. When the excavation locations and the samples in each cluster are examined from Table 3.1, there might be a relation between them. The reason is that the samples in each cluster excavated generally from the same location. This relation may imply about localized factors such as the soil composition, water availability, and land- use by human societies at that time. For example, nearly half of the ceramic samples we received (12 pieces) were from 95.555 excavation location (Table 3.1). This might be interpreted to suggest there were more human activities like cultivation of certain plants such as *Apium* which was the most common genus for Cluster 1.b, in that area. Dawson *et al.* (2004)

demonstrated that plant- derived hydrocarbons in soil organic matter can serve as biomarkers for vegetation identification. That means this method can be used to identify plants in the absence of morphological plant parts. To be sure, this technique can be performed on the ceramic samples since this approach is particularly useful for identifying historical vegetation and understanding previous ecological conditions.

The research conducted by Niemeyer *et al.* (2017) compared sedDNA and pollen data from lake sediments in Siberia. They discovered that sedDNA was more effective at capturing a range of plant species and documenting site-specific richness when compared to pollen analysis. In a study published by Liu *et al.* (2020), sedimentary ancient DNA (sedaDNA) was found to offer a detailed insight into historical vegetation changes and plant diversity in the Siberian treeline area compared to traditional pollen analysis methods. Building on this another study by Liu *et al.* (2021), utilized DNA to reveal that plant diversity peaked in the Tibetan Plateau during a cooler period following glacier retreat but is now at risk of decline due to warming induced loss of alpine habitats.

Furthermore, Duley *et al.* (2022)'s research showcased how soil eDNA metabarcoding could accurately measure relative plant diversity across habitats emphasizing the importance of understanding survey locations for results. This methodology proves effective for assessing plant diversity and community composition across habitat types making it valuable for restoration monitoring initiatives. In addition, a significant study conducted by Ariza *et al.* (2022) illustrated the effectiveness of soil eDNA surveys in monitoring plant biodiversity and vegetation changes on a scale over time. However, while this method excelled at detecting taxa it faced challenges, with identifying visually unrecorded species during visual surveys. By gaining insights from these studies, we can conduct analyses such as more in-depth examinations of eDNA and metabarcoding in the future.

Excavating the remains from archaeological sites requires extremely careful procedures since there are lots of contamination sources at the sites (Yang & Watt 2005). The ceramic samples from the Kaymakçı were relatively high-quality. One explanation for this is that no permanent settlement was established there after the Late Bronze Age, hence no buildings or disturbances were made to the site by later civilizations. Because of this, the original layers and remains have been rather well conserved, making it possible for archaeologists to excavate and study them successfully (Roosevelt *et al.*, 2015; Çiftçi *et al.*, 2019). On the contrary, there are many factors that might cause contamination before, during and after the excavations. For instance, *Populus* (Ciftci & Kaya, 2019), and *Salix* (Acar *et al.*, 2020) are very common in the Manisa area. The prevalence of *Quercus* trees in the Manisa region was not surprising as they are widely recognized as the most common type of oak tree there (Aykut *et al.*, 2017). As a result of the metagenomic analysis, it was expected that these three plant genera would be quite common in the area. Also, the pollens from trees can be carried by rivers and ending up settling near bodies of water and along their banks (Kerienė *et al.*, 2023). This also means that cross-contamination from the environment should be considered. Groundwater could lead to contamination as clay, which carries a negative charge, in salty water situations attracts cations to balance its neutrality. Moreover, the movement of groundwater may carry out DNA from other plants potentially causing contamination (Deng, 2021).

Apart from the surface- and cross-contamination, the plant genera observed in this study might have various uses. It is likely that these plants were preserved for necessities such as food (like *Phaseolus* (Corrado, 2022), *Triticum* (Arzani & Ashraf, 2017), *Hordeum* (Kajla *et al.*, 2023) and *Secale* (Saldivar, 2016)), aromatic herbs and spices for cooking (such as *Salvia* (Anwar & Qadir, 2021), and *Melissa* (Miraj *et al.*, 2016)), religious rituals, agricultural activities and environmental management well as for decorative or aesthetic reasons. The diverse assortment of stored plants indicates an understanding of their environment. This suggests the

important roles these plants played in the daily lives of the inhabitants by providing sustenance, medicinal remedies, and materials for various traditions.

Studying samples collected from sources such, as soil and sediment through metagenomics analysis allows researchers to uncover information, about plant species aiding in the comprehension of ecosystems (Sarkissian et al., 2021).

CHAPTER 6

CONCLUSION

This study successfully utilized the *rbc*L gene to identify plant genera in ancient ceramic samples from the Kaymakçı archaeological site through metagenomics analysis. The similarity between clay and soil facilitated effective DNA extraction using soil DNA isolation kits, highlighting the potential of DNA metabarcoding for rapid and accurate assessment of historical plant biodiversity.

The findings revealed 95 different genera, forming distinct clusters that correlated with excavation locations. This suggests localized environmental factors and historical human activities influenced plant distribution. The high quality of the ceramic samples, preserved due to minimal post-Bronze Age disturbances, enabled accurate analysis despite potential contamination risks from local vegetation and groundwater.

The diverse plant genera identified indicate their varied uses, such as food, aromatics, and other daily necessities, underscoring the inhabitants' understanding of their environment. This study demonstrates the value of metagenomics in reconstructing past environments and provides a foundation for future research in archaeological and ecological contexts. By leveraging DNA-based methods, researchers can gain deeper insights into historical plant diversity and ecosystem dynamics. Moreover, the success of this study underscores the importance of meticulous sample handling and contamination control in archaeological DNA research. Future studies should continue to refine these techniques, potentially integrating additional molecular markers and more advanced bioinformatics tools to further enhance the resolution and accuracy of plant identification.

In conclusion, the application of DNA metabarcoding in archaeological research offers a powerful tool for unraveling the complexities of past ecosystems. This approach aids in understanding historical biodiversity. As molecular techniques continue to evolve, they will undoubtedly play an increasingly significant role opening new avenues for interdisciplinary research and discoveries.

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APPENDICES

1. DNA concentrations of isolated ceramic samples

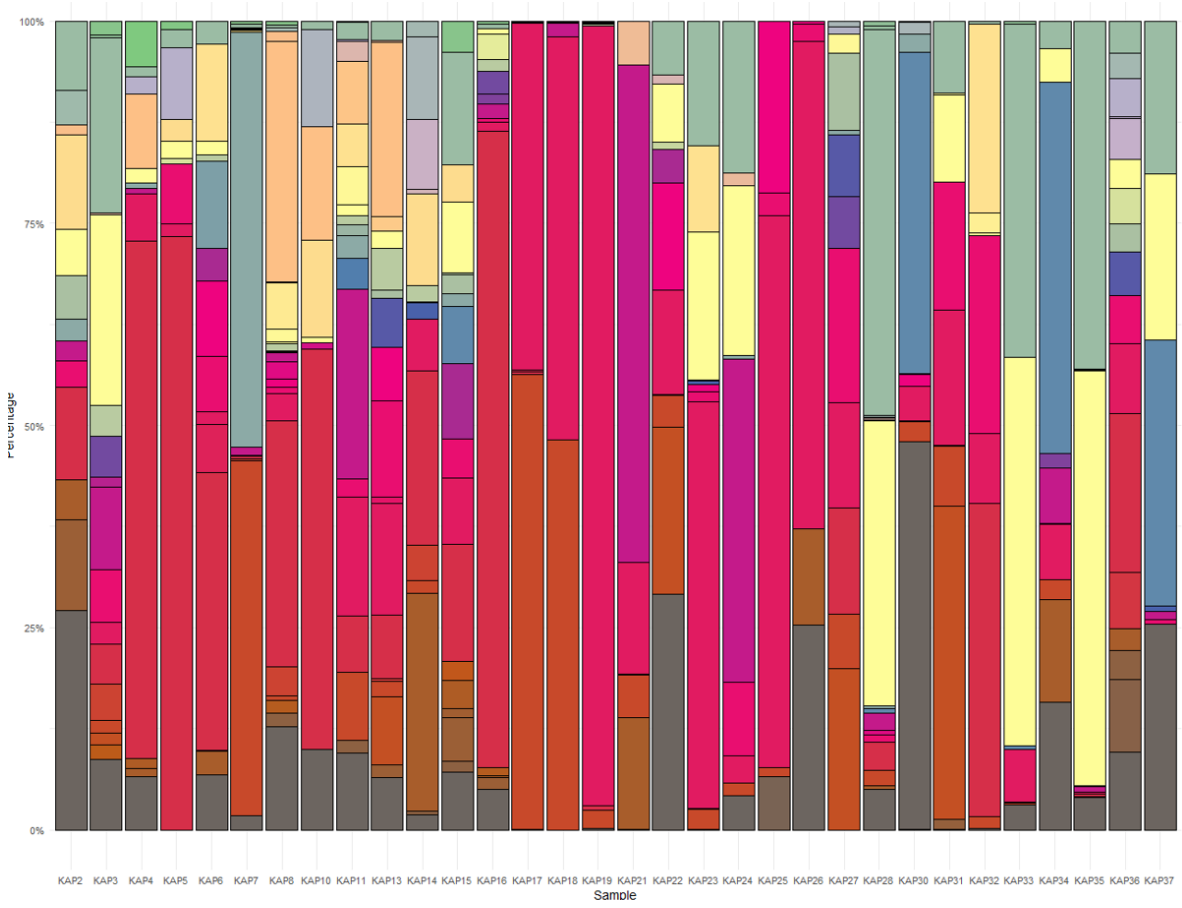
Appendix 1.a DNA concentrations of isolated ceramic samples

Sample No.	Weight(g)	DNA Conc. (µg/ml)	A260/230	A260/280
KAP02	0.25	5.671	0.586	2.123
KAP03	0.25	6.721	0.627	1.806
KAP04	0.25	7.004	0.159	2.331
KAP05	0.25	12.40	0.530	1.676
KAP06	0.25	4.952	0.553	3.204
KAP07	0.25	7.128	0.471	3.350
KAP08	0.25	3.706	0.236	2.339
KAP10	0.25	8.954	0.508	2.265
KAP11	0.25	10.34	0.532	2.383
KAP13	0.25	8.979	0.473	2.256
KAP14	0.25	6.724	0.457	2.469
KAP15	0.25	5.831	0.422	3.185
KAP16	0.25	6.143	0.56	2.687
KAP17	0.25	10.19	0.481	1.964
KAP18	0.25	2.934	0.295	3.141
KAP19	0.25	4.592	0.532	1.771
KAP20	0.25	10.62	0.841	1.605
KAP21	0.25	4.407	0.503	1.977
KAP22	0.25	5.66	1.546	2.128
KAP23	0.25	3.193	0.761	2.677
KAP24	0.25	13.5	0.659	1.979
KAP25	0.25	4.227	0.32	1.898
KAP26	0.25	1.995	0.333	2.006
KAP27	0.25	6.279	0.511	1.915

Appendix 1.a.(continued)

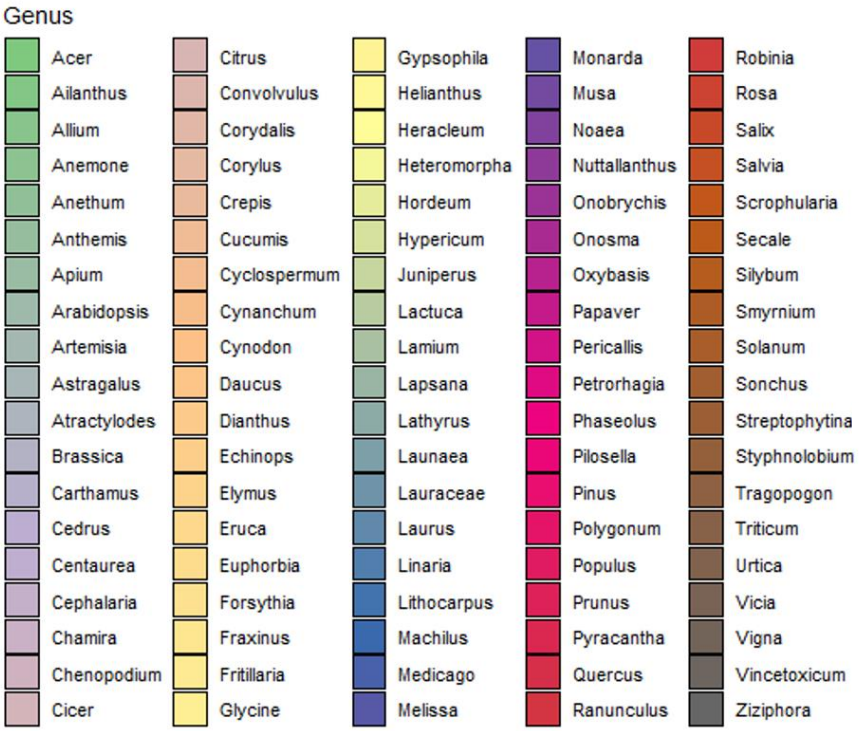
KAP28	0.25	2.774	0.259	2.343
KAP30	0.25	0.406	0.113	1.000
KAP31	0.25	3.804	0.322	2.109
KAP32	0.25	30.66	0.605	1.643
KAP33	0.25	2.689	0.305	3.109
KAP34	0.25	13.32	0.477	1.819
KAP35	0.25	2.859	0.374	2.457
KAP36	0.25	3.336	0.81	2.497
KAP37	0.25	3.38	0.717	1.491

2. Enlarged form of the Figure 4.2 (for plant genera and their frequencies)



Appendix 2.a.

Enlarged form of the Figure 4.2. Each color represents distinct genus.



Appendix 2.b. Legend for cumulative percentage distribution of genus composition across all 32 KAP samples. Each color scale represents the plant genus.