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WHOLE GENOME SEQUENCING APPROACH REVEALED SPECIES IN MYCENAEAN PERIOD ASSOCIATED RESIDUAL PLANT BIOMASS: FIRST RESULTS

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ABSTRACT

New technologies of DNA sequencing can provide valuable insights into the identity of archaeobotanical remains, early human-environment interactions and an understanding of social and religious activities. In the Late Bronze archaeological site of Kastrouli, Phokis (Greece), plant biomass remains in a ceramic vessel of the Mycenaean period are analysed. To determine the identity of the biomass, a high throughput sequencing technology was used to obtain whole genome sequencing (WGS) of plant, bacterial and fungal genomes. Whole genome analysis (WGS) of ancient DNA (aDNA) showed that despite the small DNA fragments recovered from the plant remains several reads were assigned to reference plant genomes The molecular analyses in the archaeobotanical and medicinal plant remains identified 10 agronomically important plant species (oak, *Eucalyptus*, tobacco, grape, cotton, cowpea, conyza) and characterized a diverse microbial community associated with the plant biomass. Surprising is the detection of wild tobacco, tomato and cotton. The taxa represented by plant parts, provide socio-cultural insight on plant use by the inhabitants of the settlement. A complementary microscopic analysis of the plant biomass revealed floral parts and leaves of different morphologies belonging to different taxa. In particular, the microbial community within the vessel environment had distinct associations with plant, rhizosphere, soil, marine and water ecosystems; which are evident in Kastrouli environs. The sequencing information recovered from the approaximately 3000-year-old biological samples revealed unexpected findings on biocultural diversity, early agriculture and species domestication in this corner of the Mediterranean basin.

KEYWORDS: archaeobotany, ancient DNA, microbial community, whole genome sequencing, Mycenaean, ceramic, environment, species, next generation sequencing

1. INTRODUCTION

Studies from plant remains retrieved from archaeological sites are limited but they can be informative regarding ancient crops and past habitats in a given region, trade goods as well as plant impact on human settlements. This evidence together with archaeogenetics and archaeometry provide a holistic approach in reconstructing the socioenvironmental history of the archaeological site. The present study is a first focus to the systematically excavated Kastrouli settlement, a Late Bronze site near Delphi in southern Phokis, Greece. This project is a first enterprise of archaeological dig initiated by an archaeometry triggered concept introducing a truly interdisciplinary and multiscientific work; which directs the archaeological dig involving most contemporary scientific tools for choosing where to dig, and retrieving maximum data information of all the finds through a holistic process (Liritzis 2021).

The origin, historical and recent advances in the field of palaeomicrobiology, along with some results of the most notable ancient pathogenic microorganism studies have been reviewed (Arriola *et al*., 2020) and provide perspectives on how the next generation sequencing (NGS) and whole-genome information from ancient microorganisms contribute to our understanding of bacterial evolution on a broader scale. NGS has unlocked the potential to identify a wide range of non-cultivable microorganisms, including those present in the ancient past.

In the era of high-throughput sequencing, microbial archaeology is growing fast, exposing the agents behind harmful historical plagues, discovering the cryptic intrusion routes of viruses in prehistory, and reconstructing the human microbiota's ancestry. Particular emphasis is currently given to the process of identifying, validating, and authenticating ancient microbes using high-throughput DNA sequencing data. This novel field at its take-off has a promising future (Warinner et al., 2017).

Next-generation sequencing has become more prevalent and can benefit the genomics exploration of aDNA, while environmental metagenomics can play an important role in uncovering microbial communities within various archaeological sites and specimens (Kazarina et al., 2019). However, substantial challenges remain that hinder genetic analyses, including contamination, decomposition, alteration of the environmental niches and fragmentation of the genetic material. Thus, proper standards and precautions to guide the field's future are a sine qua non condition for reliable and safe results. Often, the majority of DNA usually stems from the environment, while aDNA is present in less than 1% of the extracted sample and is usually poorly preserved (Spyrou et al., 2019). The identification of the organisms in archaeological samples facilitates the uncovering of biological diversity in the site and helps to define the relationship between the identified organisms and environmental conditions (e.g., salinity, temperature, trophic and cultural state). This in turn, provides a deeper understanding of species evolution and provides an integrated view of the roots of the modern world.

Recent technology advancements such as whole-genome sequencing and metagenomics have already begun to revolutionize DNA research (Gilbert et al., 2007). Soil metagenomics and microbial community analysis can complement soil archaeometry studies that examine soil physical and chemical properties (Margesin et al., 2017).

Kastrouli, as an archaeological site, appears to have been an important city of the Late Bronze Age (ca. 1300-1100 BC), a period also known as 'Mycenaean' or Late Helladic III B-C (Liritzis, 2021) (Fig.1). The rich material culture ranges from tombs, buildings, artifacts and bone finds. The archaeological-archaeometrical investigations conducted in the Kastrouli Project included archaeological excavation (www.kastrouli.org; Sideris et al., 2017; Levy et al., 2018; Sideris and Liritzis 2018; Koh et al., 2020), established and newly developed archaeometrical techniques, such as bone diagenesis, palaeogenetic and a palaeoproteomic study (Kontopoulos et al., 2019; 2020), archaeo-geophysical prospection (Levy et al., 2018; Savvaidis et al., 1999), luminescence dating of stone and ceramics using single aliquot techniques (Liritzis et al., 2016, 2019, 2020a, 1997, 1996), digital applications (Levy et al., 2018; Hatzopoulos et al., 2017), characterization and provenance of ceramics (Xanthopoulou et al., 2021; Liritzis et al., 2020b; Xanthopoulou et al., 2020; Bratitsi et al., 2018). These studies provided new data for the monumental, fortified walls and large buildings that may have belonged to a little Mycenaean palace; including clay figurines, stirrup jars, pottery and metal finds. Based on these and the surface finds in the environs of Kastrouli an apparent model emerges that of central citadel-to-communal clusters evolution.

This study is a first attempt focusing on whole-genome and metagenomic analysis in collected archaeological residues found in Mycenaean Kastrouli.

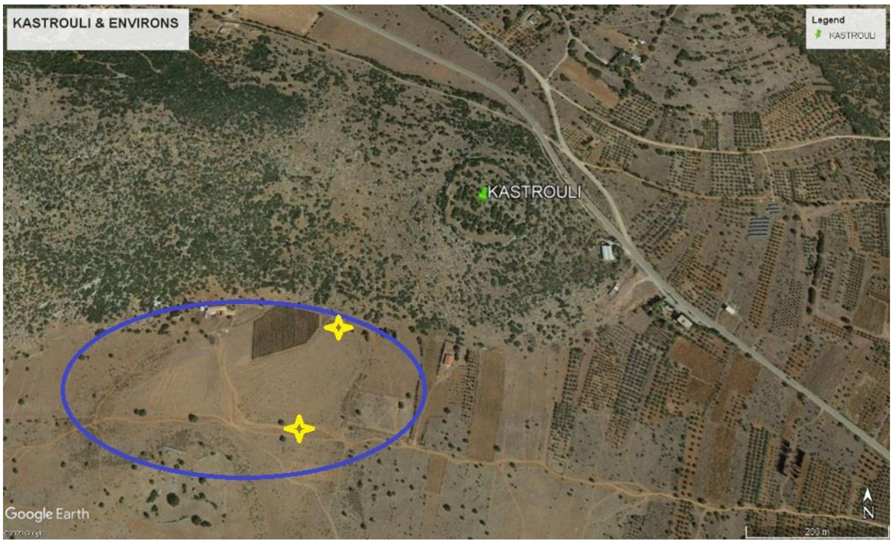

Figure 1. Kastrouli citadel and environs seen from google earth (N 38º 23′ 56.7′′, E 22º 34′ 30′′, 550 m asl). Note the fortified wall circularly traced by crops. The settlement is around 50 meters above the southwestern flat plain. A contemporary flooding flat area (<5º slope in blue) in the SW with the two man-made sink holes (stars) of unknown age, presumably engineered in prehistoric times (Liritzis et al., 2021, work in progress).

2. MATERIALS AND METHODS

2.1. *Sampling and context*

The excavation and collection of the samples took place in the archaeological site of Kastrouli in Phokis region of central Greece near the modern village of Desfina, not far from Delphi (Liritzis, 2021). Plant biomass remains that were the fills of an ancient vessel, collected and packed in sterile plastic bags and transported to the laboratory for analysis (Fig. 2). The sample derives from the Building 1, vessel filling wet sieving (leaves, branches, plants) during the 2017 excavation season.

Figure 2. Upper: Building 1 of LH IIIB - LH IIIC date, looking from the southwest corner; and the entrance and a stone base or foothold of a wooden column (approx. 25 cm thick) holding the ceiling (based on Sideris and Liritzis 2018), Lower: Ceramic vessels-oenochoe, hydria, crater - found around the center of the building (see, Sideris and Liritzis 2018; https://archsci.aegean.gr/index.php/en/).

During the excavation of a 2 m wide trench inside and along the S-W wall of Building 1 in a depth of ~15 cm from the surface, a destruction layer characterized by high density of charcoal (but no ashes), several pottery sherds, and some small finds, mostly close to the entrance, were found.

The clay had an intensive red color, almost no inclusions, but it was very friable. More fragments of the wall coating have been found on the floor, among the charcoal and soil mixture. Beneath this layer of car-bon, pottery sherds, and soil, which measured approximately 15 cm thick, sections of the latest floor before the destruction was discovered, similar in texture and color to the fragments of clay wall coating. The floor, no doubt, was made by pressed soil and the walls had unfired clay coating. Both, however, floor and walls, have suffered very high temperatures during the destruction of the building by fire, a fact that not only led to their baking and consequently to their partial preservation, but it also gave them the characteristic intensive red color. Finds include: a slightly concave mortar of Gray stone in depth 10-25 cm of 51 x 20 x 9 cm and grinders; an hydria, an oenochoe and cylix partly preserved in several fragments, both reassembled by conservators (www.kastrouli.org) (Fig.2); small finds, mostly clay and stone spindle whorls of various sizes, some other stone tools, as well as several sherds of undecorated household pottery, mostly pottery sherds, stone beads, two small metal artifacts (bronze and lead lamella with its 'nail'), repairing ligament for broken pottery, animal bones, as well as, sea shells. spherical stone crushers and a fragment of a stone axe (Sideris and Liritzis 2018; Liritzis 2021).

2.2. *Microscopic observations*

Phenotypic observations of plant biomass were conducted through a fluorescence stereoscope (Leica MZ10F, Taiwan) equipped with objective Planapo 1.0x and Leica camera DFC 310 FX. In addition, a Zeiss Axioscope 40 epifluorescence microscope was used, equipped with a ProgRes CF cool camera (Jenoptik, Jena, Germany), and ProgRes Mac Capture-Pro software (Jenoptik, Jena), different objectives (A-Plan 40x/0.65na Ph2 Var2, A-Plan 5x/0.12) and excitation and emission filters for FITC (519 nm) for autofluorescence detection. To facilitate plant morphology identification, a bright-field image was acquired.

2.3. *DNA extraction and sequencing*

For the extraction of plant biomass remains, 100-150 mg were ground under liquid nitrogen with a mortar and pestle and processed with NucleoSpin Plant II Genomic DNA (Machery-Nagel, Germany). The final DNA concentration was quantified with Qubit Fluorometer 4 (Thermo Fisher Scientific, US) by using the 1X dsDNA HS (High-Sensitivity) Assay Kit (Life Technologies, United States). The extracted DNA was amplified with a non-targeted approach with the REPLI-g UltraFast Mini Kit (Qiagen, USA) and purified with magnetic beads (Agencourt AMPure XP, USA). The sequencing library was prepared with the Rapid Barcoding Kit (Oxford Nanopore, UK) and loaded into an R9.4.1 flow cell on a MinION Mk1C (Oxford Nanopore, UK), according to the manufacturer's recommendations.

2.4. *Bioinformatic analysis*

Basecalling was performed with minKNOW software, while microbial communities were identified through an EPI2ME 16S and WIMP analysis workflow software (Oxford Nanopore, UK). The EPI2ME cloud-based software did not provide functionalities for statistical data evaluation. The taxonomic classification and quality of barcoded reads were downloaded as a

CSV file from the EPI2ME dashboard for further processing. This file included information on run and read IDs, read accuracy, barcodes, and NCBI taxonomy IDs for classed reads. The CSV file was processed with python scripts for generating root level OTU tables, by matching NCBI taxa IDs to lineages and counting the number of reads per NCBI taxa ID. Another bioinformatics pipeline based on DIAMOND (Buchfink et al., 2015) alignment algorithm. Similarity filter >85% applies to identify plant species in vessel biomass remains through WGS analysis. Further interpretation was facilitated by machine learning libraries and proprietary software modules at DNASequence SRL HELLAS.

3. RESULTS AND DISCUSSION

Delphi, a Classical settlement with Mycenaean presence, is located on the steep slope of Mt. Parnassus in Phocis region of central Greece and was the most famous Greek temple and oracle in the 7th-6th centuries BCE. Initially, the oracle of Delphi was dedicated to Mother Earth (Gaea), later to Apollo and much later to Dionysus. Only 11 Km away from Delphi, lies the Kastrouli Late Mycenaean settlement, a small citadel with cyclopean fortifications of circular shape. The location of the citadel at the top of a 700 m hill is strategic, as it overlooks the Corinthian Gulf and controls the entrance to the hill from South and East-West. Based on Homer's descriptions in the Book 2 of the Iliad (*Iliad*. 2.521) and the Catalogue of Greek Ships that participated in the Trojan War, warriors from Kastrouli fort participated with 40 ships. Given its location, including the war and navigation skills of its inhabitants, it is likely that the citadel of Kastrouli ensured the defense and protection of other coeval settlements of the surrounding area (Fig. 3). Nevertheless, it is still limited the information regarding the Kastrouli community and its cultural development, or how they interacted with the so far six Mycenaean sites in southern Phokis (Kastrouli, Sykia, Steno, Vroulia, Antikyra/Kyparissos, Medeon acropolis and cemetery) (see, Sideris et al., 2018). Recent excavations (Sideris et al., 2017; Levy et al., 2018; Koh et al., 2020) have shed light on various aspects of life inside this ancient society with the discovery of interesting artifacts such as small clay female figurines (possibly religious votive offerings or representations of female deities), osteological remains, sea shells, pottery sherds, stone mortars, grinders, spindle whorls, stone beads and a ceremonial vessel, which contained plant offerings, thus indicating a religious connection of the people through plants to Earth and possibly to the spiritual world (through smoke).

Figure 3. Kastrouli and environs with accessibility to the sea. Steno coastal Late Helladic III settlement on the right lies an excellent leeward port, highly possible a seaport of Kastrouli. The western Itea-Kirrha port denotes the presence of an Early-Middle beginning of Late Helladic settlement. Up right is Parnassus mount, above the seismic fault passing from Delphi. Note the geomorphological relief (terrains, torrents, plains) and the strategic positioning of the settlement with access to the sea.

3.1 Description of Plant Biomass

In general, the anatomical and morphological features of plant remains retrieved from archaeological sites can provide information regarding their identity and/or botanical taxonomy. This type of archaeobotanical investigation is aiming to address questions concerning past interactions of humans with their environment, plant uses, and the cultural context of the plant remains. Furthermore, it tries to determine how and why the plant remains were brought to the site and if possible, to reconstruct the history of the past surrounding habitats.

In this study, we examined plant remains collected from Building 1 in the excavated Kastrouli archaeological site and associated with the Mycenaean period. To differentiate the plant species based on plant structure and morphology non-destructive high-resolution microscopy was used. According to its design, the vessel was presumably used in social or religious rituals. A close examination of the plant remains by microscopy revealed that the plant biomass was not a mono-specific type but a mixture of a few different taxa (e.g., species). The sample from the fills of the vessel contained leaves (a rare find in archaeological plant remains), small twigs, and calyces of flowers. While the pristine condition of these delicate tissues suggests little transportation and in situ preservation. Specifically, among the finds were decomposed simple flat leaves of rhomboid shape and acute at the apex in an alternate leaf arrangement, where one leaf emerged in each node along the stem (Fig. 4A). The leaf was attached to a solid erect stem through a petiole (Fig. 4B). Typically, leaves from terrestrial plants are thicker and expanded compared to submerged leaves of aquatic plants, which are thin and narrow (Wells & Pigliucci, 2000). It is apparent that parts of the leaf blade decomposed except the venation structure, which was well preserved. The leaves have a raised midrib and reticulate venation pattern characterized by veins arranged on the two sides of the midrib in a net-like fashion. This type of leaf venation is typical of dicot plants. Although the primary function of leaf venation function is to transport water and photosynthetic assimilates from the petiole across the lamina, it can also provide mechanical support to the leaf (e.g., leaf strength). In the present study, the litter leaves show adaptations for floating on water due to the thick cylindrical structure of the veins, which is a sign of increased buoyancy in water. Also, the development of unbranched adventitious roots on the ventral side of the leaf, might serve for absorption of water and minerals (Jones, 2012).

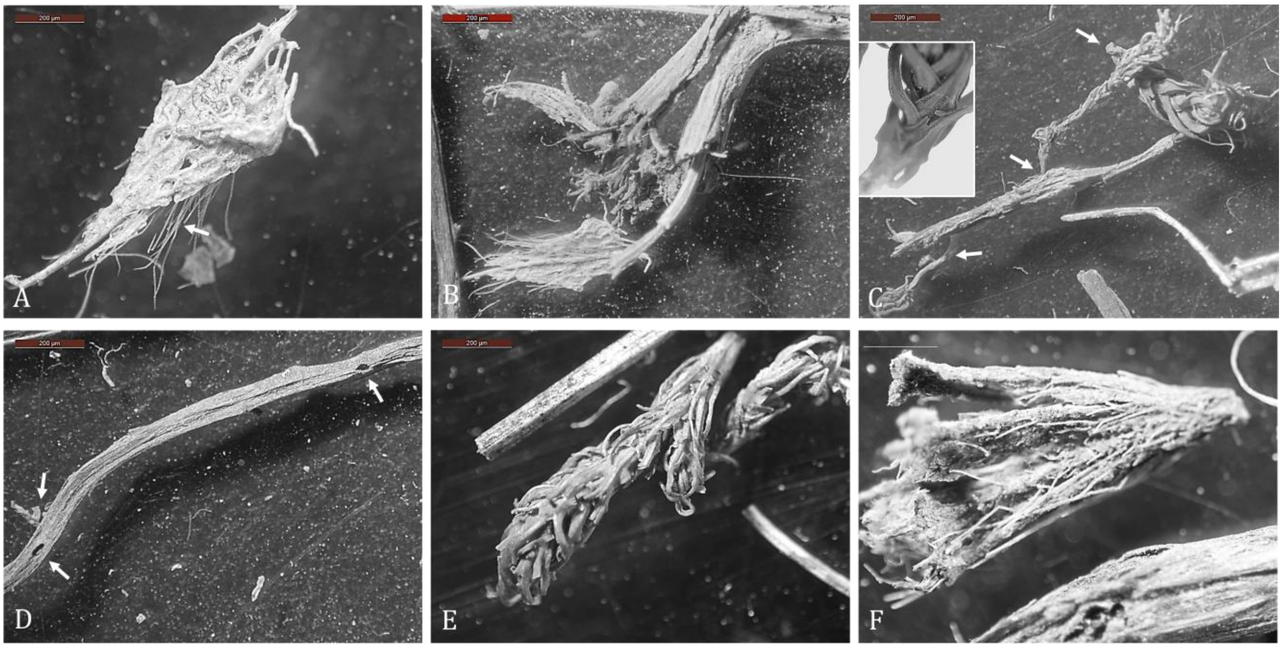

Figure 4. Bright-field micrographs of plant biomass remains. White arrows show micro morphological features. Inset shows a larger magnification of the specimen. Scale bar, 200 µm.

The solid thick stem in **Fig. 1B** has secondary growth, a characteristic of the dicots, which agrees with the leaf venation pattern. Further, the presence of a rigid stem, that is more recalcitrant to decomposition than the leaves, suggests terrestrial growth and rich lignin content. Stems have higher lignin than the leaves and decompose slower because of lignin's complex structure. On the contrary, leaves contain more cellulose that can be more easily decomposed by microorganisms. This is the reason why leaves are more susceptible to decomposition and are rarely found in archaeological specimens. Of note, the presence of roots in the leaves is an adaptive feature, which clearly shows that the plant is amphibious occupying a niche in the terrestrial-aquatic interface where fluctuating water levels force it to develop specialized structures in leaves floating at the water's surface for survival. Similarly, the vessel also contained plants with hollow and thin stems of polygonal shape lacking secondary growth that are presumed to be aquatic (Figs. 4C-E). In these plants, lateral shoots display indetermined growth generated from superficial and scattered positions along the shoot (Figs. 1C-1D). Notably, the morphology of the juvenile leaf-like structures at the base of the plant was different from that of the adult leaves (Fig. 4E) in terms of size and shape, a phenomenon called heteroblasty. Collectively, the morphological traits of the aquatic plants resemble those of bryophytes (amphibians of plant kingdom) like mosses and liverworts, which have leaf-like organs called phyllids. The presence of aquatic and amphibious plants in the vessel may have come from water transported from a marsh or lake, or during the collection of other plant species from the nearby area (see, Liritzis 2021, Fig.4). Still, small floral archaeobotanical remains were discovered in the sample with tubular calyces, symmetrical flowers with trichomes in sepals, all fused together (Fig. 4F) These floral parts were found detached from the plant, so information on stem and leaf morphology could not be used. The flowers with the showy tubular floral structures could be Hellebore (*Helleborus spp*.) flowers of the *Ranunculaceae* family that are unique in that petals evolved into short, tubular structures (nectaries) producing nectar to attract insects around the time of anthesis. In Greece, hellebore blooms from late autumn to early spring and it was the sacred plant of Saturn. In antiquity, there was also hellebore wine, a wine which contained hellebore, and which was used as an emetic drug but also against insanity. The myth says that Antikyra, the founder of the homonymous city of Boeotia in the Corinthian gulf, healed Hercules when he had once reached a point of insanity, with this plant that was overgrown in the area. The therapeutic use of the black hellebore against epilepsy was found later in 'On medical matter' of Dioscouridis (c. 40-90 AD). Though in antiquity this is known its rather presence in Kastrouli context may indicate a traditional knowledge and use of this to later periods.

Additionally, UV-light-induced fluorescence was used to detect lignin autofluorescence from the plant specimens and to study special anatomical features that could facilitate their taxonomic identification. Degeneration of organic compounds like lignin during tissue senescence and decomposition. Differences in the fluorescence properties were taxon-specific (Figs. 5A-C). A simple linear leaf with a nearly parallel side and penni parallel vein pattern can be seen in Figure 2B. Detailed observation of fluorescence that is associated with the arrangement of the rectangular cells (Figure 2B, inset) in the polygonal stem is well preserved and resembles the monocot type of stem seen in *Elodea* aquatic weed. A flower with tubular calyx bearing dense long hairs exhibited remarkable fluorescence (**Fig. 5C**). The flower resembles aromatic herbs of the *Lamiaceae* family like *Thymus vulgaris* (thyme) and *Kalamintha nepeta* (mountain mint), which thrive in the Mediterranean region (Alan and Ocak, 2009). Ancient Greeks used to burn thyme as incense in their temples believing it was bringing courage (Staszek et al., 2014).

3.2 DNA analysis and identification of the plant specimens

The material analyzed from the insides of a vessel buried and sealed by soil at the Kastrouli excavation site contained ancient botanical remains. Samples ranging in weight from 100 mg to 150 mg were ground in 1.5 ml micro centrifuge tubes in the presence of liquid nitrogen using acid-washed plastic pestles. Following the extraction of the genetic material, authentic genomic DNA was retrieved from the plant remains, which were more than 3000 years old. In the present study, the archaeobotanical remains were characterized by fragmented DNA, which made their analysis challenging as short ≤100 DNA fragments were recovered especially from plant tissues. On top of this challenge, these short DNA fragments must be species-specific for identification. When this aDNA was subjected to untargeted WGS analysis, unexpected findings were revealed. Specifically, from the fill of the vessel in Kastrouli a total of 10 plant species were identified with high sequence identity (86.7-96.2%) to modern reference genomes (Table 1) providing unique taxonomic information. These finds include upland wild cotton (*Gossypium hirsutum*), horseweed (*Conyza canadensis*), cowpea (*Vigna unguiculata*), rosary pea (*Abrus precatorius*), wild tomato (*Solanum pennellii*), wild tobacco (*Nicotiana tomentosiformis*), wild tobacco (*Nicotiana attenuata*), river grape (*Vitis riparia*), *Eucalyptus* (*Eucalyptus grandis*), and oak (*Quercus lobata*).

Table 1. Plant species identified in vessel biomass remains through WGS analysis.

Species name	Family name	Common name	% Identity
Gossypium hirsutum	*Malvaceae*	Upland cotton	96.2
Conyza canadensis	*Asteraceae*	Horseweed	96.2
Vigna unguiculata	*Fabaceae*	Cowpea	89.3
Abrus precatorius	*Fabaceae*	Rosary pea	96.2
Solanum pennellii	*Solanaceae*	Wild tomato	96.2
Nicotiana tomentosiformis	*Solanaceae*	Wild tobacco	92.6
Nicotiana attenuata	*Solanaceae*	Wild tobacco	91.3
Vitis riparia	*Vitaceae*	River grape	90.0
Eucalyptus grandis	*Myrtaceae*	Eucalyptus	86.7
Quercus lobata	*Fagaceae*	Oak	86.7

The identified species show a taxonomic diversity, considering the small size of the sample, as they belong to 7 important plant families and cover different plant categories: food crops (grapes, cowpea), fiber plants (cotton), medicinal plants (horseweed, rosary pea) (Garaniya and Atul, 2014), intoxicant/psychoactive plants (tobacco) and trees with wood for construction and shipbuilding (*Eucalyptus*, oak). Notably, three identified species *N. tomentosiformis*, *N. attenuata* and *S. pennellii* are among the few members of the *Solanaceae* family that have been sequenced so far but overall, the family comprises about 3000 species. This practically means that the remaining species of this family will be identified by comparing them with the family references. Considering that species such as *N. tomentosiformis* and S. *pennellii* are known to be endemic to Latin America, a distant region from the Mediterranean and the reads from the sequenced sample were mapped to those species with high identity (>91%), it is implied that these reads were mapped to the correct genera but may belong to other taxa relative to tobacco and tomato. The limited number of reference genomes especially for plant families with numerous members and the scarcity of molecular analyses on archaeobotanical remains, pose a significant challenge for the accurate identification of alien plant species found in a geographic area.

In general, ceremonial smudging or fumigation involved lighting fresh or dried plant materials, whole, or shredded such as tobacco, sage, cedar, or sweet grass and letting the plant-derived smoke spread over and around people and sacred sites for ritual or therapeutic purposes (cleansing smoke). An elaborate ritual involved the use of psychoactive plants to alter states of consciousness. For instance, *Datura stramonium* (jimsonweed), a potentially poisonous, sacred and psychoactive plant of the *Solanaceae* family was frequently used by the priestess of Delphi oracle (Gaire 2008).

The surprising discovery of two wild tobacco species among the contents of the vessel, namely, *N. tomentosiformis* (109 reads, 95.8% identity) and *N. attenuata* (91.3%), which both belong to the *Solanaceae* family and contain the psychoactive alkaloid nicotine, suggests the possible use of nicotine by the Kastrouli inhabitants. Notably, *N. tomentosiformis* with 109 mapped reads had the highest representation among the other identified species. Based on recent genetic evidence, the perennial herbaceous plant *N. tomentosiformis* is one of the parent species of the modern domesticated and commercial tobacco (*N. tabacum*) (Ren et al., 2001), which was introduced to America by European fur traders and settlers after the 1790s. However, Native North Americans used wild tobacco or sacred tobacco as an offering in religious and ceremonial practices prior to the introduction of domesticated tobacco in the Americas. The ritual practices of the American Indians likely involved the cultivation of indigenous tobacco species like *N. quadrivalvis* or *N. attenuata*, which are characterized by lower nicotine content compared to domesticated tobacco (Winter 2000). Recently, archeologists at the Wishbone site in northwestern Utah, recovered tobacco seeds from a hearth, which are approximately 12,300 years old and resemble *N. attenuata*, providing, thus, evidence for human use of tobacco much earlier than previously thought (Duke et al., 2021). Although there is great biodiversity in the Greek landscapes, and the domesticated tobacco can grow successfully today, no wild tobacco species are known. A wild type of tobacco could be cultivated by the Kastrouli inhabitants judging by the surrounded farmland and possible engineering works. But it is unknown if was commonly found in the area as a type of weed, or if the seeds and leaves were imported from other geographical areas. In support of this, it is documented that the Mycenaean Greeks were active seafarers, who seek commercial and cultural interactions with the following Bronze Age people: Canaanites, Kassites, Mitanni, Assyrians, and Egyptians (Cline 2007; Stubbings 1951, IV; Petrie, 1894). The economy of Mycenaean Greeks relied heavily on trade across the Mediterranean Sea. Based on archaeological records, Mycenaean pottery spread throughout the Mediterranean, as far as Egypt and the Levant (historical geographical term corresponding to present-day Syria, Lebanon, Jordan, Israel, Palestine, and most of Asia Minor today named Turkey). In Egypt, for example, Mycenaean vessels were found in several major centers (Kelder 2009).

From the molecular analyses, wild tomato (*Solanum pennellii*), another plant taxon of the *Solanaceae* family, was also identified with high identity (96.2%). The wild species of *S. pennellii* (formerly *Lycopersicon pennelli*) thrives in Peru (South America) and can grow in coasts, valleys, islands from the sea level up to 3000 meters altitude and in arid habitats (Warnock 1991). Furthermore, the sequencing of its genome has been completed (Bolger et al., 2014). In general, *S. pennellii* has a high level of tolerance to salinity, drought and possesses phenotypes divergent from modern tomato (*Solanum lycopersicon*) in terms of fruit size, fruit development, maturation and metabolism. Interestingly, the locals in the wider Kastrouli region in particular the Kopais basin in Boeotia province, recall anecdotally that at the beginning of the 20th century a wild tomato with tiny red fruits was growing among cotton plants. The wild tomato plant with the round hanging fruits was named 'scoularikates' (earing-like) and could be a relative species of the same *Solanum* genus to the archaeobotanical specimen. Notably, the ripe fruits of this wild species were red and edible although they did not have much placenta (a mucilaginous substance). However, tomatoes arrived in Greece after the 15th c especially beginning of 19th c. This wild tomato species seems to grow in that area much earlier. It is not certain at the moment if this tomato was growing in Desfina plain those times or it is a transferred from Kopais with trade

Another agronomically important plant species identified in Kastrouli archaeobotanical remains was wild upland cotton (*G. hirsutum*). The identification of the early spread of cotton cultivation in ancient Greece is uncertain and only based on ancient written sources since very few archaeological textile artifacts were found because of cotton's fragile nature (Margariti, 2010). Zissis (1955) reports that in Samos and other Greek islands of the Aegean Sea there was a native cotton species with very fine fibers. In the *Malvaceae* family, early radiation in the evolution of the genus *Gossypium* resulted in the creation of 50 species. Different cotton genome groups are associated with different geographical regions. Long-distance dispersal of the African-Asian D-genome (~10 million years old) into the Americas resulted in the inter-genomic hybridization between the New World A-genome and the introduced D-genome for the creation of the AD-allopolyploids such a *G. hirsutum* and *G. barbadense* (Wendel et al., 2010). The present study identifies the Mycenaean cotton as a close taxon to *G. hirsutum* (96.2% identity). This cotton would have been originated either from a wild relative species established in the natural vegetation of Greece or a non-native species that was introduced, for example, through either Upper Egypt/Africa or Arabia routes. In support of this, artifacts from the Building 1 of Kastrouli settlement include plethora of different sizes of spindle whorls, providing evidence for may be an ancient spinning tool used by women to create cords, yarn or thread from raw plant material or wool. The wooden shaft usually decays and is rarely found in archaeological artifacts. Moreover, a unique clay fragment incised with what is possibly a Linear B logogram for cloth, was found in Building 2 (Koh et al., Fig. 32a). In antiquity, cotton was used mostly to make threads and textiles. For smudging practices, plant parts are wrapped with a natural yarn, which is often a cotton thread, to create a bundle that burns slowly and steadily. Greece's environmental conditions are ideal for cotton production and the country today is the main producer of cotton in Europe. In Kopais plain, not far from Kastrouli (~40 km), the cotton cultivation is enormous at least for the past about 150 years. Initially during Mycenaean times Kopais was a lake. The attempt to drain the Kopais Lake was one of the most impressive and ambitious technical works of prehistoric times in Greece, inspiring myths and traditions referring to its construction and operation (Kountouri et al., 2013). The concern of drying up was that of malaria which afflicted the settlements round the lake and that of creating farmland to provide adequate food. A similar engineered hydraulics seem to have taken place at Kastrouli too (Liritzis et al., work in preparation). Early biogeographic documentation of cotton use by humans, comes from a Neolithic burial at Mehrgarh in central Balochistan, Pakistan, which dates to the first half of the 6th millenium BCE (Moulherat et al., 2002). Later evidence for cotton use comes from Dhuweila in Eastern Jordan during the Chalcolithic or Early Bronze Age (4450-3000 BCE) (Betts et al., 1994) while African evidence of cotton comes from Nubia in the 3rd millennium BCE (Moulherat et al., 2002).

The Mediterranean region experienced great climatic changes since the Middle Miocene epoch, with the sharp drop in global temperatures, which affected the evolution of Mediterranean flora. Based on old descriptions, the ancient Greeks knew about 1000 species from the 6,000 species of plants and developed a certain familiarity with botanical remedies to treat human and animal ailments and wounds. *Conyza canadensis* (or *Erigeron canadensis*) was another taxon identified (96.2 % identity) in plant remains. *Conyza* is a weed species of the Asteraceae family with the common name horseweed. The plant has broad geographic distribution and is common in temperate and Mediterranean zones. The *Conyza* plant is mentioned in a messianic prophecy of Isaiah (8th century BC) in the Old Testament. It is predominantly an annual herbaceous weed of small to medium size (height up to 1.50 m.) with erect branches in a dense arrangement. The narrow lanceolate leaves are smooth on the upper surface and fluffy in the lower. The leaves and shoots have a sticky substance and a carrot-like characteristic aroma. The plant blooms during the summer months and in early autumn. The medicinal properties of the plant have been known to humans since ancient times and many ancient writers refer to it. Theophrastus in his book Enquiry into Plants or Historia Plantarum, describes the plant phenotype, reproduction and growth. In addition, Dioscouridis, founder of pharmacology, reports *Conyza*'s medicinal properties and uses in his book '*On medical matter*'. He also mentions that burning branches with the leaves of the plant creates smoke that repels mosquitoes, snakes, and kills the fleas. The *Conyza* described in ancient Greece, is not clear in which taxon was exactly referred to. Probably, they are not the ones introduced the last centuries. Thus, this plant may have been intentionally added to the vessel along with other plants for keeping insects away from the ceremony site, but also to provide gratitude to a deity for the availability of this medicinal plant.

In the current study, cowpea (*Vigna unguiculata*) was identified (89.3% identity) as one taxon from the contents of the vessel in a ritual context. Perhaps, the plant has played an important role in the diet of the Kastrouli inhabitants, and its availability called for gratitude to be ritually expressed to the responsible deity. The genome of cowpea was sequenced recently (Lonardi et al., 2019). Cowpea or black-eyed pea is an annual herbaceous plant of the *Fabaceae* family. The plant is native to Africa and Southeast Asia and produces long pods with medium size beans of cream color with a black-purple spot on the seed. In ancient Greece, Theophrastus in his book 'Enquiry into Plants' mentioned this bean as 'dolichos', which means long, probably for its characteristic long pods. During Antiquity, the legume was well known in Greece and the Mediterranean basin. Even today, this type of beans is popular in Greece with the name 'mavromatika', which means black-eyed. The green (fresh) bean is produced in the warm seasons of the year (late Spring - Autumn). The edible bean can be consumed by humans and livestock (as cattle feed, likely responsible for its name). In view of our findings, it appears that cowpeas or similar taxons were known to Kastrouli's farmers. Of note, the dry bean was a convenient and nutritious food for ancient Greek warriors as it was easy to carry with them.

The rosary pea (*Abrus precatorius*) (96.2 % identity) was also identified among the plant remains. This is a small woody climbing vine of the *Fabaceae* family with ornamental red seeds with a black spot, which are highly toxic as they contain the toxin abrin. The plant has broad geographic distribution and is commonly found in Southeast Asia South Africa, China, Islands, West Indies, India, Brazil and other regions. Leaves, roots and seeds are known for their medicinal properties and veterinary uses (bone fractures) (Garaniya & Bapodra 2014). The generic name is derived from the Greek word 'habro', which means delicate in reference to the pinnate leaflets (Lewis et al. 2005). The specific epithet, precatorius, is a Latin word meaning petitioning or praying because of its use in rosaries (Masupa 2009). The plant was also traditionally used to treat tetanus, and to prevent rabies (virus caught from the bite of an infected animal).

The wild river grape (*Vitis riparia*), a perennial vine with small berry size of the *Vitaceae* family can be found in Greece. The god Dionysus was linked to the grape vine, a plant that in its wild form depends on climbing an adjacent tree. The draft genome of *Vitis riparia* was sequenced recently (Patel et al., 2020). To reconstruct the evolution of *Vitis* across different geographical regions and continents a phylogenetic analysis was performed in 48 from the 60 species of the plant family (Wan et al., 2013). Based on these results, *Vitis riparia* evolved before the divergence of Eurasia from North America, which is estimated 11.12 million years ago. Wild grape vines are found throughout the Mediterranean coast, except for the Sahara. Archaeological excavations have uncovered seeds of cultivated grapes in Egypt and Central Asia since 4000 BC. Grape remains of the Bronze Age have been found in Mycenae and other Greek cities. The most remarkable finding however was the discovery of thousands of seeds in the archaeological site of Philippi in northern Greece that were coming from a prehistoric settlement of the Middle Neolithic to Early Bronze Age (6th to 3rd millennium BC) that were probably pressed for wine production (see also Koh and Betancourt 2010).

In ancient Greece, trees were important for the indication of an oracle, something that has its roots in the Mycenaean tradition. Most oracles had their characteristic tree. In a ritual setting, burning woods from trees apart from the symbolic attribution to a certain deity had a cleansing and purifying effect for the audience. In Delphi oracle, the laurel was the sacred tree of the god Apollo and Pythia chewed its leaves on the tripod to utter the sacred oracle, which was interpreted by the Apollonian priests. The special connection of the people with the trees is one of the characteristic elements of Greek mythology. The Nymphs, which were female figures who usually accompanied the dance of gods, such as Pan, Hermes, Apollo and

Artemis, lived in the forests. The tree was a symbol of the human path, from the material (roots) to the spiritual world (canopy). Branches and leaves from the trees were used in initiation ceremonies and symbolized the initiation rebirth of the mystic.

Eucalyptus (*Eucalyptus grandis*) (86.7% identity), a fast-growing large tree that belongs to the *Myrtaceae* family and grows in temperate regions was one of the identified taxa in the plant remains. The name of the genus *Eucalyptus* comes from the ancient Greek prefix 'eu' meaning 'good or well' and 'kalupto' meaning 'cover', which refers to the calyptra (cap-like structure) that covers the floral buds before anthesis. *Eucalyptus* is one of the largest genera with about 660 species. The fossils of *Eucalyptus* from the Deccan Intertrappean beds of central India, which dates in Late Maastrichtian–Danian age (Shukla et al., 2012) and from Patagonia, Argentina (Hermsen et al., 2012) suggest a broader geographic distribution for this group, which was previously thought to be native to Australia. The genome sequence of the subtropical *E. grandis* and a representative of the temperate *E. globulus* are available (Myburg et al., 2014). These large trees can be planted in problematic areas like marshes as they can absorb huge amounts of water and repel mosquitoes as well (anti-malarian properties). *Eucalyptus* wood is hard and solid and can be used in shipbuilding as well as in heavy and light constructions. A *Eucalyptus* taxon could have been imported to Kastrouli to serve both as timber wood for shipbuilding and/or to dry up the neighboring swamp.

The oak tree (*Quercus lobata*) (86.7% identity) was the second large tree species identified in the plant remains. The genus *Quercus* of the *Fagaceae* family contains about 500 species. *Quercus* was recently sequenced (Plomion et al., 2018). The tree usually is found in the Eastern Mediterranean lowland areas, as well as at the foot of the mountains. In Greece, many oak species (*Q. ithaburensis*, *Q. robur, Q. trojana, Q. petraea, Q. pubescens, Q. aucheri*) thrive today in mountainous and lowland areas. The oak was the sacred tree of the most powerful god of Olympus, Zeus. The oak, with its majestic and imposing development (it reaches 30 meters in height), was a symbol of power and supremacy. In the oracle of ancient Dodoni the priests interpreted the rustling of the leaves of a large oak as a message from the god. The first botanical report for *Quercus* as 'ημερίς' comes from Theophrastus in his book 'Enquiry into Plants' or 'Historia Plantarum'. The leaves of the oak wreath, of King Philip II of Macedonia, found in his tomb in Vergina and dates back to the 4th century BC, were lobed. Ancient Greeks had a fascination with lobed leaves like those of oak and frequently depicted them in ancient coins (Riddle 1995; Baumann 1993).

The wood of oak is aromatic, hard, and dense, does not rot easily and can handle the ravages of saltwater. In general, the naturally curved oak branches are ideal for construction and shipbuilding and it could be concluded that this wood may have played an important role in the everyday life of Kastrouli inhabitants in their agricultural and shipbuilding activities. Kastrouli may have played a significant role in maritime activities through its port at Steno (see, Fig.3 above and Liritzis 2021; Koh et al., 2020; Sideris et al., 2017; Levy et al., 2018).

3.3 Metagenomic analysis reveals the microbiome associated with the plant biomass

Although the metagenomic analysis revealed microbial richness associated with the plant remains (Fig. 6), we shifted through the microbial data and focused only on species from non-human hosts (Table 2). The presence of other microbial species could be attributed to contamination of the biological specimens during excavation or subsequent handling. High-throughput sequencing identified several microbes associated with marine environments were also detected (Table 2), providing additional information about the lifestyle and food consumed by the Kastrouli inhabitants. For instance, a known shrimp pathogen such as *Vibrio nigripulchritudo* (Le Roux et al., 2011), a common freshwater fish pathogen *Flavobacterium columnare* (Tekedar et al., 2012), and the causal agent of nocardiosis in multiple fish species *Nocardia seriolae* (Shimahara et al., 2008) were identified. A known alpha proteobacteria species was also found, the versatile *Rhodopseudomonas palustris* that can switch between different food sources, while it is often isolated from marine coastal sediments, ponds, and even earth-worm droppings (Larimer et al., 2004). Several other microbes associated with seafloor sediment and sponges have been detected in this study, including *Pelagibaca abyssi* previously found in deep seawater (Lin et al., 2014), *Micromonospora craniellae* and *ASO4wet (Streptomyces bathyalis)* in marine and deep sea sponges (Li et al., 2019; Risdian et al., 2021), *Emcibacter congregatus* in marine sediment (Zhao et al., 2018), and *Petrocella atlantisensis* in subseafloor rocks (Quéméneur et al., 2019).

Table 2. Microbial species associated with plant biomass discovered through WGS.

Marine-related	Plant-related	Plant rhizosphere-related	Soil-related	Extreme environment-related
Vibrio nigripulchritudo	*Talaromyces rugulosus*		*Terricaulis silvestris*	
Rhodopseudomonas palustris		*Dickeya solani*	*Echinicola* sp.	*Psychromicrobium lacuslunae*
Flavobacterium columnare	*Acetobacter pasteurianus*	*Azospirillum oryzae*	*Bdellovibrio* sp. *Bacillus pumilus* *Runella* sp.	
Pelagibaca abyssi	*Arthrobacter dokdonellae*	*Ensifer mexicanus* *Pseudomonas viciae* *Pseudomonas aeruginosa* *Kibdelosporangium phytohabitans* *Phytohabitans flavus* *Mesorhizobium* sp. *Stenotrophomonas maltophilia* *Mucilaginibacter ginsenosidivorans* *Rhizobium leguminosarum* *Hartmannibacter diazotrophicus* *Caulobacter rhizosphaerae*		
Micromonospora craniellae *Streptomyces* sp. *ASO4wet* *Emcibacter congregatus* *Nocardia seriolae* *Petrocella atlantisensis*				

Plants and their products are frequently associated with microbiota in nature. In this study, microbial species associated with biodegradation of plant tissues such as *Talaromyces rugulosus, Acetobacter pasteurianus* and *Arthrobacter dokdonellae* were identified. Specifically, *T. rugulosus* an infectious fungus was previously isolated from decayed fruits like grapes (Xu et al., 2021) and pears (Stošić et al., 2021). The fungus was also isolated from the Mediterranean sponge *Axinella cannabina* (Küppers et al., 2017), providing evidence for the adaptability of the fungus to survive in diverse environment. An acetic acid-producing bacterium, *A. pasteurianus,* frequently associated with plants and fruits rich in sugar, was isolated from discarded/spoiled apples (Vashisht et al., 2019) while *A. dokdonellae* bacterium was isolated from a plant of the genus *Campanula* (Koh et al., 2019). These findings agree with the identification of decomposed plant tissues and grape tissues among the plant remains.

Rhizosphere, the narrow zone of soil that surrounds plant roots contains many microorganisms including bacteria, fungi, viruses, protozoa, algae and nematodes. In the rhizosphere, interactions between plant roots and soil organisms take place. Authentic aDNA from microbes associated with the plant remains and soil components in the vessel was retrieved. The WGS approach yielded reads that aligned to reference genomes and positively identified species that were distributed in 13 rhizosphere-related genera: *Dickeya*, *Azospirillum*, *Ensifer*, *Pseudomonas*, *Kibdelosporangium*, *Phytohabitans*, *Mesorhizobium*, *Stenotrophomonas, Mucilaginibacter, Rhizobium, Hartmannibacter, Arthrobacter* and *Caulobacter* (Table 2). Bacteria from the genus *Dickeya* belong to the family Pectobacteriaceae and inhabit several ecological niches such as soil, water and association with plants. *D. solani* has been identified as plant pathogen that secretes pectinolytic enzymes that cause the degradation of plant tissues. Diseases such as soft rot in many angiosperms (Ma et al., 2007) and in potato (Golanowska et al., 2016) have been attributed to this species.

Among the plant species identified in our molecular analysis were two legume species: *V. unguiculata* and *A. precatorius*. Legumes form symbiotic relationships with soil bacteria called rhizobia. This beneficial symbiosis is very important for the processes of atmospheric nitrogen fixation and the nitrogen cycle on earth. Biological nitrogen fixation is important for plant growth and production. Three of the identified species in our analysis, namely *Ensifer mexicanus, Mesorhizobium sp.*, *Rhizobium leguminosarum* are nitrogen-fixing bacteria. Previous studies have identified *E. mexicanus* with many legumes (Gehlot et al., 2012; 2013; Lloret et al., 2007), *R. leguminosarum* with common beans (Mulas et al., 2011) and other leguminous and nonleguminous plants (Schloter et al., 1997). The *Mesorhizobium* genus associations to different legumes were reviewed (Laranjo et al., 2014). Another nitrogen-fixation species, *Azospirillum oryzae* of the Azospirillaceae family was associated with the roots of rice, a nonleguminous plant (*Oryza sativa*) (Xie 2005).

The species *P. viciae* was isolated from the rhizosphere soil of broad bean (Zhao et al., 2020). Moreover, *P. aeruginosa* was isolated from the roots of the reed *Phragmites australis (*Wu et al., 2018) while *P. flavus* has been previously isolated from orchid roots (Inahashi et al., 2012). *S. maltophilia* was isolated from rhizosphere of oilseed rape (Berg et al., 1996). The species *M. ginsenosidivorans* was previously isolated from soil where ginseng was growing (Kim MM et al., 2017). The species *H. diazotrophicus* was isolated from tissues of coffee plant and rhizosphere soils (Jimenez-Salgado, T et. Al., 1997) as well as, from *Plantago winteri* rhizosphere growing in a salt meadow (Suarez et al., 2014). Species of the genus *Arthrobacter sp.* were isolated from tomato rhizosphere (Banerjee et al., 2010). *C. rhizosphaerae* was isolated from rhizosphere of cultivated watermelon (*Citrullus lanatus*) (Sun et al., 2017). Actinomycetes are microbes mostly found in soil and plant remains where they decompose complex mixtures in dead plants (Bhatti et al., 2017). The actinomycete *K. phytohabitans* was isolated from the root of the oilseed plant *Jatropha curcas* L. (Xing et al., 2012).

In addition, our metagenomic analysis revealed bacterial species with habitats in soil, wastewaters, and sludges (Table 2). These species were: *Terricaulis silvestris* previously found in forest soil (Vieira et al., 2020), species of the genus *Echinicola* sp., which are involved in decomposition of polysaccharides and they can be found in alkaline hypersaline environments (Xing et al., 2020; Zhao et al., 2021), species of the genus *Bdellovibrio* sp. that can be found in soil, mud and sewage (Wehr & Klein, 1971), *Bacillus pumilus* found in soil (Panbangred et al., 1983) and coastal environments (Parvathi et al., 2009) and finally species of the genus *Runella* sp. that can be found in wastewater, sludge and environmental waters (Furuhata et al., 2008; Copeland et al., 2012).

An extreme environment species, *Psychromicrobium lacuslunae,* was identified in our metagenomic analysis. This species was previously found in a high-altitude lake (Kiran et al., 2018). Overall, the microbial data provide support for the plant diversity (e.g., legume-related microbial associations) identified in the plant biomass, the local topography (heterogeneity) of the settlement and earlier excavation finds from Building 1 related to edible seashells of the following species: *Cerithium vulgatum*, *Crepidula fornicata*, *Mytillus* sp., *Monodonta turbinata*, *Ostrea edulis*, *Tarantinea lignarius*, *Spondylus gederopus* and *Pina nobilis* (Sideris & Liritzis, 2018).

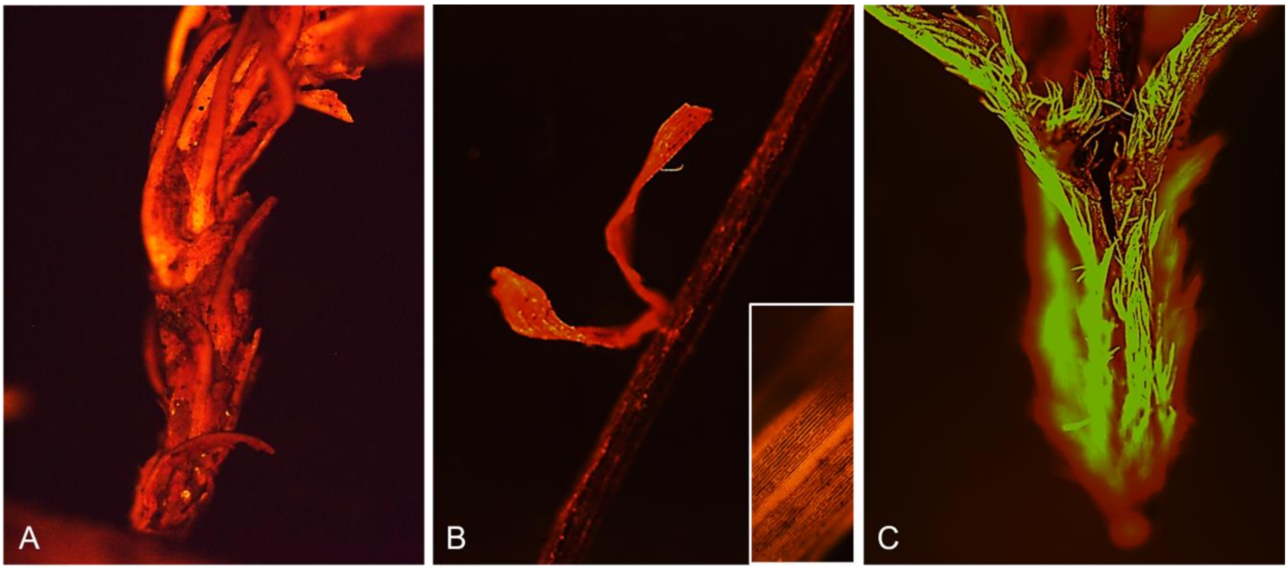

Figure 5. Epifluorescence micrographs of the plant biomass showing morphological features (arrangement of leaves, polygonal stem and inset showing stem cell arrangement, tubular calyx with dense long hairs even in the inside of the calyx). 5X magnification.

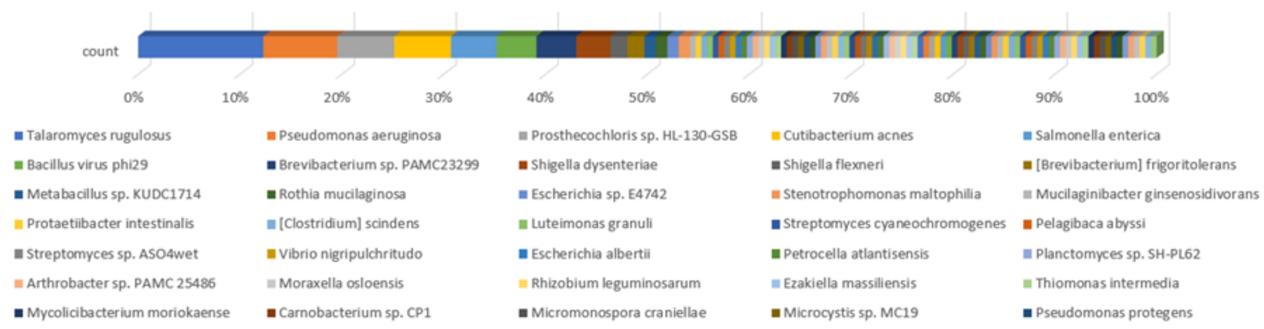

Figure 6. Taxonomic diversity of microbial species associated with the plant remains collected from the vessel. The Relative abundance (% of total abundance) of reads in the sample are presented as a bar graph.

4. CONCLUSION

Significant information has been gained on the identity of plants used in sacrificial ceremonies by the inhabitants of the Mycenaean settlement by employing microscopic and genetic analyses. The confinement of plant remains in the vessel at the excavated site conserved morphological features and facilitated plant documentation on the level of growing habitat and plant family. The application of WGS analysis on the vessel contents overcame the usual difficulties with the fragmentation of aDNA and successfully identified agronomically important species but also forest, psychotropic and other potentially poisonous species. In addition, WGS revealed the identity of the microbial species associated with the vessel plant biomass, providing thus a perspective on microbial origin from other ecological settings for some or all the plants and their possible transportation from nearby areas.

From the information now available, a first reconstruction of the past plant use and the Kastrouli environment as a near lake or marsh and shore environment, has been initiated. The presence of aquatic and amphibious plants in the vessel may come from water transported from a marsh or lake. The discovery of important agronomic species such as the river grape and upland cotton suggest the development of agriculture. The river grape may be grown locally but the cotton may have been imported or introduced as a novel cultivation from other Mediterranean regions. In addition, the DNA analysis identified the oak and eucalyptus trees, two important timber woods that are resistant to rot and decay and can be used for construction, agricultural beams, and shipbuilding.

AUTHOR CONTRIBUTIONS

Conceptualization, I.L., C.K; methodology, Z.H., C.K; software, C.K.; validation, Z.H, D.V, A.A, V.R.; formal analysis, Z.H, D.V, A.A, V.R.; investigation, I.L, Z.H, C.K.; resources, C.K.; data curation, Z.H, I.L; writing—original draft preparation, I.L, Z.H.; writing—review and editing, I.L, Z.H. C.K,; visualization, I.L,; Z.H.; supervision, I.L.; project administration, I.L, C.K.. All authors have read and agreed to the published version of the manuscript.

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