RESEARCH ARTICLE

Plants People Planet PPP

Tree Forensics: Modern DNA barcoding and traditional anatomy identify roots threatening an ancient necropolis

Gilad Jakoby[1] | Ido Rog[1] | Ilana Shtein[2] | Inbar Chashmonay[3] | Dror Ben-Yosef[3] | Amram Eshel[4] | Tamir Klein[1]

[1]Department of Plant & Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel

[2]Eastern R&D Center, Ariel, Israel

[3]North District, Israel Nature & Parks Authority, North District, Israel

[4]School of Plant Sciences, Tel-Aviv University, Tel-Aviv, Israel

Correspondence
Tamir Klein, Department of Plant, & Environmental Sciences, Weizmann Institute of Science, 234 Herzl St., Rehovot, Israel.
Email: tamir.klein@weizmann.ac.il

Societal Impact Statement

Ancient burial caves represent some of the most important sources of information on human history. In a world heritage site in Israel, such caves are under threat due to tree root growth penetrating from the ceilings and causing a risk of cave collapse. To facilitate historical conservation, while avoiding cutting of the mixed forest above the caves, we identified the tree species responsible for the damage, facilitating their specific cutting. Accurate identification of tree roots balances the needs to protect human structures on one side, while conserving the majority of vegetation on the other. Our approach is applicable to the management of ancient sites, as well as to urban management more broadly.

Summary

Tree roots have penetrated the ceiling of burial caves in a ~1,800-years-old necropolis in the Galilee, Israel, damaging the antiquities and risking the catacombs with collapse. Root identification was needed to enable selective cutting (at the species level), facilitating the conservation of the world heritage site, while maintaining the majority of trees growing on top of the burial caves. Samples of roots penetrating the cave ceilings were collected and identified both by using DNA barcoding, which has become a standard method for the reliable identification of organisms in their natural environment, and also by traditional morpho-anatomical methods. However, woody plant species of the Mediterranean region are under-represented in DNA databases. Therefore, we added relevant species to the ITS2 database by sequencing the internal transcribed spacer (ITS)2 of the 18S-26S rDNA from sampled leaves of 19 woody species of the Mediterranean maquis. The identification of these tree species facilitated their selective removal, balancing antiquities, and nature conservation needs.

KEYWORDS

antiquities, Beit She'arim, conservation, ITS2, tree roots

1 | INTRODUCTION

This investigation was conducted as part of conservation activities in the necropolis of Beit She'arim UNESCO World Heritage site in Galilee, Israel. According to Josephus Flavius, Beit She'arim ("Besara") was founded at the end of the 1st century BCE, as the administrative center of the estates of Queen Berenice in the Jezreel Valley (Avigad, 1971). By the 2nd century AD, the Sanhedrin

(supreme Jewish council) moved to Beit She'arim and the town was mentioned in rabbinical literature as an important center of Jewish learning. Rabbi Judah the Prince (Yehudah HaNasi), head of the Sanhedrin and compiler of the Mishna, lived and was buried there. This led many Jews from all over the country and from the Jewish diaspora, from nearby Phoenicia to far-away Yemen, to come there in order to be buried in the vicinity of his grave. As a result, there are dozens of burial caves in this area (Avigad, 1971; Mazar, 1957; Rosenfeld, 1995; Weiss, 1992). The Jewish town was destroyed in 351 AD and the earthquake of 363 AD affected the area as well. The place was later occupied by a Byzantine village until the 7th century AD (Mazar, 1957; Meyers, 1999).

The soft chalk rock, into which the tombs were hewn, undergoes accelerated erosion by roots and water. The roots penetrate into the caves' cavities at a depth of about 20 meters. In recent years, roots have protruded into the burial caves through the soft limestone ceiling. As root penetration into the necropolis intensified, maintenance of the ceiling structure was compromised, and the risk of collapse emerged. The need arose to identify the plants responsible for this damage to the caves in order to find ways to prevent worsening of the situation.

Identifying organisms in the field has been the cornerstone of ecological research ever since its inception. For over 200 years, scientists have been collecting biological samples systematically in the field, assigning them to known taxa. In recent years, careful morphological and anatomical practices traditionally used for identification are being complemented by established genetic methods, taking advantage of the DNA sequence as a fundamental identifier. Sufficient variation between taxa in specific DNA regions laid the foundation to the barcoding approach. Since 2003, DNA barcoding has become a standard method for the reliable, relatively simple, identification of organisms (Hajibabaei et al., 2007; Moritz & Cicero, 2004). In fact, standard procedures for DNA barcoding have become common tools in genomics, with implications to molecular phylogenetics and population genetics (Hajibabaei et al., 2007).

In addition to the well-established use of DNA barcodes for the identification of microbiota such as bacteria and fungi, a need for the barcoding of land plants has also emerged (C. P. W. Group, 2009; Kress et al., 2005). The applicability of several plastid DNA regions has been tested (C. P. W. Group, 2009; Lahaye et al., 2008), as well as the whole-chloroplast genome sequence (Li et al., 2015), and the use of the nuclear internal transcribed spacer region (ITS) of the 18S-26S rDNA has proved most effective (Kress et al., 2005; Li et al., 2015). The ITS was the most commonly sequenced locus used for plant phylogenetics, showing high levels of interspecific divergence (Kress et al., 2005). In a comparative study of seven candidate DNA barcodes, ITS2 showed the highest suitability for DNA barcoding applications, with 93% success rate in species identification among 4,800 plant species (Chen et al., 2010). Plant DNA barcoding is of particular interest when studying plant parts which are hard to identify, such as roots and ancient pollen. DNA barcoding has been used in archaeobotany to reconstruct past plant use, agriculture, diet, and

vegetation (Schlumbaum et al., 2008). In this respect, the ITS markers are advantageous, since the high copy number of rRNA increases the probability of detection in small amounts and ancient DNA samples. Seeds found in Neolithic site in Italy were identified as *Olea europea* and *Cornus mas*, shedding light on the diet of its inhabitants (Gismondi et al., 2012). The utilization of DNA barcoding in the field of plant ecology is advancing fast. For example, over 1,000 Mesoamerican orchid species have been identified in a single study (Lahaye et al., 2008). Other biodiversity inventories have been compiled in additional projects (Valentini et al., 2009). DNA barcoding is also essential where only the remains of organisms are found, much like in modern-day forensics (Valentini et al., 2009). As in many other research fields in ecology and biology, plant DNA barcoding techniques were applied in Europe, North America, and Japan, i.e., to plant species of the temperate region. At present, woody plant species of the Mediterranean region are under-represented in DNA databases.

In spite of the recent advances mentioned above, the most obvious, classical way to identify plants is by using structural characteristics. Numerous atlases and books exist (Carlquist, 1988; Schweingruber et al., 2011; Crivellaro & Schweingruber, 2013; Fahn et al., 1986), some of which are specific for Mediterranean woody species (Crivellaro & Schweingruber, 2013; Fahn et al., 1986) that can be used in order to identify woody plant species. However, while studies on Mediterranean wood anatomy are prevalent (Crivellaro & Schweingruber, 2013; Fahn et al., 1986; Mrak & Gricar, 2016), very few deal with root structure (Rewald et al., 2012), and none deal with roots of Mediterranean species. That is not surprising, as root identification presents quite a few challenges (Maeght et al., 2013; Rutherford, 1980). While woody roots possess some distinct structural characters, the very thin roots are difficult to identify, although such identification is still possible (Rewald et al., 2012). Also, root morphology and anatomy vary with soil type and growth conditions. Moreover, root anatomy often differs from stem anatomy, and separate identification keys must be obtained. Nonetheless, root morphology and anatomy can be successfully used for identification (Uma & Muthukumar, 2014; Battipaglia et al., 2011). Interestingly, some identifying characters used include root odor, crystals, and mycorrhiza.

In this study, we combined ITS2 DNA barcoding with traditional morpho-anatomical identification methodologies to facilitate two distinct, yet intimately linked, purposes: (a) To identify roots protruding into ancient burial caves in order to pinpoint the species involved and settle conflicting needs for vegetation conservation and antiquities preservation, and (b) to add Mediterranean woody plant species to the existing barcoding database, thereby facilitating future research in the region.

2 | MATERIALS AND METHODS

2.1 | The research problem

In recent years, staff of the Israeli Nature Parks Authority which manages the Beit Shea'rim Park that includes the ancient burial

caves noticed an ongoing process of plat root penetration through cracks in the cave ceilings (Figure 1). In some places, parts of the caves' ceilings and walls were on the verge of collapse. To ensure the conservation of the heritage site antiquities, an experts meeting was conducted at the site, on 19 December 2017. An immediate need to remove the trees growing in the mixed forest on top of the necropolis was agreed upon. One of the suggested solutions was to cut down the trees and bushes, wait until the root dried and then fill the gaps with cement preserves. Yet, this need conflicted with Israel's nature conservation legislation, prohibiting the removal of mature trees anywhere without a special permit. The main challenge we were faced with was identifying which of all the plants are responsible for damaging the ancient caves. It was, therefore, decided that a selective tree removal (at the species level) will be performed, once the tree species, to which the roots belong to, are identified. A 'tree forensics' project was initiated, using both traditional morpho-anatomical and modern DNA barcoding techniques.

2.2 | Site description

The Beit She'arim UNESCO World Heritage site is located in the Galilee region of Israel, 20 km West of Nazareth (32° 42′ 12.8124" N 35° 7′ 44.5656" E, elevation 120 masl). Climate at the site is thermo-Mediterranean, with an annual precipitation of ~530 mm, and average minimum, mean, and maximum temperatures of 16°C, 21°C, and 25°C, respectively. The soil is shallow rendzina, overlying a chalk-limestone bedrock. The town's vast necropolis, carved out of soft limestone, includes more than 30 burial cave systems. Only a portion of the necropolis has been excavated. Its catacombs, mausoleums, and sarcophagi are adorned with elaborate symbols and figures as well as an impressive quantity of incised and painted inscriptions in Hebrew, Aramaic, Palmyrene, and Greek, documenting two centuries

of historical and cultural achievement. The wealth of artistic adornments contained in this is the most ancient extensive Jewish cemetery in the world, and unparalleled anywhere. Excavations at the necropolis by B. Mazar and N. Avigad started in 1936 and took place until 1959 (Avigad, 1971; Mazar, 1957). Aaronshon, a noted botanist, visited the area in 1904 and described it as a rich grazing ground covered by grasses with a handful of oaks. Many shepherds were bringing their sheep to this lush grazing ground from the whole region of Jezreel Valley (Aaronsohn, 1940; Patishi, 1983). At the time of the archaeological excavations, root penetration to the ancient caves was not mentioned at all. In pictures taken at the time, the area was clearly devoid of large trees (Avigad, 1971; Mazar, 1957). At the end of Avigad's book, he acknowledged the Gardening Department personnel and its director Mr. Yanai for planting decorative trees and shrubs in the area.

2.3 | Site recent history

The woody vegetation that currently surrounds the burial caves includes common Mediterranean maquis species such as *Pinus halepensis*, *Quercus calliprinos*, *Pistacia palaestina*, *Rhamnus lycioides*, *Cercis siliquastrum*, *Cupressus sempervirens*, and *Rosmarinus officinalis*. The archaeological preservation at Beit She'arim is a serious professional challenge. A question arose regarding the origin of the site vegetation, in view of the fact that the trees at the site seemed mature but included a mixture of local and introduces species. Excavations at the necropolis by B. Mazar and N. Avigad started in 1936 and took place until 1959 (Avigad, 1971; Mazar, 1957). Aaronsohn, a noted botanist, visited the area in 1904 and described it as a rich grazing ground covered by grasses with a handful of oaks. Many shepherds were bringing their sheep to this lush grazing ground from the whole region of Jezreel Valley (Aaronsohn, 1940; Patishi, (1983). At

FIGURE 1 Penetration of plant roots through the cave limestone ceilings of the Beit She'arim necropolis, Galilee, Israel. Photos taken by the authors in January 2018. (a) Entrance to one of the burial caves, showing the Mediterranean maquis ~5 m above the cave and on the hillslope, with *Cupressus*, *Quercus*, *Pistacia*, and other trees; (b) the view inside the cave with sarcophagi, roots can be seen penetrating from the ceiling; (c) roots of *Pistacia palaestina* growing through the limestone on top of the entrance of another cave; (d) view of a cave ceiling affected by roots; and (e) close-up of a cave ceiling, showing several root penetration points

the time of the archaeological excavations, root penetration to the ancient caves was not mentioned at all (Avigad, 1971; Mazar, 1957). In an aerial photograph (Figure 2) taken in 1944 of the site, it can be clearly seen that the area was devoid of large trees. At the end of Avigad's book (Avigad, 1971), he acknowledged the Gardening Department personnel and its director Mr. Yanai for planting decorative trees and shrubs in the area. The trees are now an integral part of the park landscape and highly valued by the staff and visitors.

2.4 | Sample preparation and morpho-anatomical root identification

Roots (5–7 cm long; 5–15 mm in diameter) were sampled and immediately placed in 50-ml plastic tubes and brought to the laboratory. The fresh samples were photographed for morphological identification and transferred to 70% alcohol for storage till sectioning. Hand cross-sections were made with razor blade. The sections were stained either with TBO (O'Brien et al., 1964) or with Safranin-Alcian blue (Shtein et al., 2020). The sections were analyzed under a stereo microscope (Olympus SZ2-ILST, Japan) and photographed using the microscope camera (Olympus LC20, Japan).

The preparation of species identification key was the most time-consuming part of the morpho-anatomical identification. At first we made the mistake of sampling the roots in the field, in proximity to the tree, and subsequently we realized that all the oak roots we sampled were in fact *Pistacia* roots. Thus, caution must be made when using field-collected samples for root identification keys. Our current key is at the genus level, but in the future a more extensive key could be made. Once the identification key has been prepared, the identification was straightforward. As we used free hand sections and a simple
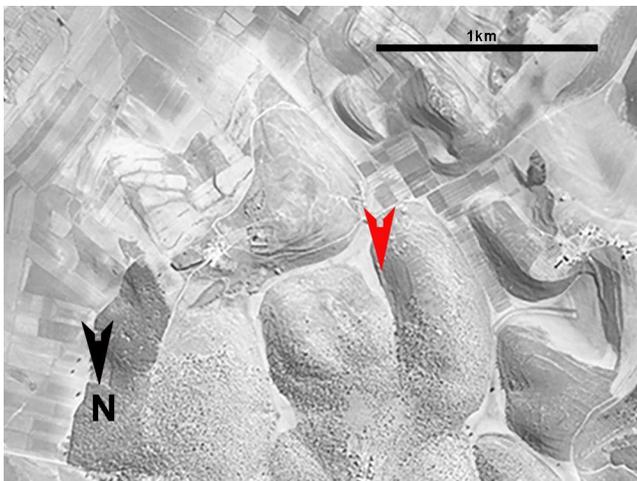

FIGURE 2 An aerial photo of the region taken on December 16th 1944. The hill at the immediate vicinity of the archaeological site (red arrow) is bare. Sparse small trees can be seen several hundred meters north (note the black arrow) of the site. Reproduced by permission from Survey of Israel, The Israel Mapping Center

double-staining procedure, the identification took about 15 min per sample. However, caution must be used, as root structure might sometimes vary with habitat and the key should be adjusted accordingly (Maeght et al., 2013). Additionally, as a broad range of characters is used for identification, a good knowledge of plant anatomy is required, as compared to a less challenging identification using molecular markers. Nonetheless, in a given habitat with a known range of species, morpho-anatomical identification is possible and could give good results.

2.5 | Site of reference plant material

To provide verifiable DNA barcodes, mature leaves of 19 Mediterranean woody plant species were collected from identified specimens in the Tel-Aviv University Botanical Garden on 21 March 2018. A sample of 5–10 leaves was collected from an individual plant of each species. Leaves were dried at 60°C for 48 hr prior to preparation for DNA extraction. In addition, tree roots were collected from potted plants at the Tel-Aviv University Botanical Garden for the preparation of a morpho-anatomical identification key.

2.6 | DNA extraction, amplification, and sequencing

Among multiple barcoding approaches, we chose the ITS2 marker, i.e., part of the nuclear internal transcribed spacer region (ITS) of the 18S-26S rDNA, which has proved most effective for plant phylogenetics, showing high levels of interspecific divergence (Chen et al., 2010; Kress et al., 2005; Li et al., 2015). We applied this approach to identify roots at the species level. DNA-based identification of plant individuals is also possible, however, requires a deeper investigation of multiple loci, which was beyond the scope of this project.

DNA extraction was based on Wang, Qi & Cutler (1993) method modified to woody plant species. Briefly, dry samples were grounded using a mixer mill (MM 400; Retsch GmbH, Haan, Germany) for six minutes at 20 frequency/second using two 5-mm stainless steel milling balls (Retsch). NaOH (400 µl 0.5 N) + 1.5% Polyvinylpyrrolidone (PVP) was added to 3 to 4 mg of dried ground leaf sample. The samples were vortexed and incubated for 10 min at 60°C, inverted gently every 150 s. Next, samples were centrifuged for five min at 20,000 RCF. From each sample, 1.5-µl supernatant was transferred to 150-µl 100 mM Tris HCl solution (pH 8.0), and mixed well. Three µl of each sample was used for PCR amplification. Three separate PCR reactions were performed for each sample using 2X PCRBIO HS Taq Mix Red (PcrBiosystems) in a total volume of 30 µl with the following program: 95°C for 30 s, 56°C for 40 s, 72°C for 60 s, 35 cycles. The primer sequences were ITS-p5 (Cheng et al., 2016) ('5-CCTTATCATTTAGAGGAAGGAG-3') and ITS-u4 ('5-GGTTTCTTTTCCTCCGCTTA-3'). Amplicons were purified by enzymatic PCR cleanup using 0.5-µl Exonuclease I (Exo I, #M0293, New England Biolabs) and 0.5-µl Shrimp Alkaline Phosphatase (rSAP, NEB, #M0371) with 5-µl PCR product in the following

program: 37°C for 15 min, 90°C for 10 min. Amplicons were sequenced in both directions with the above primers using Sanger sequencing method on a 3,730 DNA analyzer sequencer (Applied Biosystems). Generation of consensus sequences were made by contig assembly of six sequences (three forward and three reverse) using Sequencing Analysis Software v5.3 (Applied Biosystems) with minimum match percentage 90% and minimum overlap of 85 bp. Full ITS sequences were subjected to Hidden Markov Model (Keller et al., 2009) to remove the conserved 5.8S and 28S sequences and retain the correct position of the ITS2 within the full ITS sequence (Koetschan et al., 2012). Species identification was evaluated with BLAST1 method (Howard et al., 2008) on three different parts of the ITS sequence: full ITS sequence, primary structure of the ITS2, and secondary structure prediction of the ITS2 using tools from the ITS2 database (Koetschan et al., 2012; Schultz et al., 2005). Blast results were typically with 96%–97% similarity, and always > 90% similarity. All verified ITS2 sequences were provided in Table S1.

3 | RESULTS

3.1 | DNA identification of the trees responsible for the problem

Comparison of ITS2 DNA sequences from 19 woody plant species of the Mediterranean maquis against the NCBI database and the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) yielded mixed results (Table 1). Only four and ten species were accurately identified by the NCBI and ITS2 databases, respectively. Additional eleven and seven species were misidentified as related species of the same genus (i.e., identified correctly at the genus level, but not at the species level) by the respective databases. For example, *Quercus calliprinos* and *Q. boissieri* were both identified by the NCBI database as *Q. poilanei* (Table 1). This result demonstrates the importance of local projects, and specifically with endemic species, whose sequences are rarely studied. Notably, both *Quercus calliprinos* and *Q. boissieri* belong to rather distant clades from *Quercus poilanei* within the phylogeny of *Quercus* (Hipp et al., 2020). *Rhamnus*, *Crataegus*, *Clematis*, and *Tamarix* presented a similar case. Last, four and two species were not identified at all. The ITS2 database performance was consistently better than that of the NCBI. In the example of *Quercus calliprinos*, the misidentification of the ITS2 database as *Q. coccifera* was very close phylogenetically, as these two species diverged from each other only during the Pliocene, ~3 mya (Hipp et al., 2020). Therefore, we developed novel ITS2 DNA barcodes for 10 species which were missing from both databases. These sequences, along with the sequences of the other nine species, were uploaded to the ITS2 database, adhering to the database guidelines. Among the identified tree species, the two *Pistacia* species allowed for investigation of DNA polymorphisms among roots of different individuals. Within an ITS fragment sequence of 425–435 bp, we identified six single nucleotide polymorphisms (SNPs) in *Pistacia atlantica*, which were mostly related with one specific samples. Interestingly, in *Pistacia palaestina*, there was only one SNP in this fragment. The final sequences that were uploaded to the database were those representing the majority of samples from each species.

Next, root samples were taken in situ at Beit She'arim (Figure 1). Roots were sampled at three-five locations in each of three burial caves in the necropolis: The Sidonian cave, the Museum cave, and the cave of Rabbi Judah the Prince (Yehudah HaNasi). Each root sample was split into two fragments, one for DNA identification, and the other for morpho-anatomical identification.

3.2 | Morphological identification of the trees responsible for the problem

For the morpho-anatomical identification, the efficient approach was to distinguish several unique traits for each genus, rather than making a large key for each species. Root surface color, periderm structure and xylem vessel distribution were among the most useful traits, showing large and notable differences between the species, with fibers, ray structure, and secretory cavities used as auxiliary distinguishing traits. Thus, *Cupressus* was distinguished by a typical gymnosperm xylem anatomy with tracheids, and devoid of resin canals as compared to *Pinus* (Figure 3a). *Pistacia* had resin canals typical for Anacardiaceae in the phloem (Figure 3b) and usually had brown roots. One of the samples (a smooth, aromatic root) was not initially identified by anatomy. Following the DNA sequence analysis placing it in the Cucurbitaceae, it was identified as *Bryonia sp. Bryonia* had a mostly parenchymatous root with large vessels, surrounded by a complete ring of small cells (Figure 3h). To facilitate the morpho-anatomical identification, additional root samples were taken from the site. *Rhamnus* had typical yellow colored roots and undulating, diagonally arranged, xylem vessels, and rays (also termed as dendritic; Figure 3d). *Quercus* was distinguished by black roots and extensive, very thick sclereids in the stem periphery (Figure 3e).

After combining the results of both DNA barcoding and morpho-anatomical analyses, of thirteen root samples, seven were identified as *Pistacia* (four *Pistacia atlantica* and three *Pistacia palaestina*; Table 2). Three other roots were identified as the gymnosperms (two *Cupressus sempervirens*, and a *Pinus halepensis)*, and other two as perennial herbs (*Capparis spinosa* and an unidentified member of the Cucurbitaceae). Note that *Cupressus sempervirens* was existing in the databases, and did not require novel sequencing, unlike some of the species in Table 1. Anatomy and morphology methods could not verify the *C. spinosa* identification, but the Cucurbitaceae sample was identified as *Bryonia sp*. A single sample unidentified with DNA analysis was identified by anatomy as *Cercis siliquastrum*. In 10 of the cases, the DNA barcodes improved the resolution of the anatomical identification from the genus to the species level. In one case, where species-level information was missing in the DNA databases, the traditional techniques proved more useful. Among the seven identified species, five existed in the ITS database, of which two species were collected here too

TABLE 1 Leaf samples of 19 woody plant species collected at the Tel-Aviv University Botanical Garden and their identification in the ITS2 and NCBI Blast databases. Plant height is reported as an indication of size and ecological niche. Green and orange backgrounds denote existing accurate identification at the species and genus level, respectively

| Clade	Family	Species	Plant height (m)	Distribution	ITS2 Identification	NCBI Identification
Gymnosperms, Pinophyta	Pinaceae	Pinus halepensis Mill.	10–30	pan-Mediterranean	Pinus halepensis	Pinus taeda
Angiosperms, Eudicots	Fagaceae	Quercus calliprinos Webb.	3–10	Eastern Mediterranean	Quercus coccifera	Quercus poilanei
Angiosperms, Eudicots	Fagaceae	Quercus boissieri Reut.	5–12	Eastern Mediterranean	Quercus pubescens	Quercus poilanei
Angiosperms, Eudicots	Oleacea	Phillyrea latifolia L.	2–7	pan-Mediterranean	Phillyrea latifolia	Phillyrea angustifolia
Angiosperms, Eudicots	Anacardiaceae	Pistacia atlantica Desf.	5–15	Western and central Asia, Mediterranean	Pistacia atlantica	Pistacia atlantica
Angiosperms, Eudicots	Anacardiaceae	Pistacia lentiscus L.	0.5–5	Mediterranean	Pistacia lentiscus	Pistacia lentiscus
Angiosperms, Eudicots	Rhamnaceae	Rhamnus lycioides Pall.	2–4	Eastern Mediterranean	Rhamnus cathartica	Rhamnus cathartica
Angiosperms, Eudicots	Rhamnaceae	Rhamnus punctate Boiss.	1–3	Eastern Mediterranean	Rhamnus cathartica	Rhamnus cathartica
Angiosperms, Eudicots	Aceraceae	Acer obtusifolium Sm.	3–7	Eastern Mediterranean	Acer obtusifolium	Acer obtusifolium
Angiosperms, Eudicots	Lauraceae	Laurus nobilis L.	3–10	pan-Mediterranean	Laurus nobilis	–
Angiosperms, Eudicots	Ericaceae	Arbutus andrachne L.	4–12	Eastern Mediterranean	Arbutus andrachne	Arbutus canariensis
Angiosperms, Eudicots	Caprifoliaceae	Viburnum tinus L.	2–5	Mediterranean	Viburnum tinus	Viburnum opulus
Angiosperms, Eudicots	Rosaceae	Crataegus azarolus L.	3–8	Eastern Mediterranean	Crataegus laevigata	Crataegus laevigata
Angiosperms, Monocots	Liliaceae	Smilax aspera L.	Vine	Central Africa, western and southern Asia, Mediterranean	–	–
Angiosperms, Eudicots	Myrtaceae	Myrtus communis L.	0.5–3	Western and southern Asia, Mediterranean	Myrtus communis	Myrtus communis
Angiosperms, Eudicots	Ranunculaceae	Clematis cirrhosa L.	Vine	Mediterranean	Clematis recta	Clematis vitalba
Angiosperms, Eudicots	Cistaceae	Cistus salviifolius L.	0.5–1	Mediterranean	Cistus salviifolius	–
Angiosperms, Eudicots	Cistaceae	Cistus creticus L.	0.5–1.5	Mediterranean	–	–
Angiosperms, Eudicots	Tamaricaceae	Tamarix nilotica Bunge	5–10	North Africa, Middle East	Tamarix laxa	Tamarix aucheriana
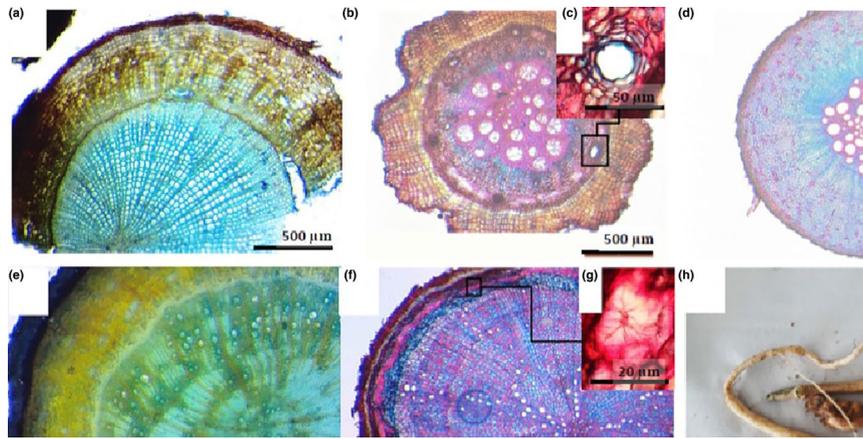
FIGURE 3 Tree species identification based on morpho-anatomical characteristics of the root. (a) Sample from the Sidonian cave entrance, identified as *Cupressus*; (b-c) samples from the Museum cave, identified as *Pistacia*. Note the secretory cavities in the phloem, characteristic of Anacardiaceae; (d) sample from the Museum cave, identified as *Bryonia*; (e) *Rhamnus* root: note the undulating, diagonally arranged, vessels, and the yellow root color; (f, g) *Quercus ithaburensis* root, note the thick sclereids in the stem periphery; and (h) thick *Bryonia* root with a smooth yellow-brown surface. A and E are hand sections, with TBO stain. B, C, D, F, and G are hand sections, with Safranin-alcian blue stain

TABLE 2 Identification of root samples which have penetrated the ceilings three burial caves the Beit She'arim necropolis

Sample	DNA barcoding ID	Morpho-anatomical ID
Sidonian Cave entrance	*Cupressus sempervirens*	*Cupressus*
Sidonian Cave #1	*Pistacia atlantica*	*Pistacia*
Sidonian Cave #2	*Pistacia atlantica*	*Pistacia*
Museum Cave entrance	*Pistacia palaestina*	*Pistacia*
Museum Cave #1	*Cupressus sempervirens*	*Cupressus*
Museum Cave #2	*Pistacia palaestina*	*Pistacia*
Museum Cave #3	*Pistacia palaestina*	*Pistacia*
Museum Cave #4	*Capparis spinosa*	?
Yehudah Cave #1	Cucurbitaceae	*Bryonia*
Yehudah Cave #2	*Pistacia atlantica*	*Pistacia*
Yehudah Cave #3	?	*Cercis siliquastrum*
Yehudah Cave #4	*Pistacia atlantica*	*Pistacia*
Yehudah Cave #5	*Pinus halepensis*	?

(*Pistacia atlantica* and *Pinus halepensis*; Table 1). Still, our work added confidence to the *Pinus halepensis* identification, which was missing at the NCBI database.

4 | DISCUSSION

The integrity of the bedrock structure was affected by both the direct cracking activity of growing tree roots, and the indirect increase in water flow through new cracks in the rock. These processes facilitated each other, increasing the risk of cave collapse. Even though

the vegetation above and around the caves was not native to the area it became a valuable part of the park landscape. The park authorities wanted to preserve as much of it as possible, while preventing continuation of the processes that endangered the preservation of the burial caves and their content.

Our ad hoc tree forensics study demonstrates the advantage of combining traditional methods with modern techniques in plant science, in service of conservation efforts. Although DNA barcoding applications have been increasing rapidly in the past decade, including many new plant families and species, barcodes for many Mediterranean plant species are still lacking. Here, nine of nineteen species (47%) were missing in the databases. The addition of these, and other species, to the public database, is an important step forward in expanding the use of DNA barcoding. In cases where only plant parts are available and identification is difficult, genetic identification has an important added value. Tracing techniques of water transport, e.g., using $H_2^{18}O$, could potentially improve the resolution of our study to the individual tree level (Maeght et al., 2013; Schenk & Jackson, 2005), yet require far more resources. Our choice of using the (ITS)2 marker system proved well in our case, in spite of its known problems, such as polymorphisms within species (observed here for *Pistacia atlantica*, but not for *P. palaestina*) and contamination by mycorrhizal DNA. It is possible that the spatial homogeneity of the samples contributed to a reduced level of such polymorphism that would otherwise complicate the identification. In addition, a visual inspection showed that mycorrhizal colonization of our roots was lower than that of tree roots in wetter biomes, e.g., in a temperate forest (Rog et al., 2020). Interestingly, ITS markers were also those which enabled the identification of ancient cedar wood in Jerusalem's temple mount (Rogers & Kaya, 2006).

In the case of Beit She'arim, the conservation was dual: risk had to be reduced from the archaeological site, while minimizing the impact on the vegetation, which is partly unique to confined regions of the

Eastern Mediterranean. Here, identification of the tree species enabled selective removal of specific trees growing on top of the necropolis, while retaining the majority of the trees in the area. Clearly, root identification at the individual tree level would have been better from this aspect. However, since rooting patterns are conserved at the species level, one can assume that multiple individuals of a certain species are either currently penetrating the cave, or are prone to penetrate it, rather than an individual tree. It was estimated that the *Pistacia* trees and shrubs comprise about ~25% of the woody vegetation at the site. Informing the Parks Authority has, therefore, saved ~75% of the maquis on top of the caves, including the prevalent *Quercus calliprinos* and other tree species which were identified aboveground but not penetrating the caves (*Rhamnus alaternus*, *Quercus ithaburensis*, *Pistacia lentiscus*, *Punica granatum*, *Ceratonia siliqua*, and *Ficus carica*).

What caused tree roots, and primarily those of *Pistacia* species, to penetrate the burial caves of Beit She'arim? In general, deep roots develop where precipitation is seasonal (rather than just overall low) (Schenk & Jackson, 2005). In mid-latitudes specifically, the occurrence of roots deeper than 2 m depends on soil structure (Schenk & Jackson, 2005). In places where shallow soil is underlain by weathered bedrock, roots tend to grow into the rock, in turn extracting water and nutrients from it (Schwinning, 2010). In our site, all three predisposing conditions exist: a shallow Rendzina soil covers a soft chalk-limestone bedrock, and rainfall is restricted to October-April, with a ~6 months zero precipitation dry season. Furthermore, woodland plant species, such as in our case, tend to allocate significantly more biomass to roots, than species of the temperate, boreal, or tropical forest (Poorter et al., 2012). Interestingly, unlike in natural caves (Adams et al., 2019; Howarth et al., 2007; Jackson et al., 1999), part of the roots in our study did not seem to extend into the space of the caves (Figure 1). One hypothesis is that the drier conditions in our site, compared with the previously reported settings, prohibited water, and nutrient uptake from the cave itself, in contrast to the rock environment (Schwinning, 2010). It has been suggested that rooting depth might be a more important plant trait than root biomass, shaping plant communities by hydraulic lift (Maeght et al., 2013), which has already been reported in a Mediterranean pine species (Penuelas & Filella, 2003). Our findings of *Pistacia*, *Cupressus*, *Cercis*, and *Pinus* roots at 5 m depth below surface shed new light on the eco-physiology of these important taxa.

5 | CONCLUSIONS

The efforts by botanists in combining novel and traditional methods for identification of subterranean roots, disconnected from the plants themselves, enabled the devising of management procedures that served both the antiquities preservation and landscape maintenance. The DNA barcoding applied here to the local Mediterranean vegetation identified certain lacunae in the database, which have been filled through analysis of verified plants from the botanical garden. Additionally, as a broad range of characters is used for the morpho-anatomical identification, a good knowledge of plant anatomy is required, as compared to a less challenging identification using molecular markers. Nonetheless, in a given habitat with a known range of species, morpho-anatomical identification is possible and could give good results.

AUTHOR CONTRIBUTIONS

The study was initiated by IC, IS, and DBY. AE, IS, and TK designed the research. GJ and IR conducted the DNA barcoding study. IS conducted the morpho-anatomical study. IC and DBY were in charge of the archaeological conservation and information. TK wrote the manuscript with contributions from all authors.

ORCID

Tamir Klein https://orcid.org/0000-0002-3882-8845

REFERENCES

Aaronsohn, A. (1940) Reliqiae Aaronsohnianae. II. Florula Cisiordanica. : Societe Genevoise d'Editoins et Impressions.

Adams, R. E., Iliffe, T. M., & West, J. B. (2019). Identifying tree roots in the caves of Quintana Roo, Mexico as a step toward ecological insights and improved conservation. *Plants*, *People*, *Planet*, *2*, 133–139. https://doi.org/10.1002/ppp3.10079

Avigad, N. (1971). Beth She'arim, vol. 3 The archaeological excavations during 1953–1958. The catacombs 12–23. : The Israel Exploration Society with assistance of Mosad Bialik.

Battipaglia, G., De Micco, V., Aronne, G., & Cherubini, P. (2011). Hidden phenology in wood: growth dynamics in Mediterranean trees and shrubs in response to climate. Geophysical Research Abstracts (ISSN: 1607–7962) pp, 1–1.

C. P. W. Group;Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., Chase, M. W., Cowan, R. S., Erickson, D. L., Fazekas, A. J., Graham, S. W., James, K. E., Kim, K.-J., Kress, W. J., Schneider, H., van AlphenStahl, J., Barrett, S. ch, van den Berg, C., Bogarin, D., ... Little, D. P. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the USA*, *106*(31), 12794–12797. https://doi.org/10.1073/pnas.0905845106

Carlquist, S. (1988). *Comparative wood anatomy: Systematic, ecological, and evolutionary aspects of dicotyledon wood*. Springer.

Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., Zhu, Y., Ma, X., Gao, T., Pang, X., Luo, K., Li, Y., Li, X., Jia, X., Lin, Y., & Leon, C. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One*, *5*(1), e8613. https://doi.org/10.1371/journal.pone.0008613

Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., & Zhou, S. (2016). Barcoding the kingdom Plantae: New PCR primers for ITS regions of plants with improved universality and specificity. *Molecular Ecology Resources*, *16*(1), 138–149. https://doi.org/10.1111/1755-0998.12438

Crivellaro, A., & Schweingruber, F. H. (2013). *Atlas of wood, bark and pith anatomy of Eastern Mediterranean trees and shrubs with a special focus on Cyprus*. Springer International Publishing.

Fahn, A., Werker, E., & Baas, P. (1986). *Wood anatomy and identification of trees and shrubs from Israel and adjacent regions*. Israel Academy of Sciences and Humanities.

Gismondi, A., Rolfo, M. F., Leonardi, D., Rickards, O., & Canini, A. (2012). Identification of ancient Olea europaea L. and Cornus mas L. seeds by DNA barcoding. *Comptes Rendus Biologies*, *335*(7), 472–479.

Hajibabaei, M., Singer, G. A., Hebert, P. D., & Hickey, D. A. (2007). DNA barcoding: How it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, *23*(4), 167–172. https://doi.org/10.1016/j.tig.2007.02.001

Hipp, A. L., Manos, P. S., Hahn, M., Avishai, M., Bodénès, C., Cavender-Bares, J., Crowl, A. A., Deng, M., Denk, T., Fitz-Gibbon, S., Gailing, O., González-Elizondo, M. S., González-Rodríguez, A., Grimm, G. W., Jiang, X.-L., Kremer, A., Lesur, I., McVay, J. D., Plomion, C., … Valencia-Avalos, S. (2020). Genomic landscape of the global oak phylogeny. *New Phytologist*, *226*(4), 1198–1212. https://doi.org/10.1111/nph.16162

Howard, A. R., Sumathi, M., & Wai, L. S. L. (2008). Testing the reliability of genetic methods of species identification via simulation. *Systems Biology*, *57*(2), 216–230. https://doi.org/10.1080/10635150802032990

Howarth, F. G., James, S. A., McDowell, W., Preston, D. J., & Imada, C. T. (2007). Identification of roots in lava tube caves using molecular techniques: Implications for conservation of cave arthropod faunas. *Journal of Insect Conservation*, *11*(3), 251–261. https://doi.org/10.1007/s10841-006-9040-y

Jackson, R. B., Moore, L. A., Hoffmann, W. W. A., Pockman, W. T., & Linder, C. R. (1999). Ecosystem rooting depth determined with caves and DNA. *Proceedings of the National Academy of Sciences of the USA*, *96*(20), 11387–11392. https://doi.org/10.1073/pnas.96.20.11387

Keller, A., Schleicher, T., Schultz, J., Müller, T., Dandekar, T., & Wolf, M. (2009). 5.8 S-28S rRNA interaction and HMM-based ITS2 annotation. *Gene*, *430*(1–2), 50–57. https://doi.org/10.1016/j.gene.2008.10.012

Koetschan, C., Hackl, T., Müller, T., Wolf, M., Förster, F., & Schultz, J. (2012). ITS2 database IV: Interactive taxon sampling for internal transcribed spacer 2 based phylogenies. *Molecular Phylogenetics and Evolution*, *63*(3), 585–588. https://doi.org/10.1016/j.ympev.2012.01.026

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the USA*, *102*(23), 8369–8374. https://doi.org/10.1073/pnas.0503123102

Lahaye, R., van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, S., Barraclough, T. G., & Savolainen, V. (2008). DNA barcoding the floras of biodiversity hotspots. *Proceedings of the National Academy of Sciences of the USA*, *105*(8), 2923–2928. https://doi.org/10.1073/pnas.0709936105

Li, X., Yang, Y., Henry, R. J., Rossetto, M., Wang, Y., & Chen, S. (2015). Plant DNA barcoding: From gene to genome. *Biological Reviews*, *90*(1), 157–166. https://doi.org/10.1111/brv.12104

Maeght, J. L., Rewald, B., & Pierret, A. (2013). How to study deep roots—and why it matters. *Frontiers in Plant Science*, *4*, 299. https://doi.org/10.3389/fpls.2013.00299

Mazar, B. (1957). Beth She'arim, Report on the Excavations During 1936–40. Vol. One: The Catacombs I-IV. : The Israel Exploration Society.

Meyers, E. M. (1999). *Galilee through the Centuries. Confluence of Cultures*. Eisenbrouns.

Moritz, C., & Cicero, C. (2004). DNA barcoding: Promise and pitfalls. *PLoS Biology*, *2*(10), e354. https://doi.org/10.1371/journal.pbio.0020354

Mrak, T., & Gricar, J. (2016). *Atlas of woody plant roots - Morphology and anatomy with special emphasis on fine roots*. Slovenian Forestry Institute, The Silva Slovenica Publishing Centre.

O'Brien, T. P., Feder, N., & McCully, M. E. (1964). Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma*, *59*, 368–373. https://doi.org/10.1007/BF01248568

Patishi, D. (1983). *A Town in the Shade of the Oak Trees: The Story of Kiryat-Tivon*. Cherikover.

Penuelas, J., & Filella, I. (2003). Deuterium labelling of roots provides evidence of deep water access and hydraulic lift by *Pinus nigra* in a Mediterranean forest of NE Spain. *Environmental and Experimental Botany*, *49*(3), 201–208. https://doi.org/10.1016/S0098-8472(02)00070-9

Poorter, H., Niklas, K. J., Reich, P. B., Oleksyn, J., Poot, P., & Mommer, L. (2012). Biomass allocation to leaves, stems and roots: Meta-analyses of interspecific variation and environmental control. *New Phytologist*, *193*(1), 30–50. https://doi.org/10.1111/j.1469-8137.2011.03952.x

Rewald, B., Meinen, C., Trockenbrodt, M., Ephrath, J. E., & Rachmilevitch, S. (2012). Root taxa identification in plant mixtures - current techniques and future challenges. *Plant and Soil*, *359*, 165–182. https://doi.org/10.1007/s11104-012-1164-0

Rog, I., Rosenstock, N. P., Körner, C., & Klein, T. (2020). Share the wealth: Trees with greater ectomycorrhizal species overlap share more carbon. *Molecular Ecology*, *29*(13), 2321–2333. https://doi.org/10.1111/mec.15351

Rogers, S. O., & Kaya, Z. (2006). DNA from ancient cedar wood from King Midas' tomb, Turkey, and Al-Aksa Mosque. *Israel. Silvae Genetica*, *55*(1–6), 54–62. https://doi.org/10.1515/sg-2006-0009

Rosenfeld, B.-Z. (1995). Rabbi Yehoshua Ben Levi and his wife Kira Mega: Interpretation of Beth She'arim Inscriptions. *Katedra*, *114*, 11–16. (in Hebrew).

Rutherford, M. C. (1980). Field identification of roots of woody plants of the savanna ecosystem study area, Nylsvley. *Bothalia*, *13*, 171–184. https://doi.org/10.4102/abc.v13i1/2.1307

Schenk, H. J., & Jackson, R. B. (2005). Mapping the global distribution of deep roots in relation to climate and soil characteristics. *Geoderma*, *126*(1–2), 129–140. https://doi.org/10.1016/j.geoderma.2004.11.018

Schlumbaum, A., Tensen, M., & Jaenicke-Després, V. (2008). Ancient plant DNA in archaeobotany. *Vegetation History and Archaeobotany*, *17*(2), 233–244. https://doi.org/10.1007/s00334-007-0125-7

Schultz, J., Maisel, S., Gerlach, D., Müller, T., & Wolf, M. (2005). A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. *RNA*, *11*(4), 361–364. https://doi.org/10.1261/rna.7204505

Schweingruber, F. H., Börner, A., & Schulze, E. (2011). *Atlas of stem anatomy in herbs, shrubs and trees: Volume 1*. : Springer.

Schwinning, S. (2010). The ecohydrology of roots in rocks. *Ecohydrology*, *3*(2), 238–245. https://doi.org/10.1002/eco.134

Shtein, I., Baruchim, P., & Lev-Yadun, S. (2020). Division of labour among culms in the clonal reed *Arundo donax* L. (Poaceae) is underlied by their pre-determined hydraulic structure. *Botanical Journal of the Linnean Society (accepted)*, https://doi.org/10.1093/botlinnean/boaa062

Uma, E., & Muthukumar, T. (2014). Comparative root morphological anatomy of Zingiberaceae. *Systematics and Biodiversity*, *12*, 195–209. https://doi.org/10.1080/14772000.2014.894593

Valentini, A., Pompanon, F., & Taberlet, P. (2009). DNA barcoding for ecologists. *Trends in Ecology, & Evolution*, *24*(2), 110–117. https://doi.org/10.1016/j.tree.2008.09.011

Wang, H., Qi, M., & Cutler, A. J. (1993). A simple method of preparing plant samples for PCR. *Nucleic Acids Research*, *21*(17), 4153–4154. https://doi.org/10.1093/nar/21.17.4153

Weiss, Z. (1992). Social aspects of burial in Beth She'arim: archeological finds and talmudic sources. In L. I. Levine (Ed.), *The Galilee in Late antiquity* (pp. 357–371). The Jewish Theological Seminary of America.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.