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Ancient DNA fragments inside Classical Greek amphoras reveal cargo of 2400-year-old shipwreck

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Abstract

The origins and spread of eastern Mediterranean civilizations 4000–2000 years ago constitute defining events in human development. Interregional connections across the sea played critical roles in building increasingly sophisticated economies and societies. Research of trade and exchange among these first centers has relied upon ancient societies' archaeological artifacts. The most ubiquitous artifacts recovered from shipwreck sites are ceramic transport jars, *amphoras*. However, for archaeologists and historians determining the original contents of these containers has been problematic, aided only occasionally by physical evidence (e.g. olive pits, resins) found inside excavated jars. Here, we investigate whether modern DNA analyses can reveal original contents of amphoras containing no visible physical remains. Using chloroplast DNA markers and PCR we analyzed the walls of two amphoras recovered from a 2400 year-old shipwreck off the Greek island of Chios. Our results show that short ($\leq$100 bp) ancient DNA fragments can be extracted from scrapings taken from amphoras' interior walls. These DNA fragments identify the amphoras' original contents. Our analyses indicate that one of the amphoras most likely contained olive oil and oregano, even though no physical traces of remains are visible inside the jar. The second amphora might have contained mastic resin; resins of various types were preservatives commonly added to ancient wine. Our analyses are the first to demonstrate that ancient DNA fragments can be extracted from the walls of amphoras recovered from underwater shipwreck sites. This opens a new field of molecular archaeology analyses, and provides a powerful tool for obtaining information about the agricultural production, contact networks, and economies of the early civilizations.
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1. Introduction

Recent decades have brought major advances in molecular biology techniques. Rapid detection and identification of very low copy numbers of DNA is now possible through enzymatic amplification of specific sequences from analyzed samples. Methods for isolation and amplification of scarce ancient DNA fragments have become increasingly important tools for scientists trying to answer a wide range of questions about evolution of species, early humans, agricultural production, origin of civilizations, etc. (Pääbo et al., 2004; Noonan et al., 2006; Willerslev and Cooper, 2005; Gugerli et al., 2005; Cavalieri et al., 2003).

Here, we use this approach to demonstrate the ability and usefulness of modern DNA methods in determining trade goods once contained inside archaeological artifacts from ancient deepwater shipwreck sites. We are particularly interested in traces of ancient non-algal botanical material, as contamination of modern terrestrial plant DNA would be unlikely in artifacts sampled from sites deep underwater. The artifacts

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discussed here are 2400-year-old ceramic transport jars, known as *amphoras*. Originally invented by the Canaanite culture in the 16th century B.C., these ceramic containers were widely used throughout the Mediterranean region as inexpensive, disposable containers for seaborne bulk commodity transport (Koehler, 1986). From the seventh century B.C., the Greeks embraced this technology. Many Greek cities and islands developed their own distinct amphora style (e.g. Rhodian, Thasian, Samian, etc.) to carry locally-produced goods within their maritime trading networks (Whitbread, 1995; Grace, 1979). Interregional connections, particularly via ships plying sea routes, played critical roles in the emergence of increasingly sophisticated economies and societies around the Mediterranean 4000–2000 years ago. Remnants of this ancient trade can today be found on the Mediterranean Sea floor in the form of amphoras on shipwreck sites. The organic remains and the hulls of the ships themselves were long ago consumed by benthic biological activity, and often the pile of amphoras is the only trace left of a wreck (Oleson and Adams, 2004).

Amphoras are known to have carried wine, olive oil, spices, fish products as well as several other liquid or semi-liquid goods (Peacock and Williams, 1991). A fundamental problem for archaeologists, economists, and historians studying the ancient world is accurately determining the volume of trade in these commodities (Lund, 2004). Ancient texts describe amphora contents generally, but direct archaeological evidence is found infrequently in amphoras excavated from sites on land or from shipwrecks. When possible, archaeologists have identified ancient amphora contents based on the occasional physical residues found inside the vessels, e.g. olive pits or resin linings. These residues can be investigated by applying gas/liquid chromatography and mass spectrometry to analyze triterpenoid composition in resins (Stern et al., 2000, 2003; Serpico and White, 2000) and detect traces of tartaric acid originating from resinated wine (Lund, 2004; McGovern, 2003; Jones, 1986; Guasch-Jane et al., 2004). Fragments of ancient DNA from *Saccharomyces cerevisiae*, the principal yeast used in fermentation processes, have been extracted from residues contained inside Egyptian jars recovered from 5000-year-old tombs. These analyses provide indirect evidence for early wine production (Cavalieri et al., 2003).

Amphoras were invented to carry goods in ships, and a substantial portion of early trade moved across water. Some fractional percentage of those voyages ended in wrecking events (Muckelroy, 1978), leaving the amphoras on the sea floor. Shipwrecks hold invaluable qualitative and quantitative information about ancient technology transfer, long-distance communication, economic and agricultural production, regional interdependence, and cultural development. Determining specific details about what was traded in various eras is of great interest to a broad spectrum of scientists. Successful amplification of ancient DNA fragments from artifacts recovered underwater would answer long-standing queries, and allow scientists to pose new questions.

By isolating and identifying ancient DNA, we here present evidence that two 2400-year old amphoras (*A-1* and *A-2*; Fig. 1) excavated from a deep water shipwreck site in the Mediterranean still hold ancient genetic plant material inside their walls, revealing their original contents. Our protocol provides an easy method for identifying amphora contents recovered from archaeological underwater sites, by isolating and amplifying short ($\leq$100 bp) ancient DNA fragments.

2. Materials and methods

The amphoras used for this study are of two distinct types contained in a shipwreck off the Greek Aegean island of Chios. This wreck site was discovered and identified in 2004 by Greek archaeologists and scientists from the Hellenic Ministry of Culture's Ephorate of Underwater Antiquities (HMC/EUA – Greece) and the Hellenic Centre for Marine Research (HCMR – Greece). One amphora of each type was collected by an HCMR robotic vehicle in 2004, and the wreck was completely surveyed in 2005 by an international team including HCMR, HMC/EUA, Woods Hole Oceanographic Institution (WHOI, USA), and Massachusetts Institute of Technology (M.I.T., USA) (Foley et al., in press). The 2400-year old shipwreck lies in water 70 m deep, and the site contains more than 350 amphoras of two forms. The first type is a fourth century B.C. style from Chios typically interpreted as a wine container (Barron, 1986) (*A-1*; Fig. 1A). The second type has an unknown origin (*A-2*; Fig. 1B), but might be from the Eurasian mainland (Carlson, 2003), or Chios itself (Arribas et al., 1987). In May 2006 we collected ceramic material (scrapings) from both amphoras' interior walls, and from the bottom of *A-2* a sample of hardened blackish-brown resin (referred to as *A-2-R* in the text for easier separation from analyses of the ceramic material). Genetic analyses were conducted in accordance with established criteria of authenticity for ancient DNA research (see Pääbo et al., 2004; Cooper and Poinar, 2000).

2.1. DNA extraction and primer design

All solutions were made fresh and pipettes and bench spaces were treated with UV-light before analyses began. Extractions were set up in a lab space that is used exclusively for pre-PCR analyses. Approximately 1 cm^3 clay or resin material was analysed in 1.5 ml eppendorf tubes. One extraction control that contained no sample material was included in the extractions and treated identically to the tubes with clay/resin samples. We added 800 µl Lysis buffer (0.1 M Tris–HCl, pH 8.5; 0.005 M EDTA; 0.2% SDS; 0.2 M NaCl) and 150 µg proteinase K before placing tubes in a water bath at 56 °C for 4 h. Samples were centrifuged for 10 min at 10,000 rpm and the supernatant transferred to new tubes. The DNA was precipitated with 40 µl NaAc and 400 µl ice-cold ethanol (99%), centrifuged for 10 min at 10,000 rpm, washed with ethanol (70%) and dried in a vacuum centrifuge. Samples were redissolved in 50 µl 1 × TE. After DNA extraction we ran 2% agarose gels stained with ethidium bromide to confirm presence of genetic material. We saw a distinct smear of material sizing up to approximately 400 bp from the *A-1*

Fig. 1. Photographs of the two amphoras used for these analyses; (**A**) Amphora *A-1*, (**B**) Amphora *A-2*. Image courtesy of the Hellenic Ministry of Culture, Ephorate of Underwater Antiquities.

and *A-2* samples but not from the extraction control. The smear from the *A-2-R* sample was very weak.

Gene-specific oligonucleotide primers targeting the chloroplast *Embryophyta* (plants) gene sequences were designed to prevent amplification of algal DNA (Table 1). The chloroplast is a plant organelle that has been widely used for phylogenetic analyses and species identification analyses (Palmer, 1991, 1985; Raubeson and Jansen, 2005). Primers were either designed to recognize several different species by placing them in regions of high conservation, or designed to amplify only one specific species (or genus) by placing them in regions of high variability between genera. Specifically, the primers were designed for the tRNA-Leu (trnL), NADH dehydrogenase subunit F (ndhF), ribulose-1,5-bisphosphate carboxylase large subunit (rbcL) and maturase K (MatK) chloroplast gene sequences. Suitable genes were selected based on two main criteria. First, the genes had to have been sequenced for several plant species and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/). Second, the genes must contain regions where even short $\leq$100 bp fragments identified a specific plant genus or, if possible, species.

2.2. PCR, cloning and sequencing

Taking all necessary precautions in order to avoid any form of contamination from modern plant DNA, we set up standard polymerase chain reactions (PCR) in a designated pre-PCR lab to target specifically the DNA of the different chloroplast regions. For all of the PCRs we used the Advantage 2 polymerase mix PCR enzyme (BD Biosciences Clontech, Palo Alto, CA, USA). PCR conditions were 1 min initial denaturation at 95 °C, followed by 42 cycles of denaturation at 95 °C; 10 s, a primer specific annealing (Table 1) for 10 s; and elongation 68 °C for 20 s ending with a single elongation step at 68 °C for 5 min. Every PCR run contained a blank (no template) to control for contamination. Amplified products were

Table 1
The plant species used for primer design, the primer sequences and annealing temperatures used in the PCR analyses

Plant species	Gene	GenBank accession no	Primer names	Primer pair sequences (5′−>3′)	Fragment size (bp)	Annealing temp. (°C)
Olea europaea	trnL-trnF	AF231866	trnL-F	GCAATCCTGAGCCAAATCCT	*VARIABLE*	62
Vitis vinifera	trnL-trnF	AF300295	trnL-R	GACTCAATGGAAGCTGTTCTAACA	(73–103)	
Nasturtium officinale	trnL-trnF	AF361930				
Sesamum indicum	trnL-trnF	AF479010				
Thymus vulgaris	trnL-trnF	AF506613				
Origanum vulgare	trnL-trnF	AF506614				
Rosmarinus officinalis	trnL-trnF	AF506616				
Mentha arvensis	trnL-trnF	AF618513				
Pistacia vera	trnL-trnF	AF677209				
Pinus strobus	rbcL	AY497219	rbcL-P-F	ATGCATGCAGTTATTGAYAGACA	119	62
Commiphora habessinica	rbcL	U39276	rbcL-P-R	GGTACYGTAGTAGGTAAACTTG		
Olea europaea	rbcL	AJ001766				
Rosmarinus officinalis	rbcL	Z37435				
Vitis vinifera	rbcL	AJ635355				
Olea europaea/*Vitis vinifera*	Maturase K	AJ429335	MatKF3	AGATATACTAATACCCCACCCCAT	94	62
		AJ429274	MatKR5	CTCTTCTTTGCATTTATTACGA		
			MatK-O-F	TCACATTTAAATTTTGTGTTAGAT	114	57
			MatK-R5	CTCTTCTTTGCATTTATTACGA		
			MatK-O-F2	TCAACATCTTCTAGAGCTCTTCTTG	110	61
			MatK-O-R	ACCAATCTATGCTTGTTCAAGGA		
Origanum vulgare/*Thymus serpyllum*	Maturase K	AY840165	MatK-TOF	CGCAACAAGAACTTGTATTCTC	111	60
		AY840173	MatK-TOR	AGAAGCGAAAAAGAACAAGATA		
Pistacia lentiscus/*Pisacia vera*	ndhF	DQ390463	Nadh-M-F	AGGACATTTCAACATTCGT	95	57
		AY677204	Nadh-M-R	GGTAAAGAAGAGCCAAAATCTA		
			Nadh-M-F3	TTGGAACATACTGAATTTAGTTG	96	57
			Nadh-M-R3	GATTGGTCATATAATCGTGC		

inserted into a TOPO cloning vector (Invitrogen™ Life Technologies, Carlsbad, CA, USA) and 10–20 clones from each amplification were sequenced using BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit on an ABI PRISM® 310 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were blasted to GenBank and manually aligned with closely related plant species (see Section 3) using the BioEdit program (Hall, 1999).

To rule out that amplified sequences originated from modern plant material (contamination after the amphoras were brought up from the ocean floor), we ran additional PCRs targeting fragments ranging from 500 bp up to 1000 bp. For these particular analyses we used primers designed to amplify fragments of the same chloroplast genes targeted previously but the analyses amplified no specific bands from any of the templates (methods not shown). The laboratory at Lund University where these analyses were performed has never before been used to extract or amplify modern DNA of targeted plant species. Overall, the analyses follow guidelines for ancient DNA detection and analyses (Cooper and Poinar, 2000; Pääbo et al., 2004) by including blank controls in extraction and in all run PCRs, sequencing of multiple clones and repeated amplifications of templates, as well as attempts to amplify longer (>500 bp) fragments. We did not run quantitation experiments to estimate the copy number of the DNA target but used multiple primer pairs targeting different chloroplast regions to identify and confirm all amplified plant material from the amphora material.

3. Results

3.1. PCR amplifications from A-1 template

The first round of PCRs using the *A-1* template together with primer pairs designed for multiple plant species resulted in the following sequences according to GenBank database: trnL-F/trnL-R amplified trnL gene sequences identical to several species within the *Thymus* or *Origanum* genera; rbcL-P-F/rbcL-P-R amplified sequences identical to species within the *Thymus* or *Salvia* genera; MatKF3/MatKR5 only amplified sequences identical to *Olea europaea* (olive). The second round of PCRs, now only using genus-specific primers, resulted in the following sequences: the primer pairs MatKOF/MatKR5 as well as MatKOF2/MatKOR both amplified MatK gene sequences identical to *O. europaea*; MatK-TOF/MatK-TOR amplified MatK gene sequences identical to *Origanum vulgare* (oregano) (Figs. 2A,B and 3a,b).

3.2. PCR amplifications from A-2 template

When template from *A-2* was used in PCRs together with the primer pairs designed for multiple plant species (see Section 3.1), no specific fragments were amplified. However, the genus-specific Nadh-MF/Nadh-MR primer pairs amplified ndhF gene fragments belonging to the *Pistacia* genus. Using a second primer pair, Nadh-MF3/Nadh-MR3, another fragment was successfully amplified and identified in GenBank database as a ndhF gene fragment from either *Pistacia lentiscus* (mastic) and/or *P. vera* (pistachio) (Figs. 2C and 3c). The high sequence similarity between these two particular species prevented us from determining from which species the fragment originated.

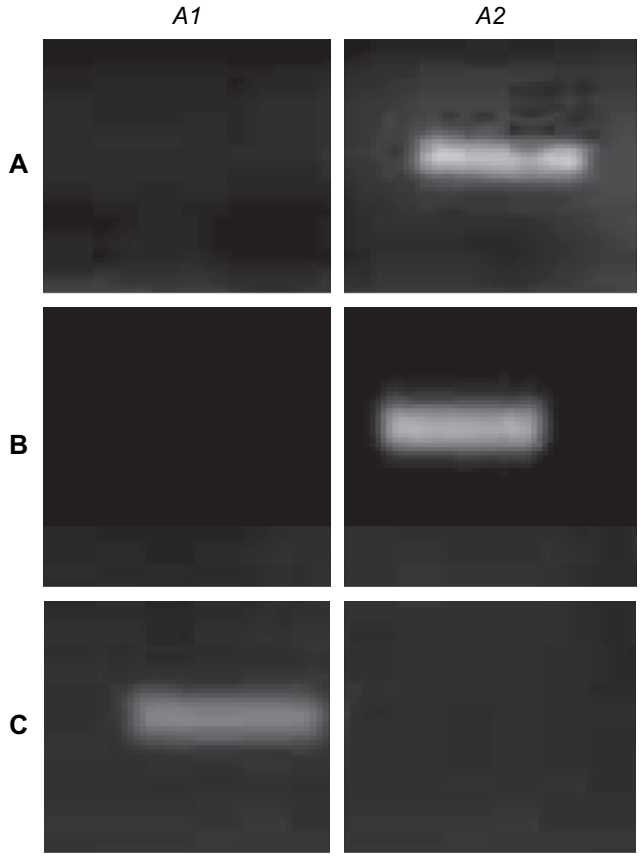

Fig. 2. Amplified bands in PCR using primers specific for: (**A**) olive (*Olea europaea*) maturase K gene. The size of the fragments is 114 bp. (**B**) oregano (*Origanum vulgare*) maturase K gene. The size of the fragments is 111 bp. (**C**) mastic/pistachio (*Pistacia lentiscus*/*P. vera*) NADH dehydrogenase subunit F gene. Fragment size is 95 bp. The 3% agarose gel was stained with ethidium bromide. *A-1*: template from amphora 1; *A-2*; amphora 2 template.

All PCRs using the genus-specific primer pairs results were confirmed by at least two additional PCR analyses, with subsequent cloning and sequencing of products. *Salvia* and *Thymus* sequences could not be amplified when species specific primers were used. No PCRs amplified sequences belonging to the *Vitis* (grape) genus. None of the performed PCR analyses produced bands in the included control, strongly indicating that the amplified fragments were not a result of contamination. No sequences were amplified from the lump of hardened resin taken from the inside of *A-2*.

4. Discussion

We show here that ancient DNA can successfully be isolated from the walls of two 2400-year old amphoras sampled from an underwater wreck site off the Aegean island of Chios. Our detection and isolation of olive and oregano fragments in the first analyzed amphora template, *A-1*, are unexpected

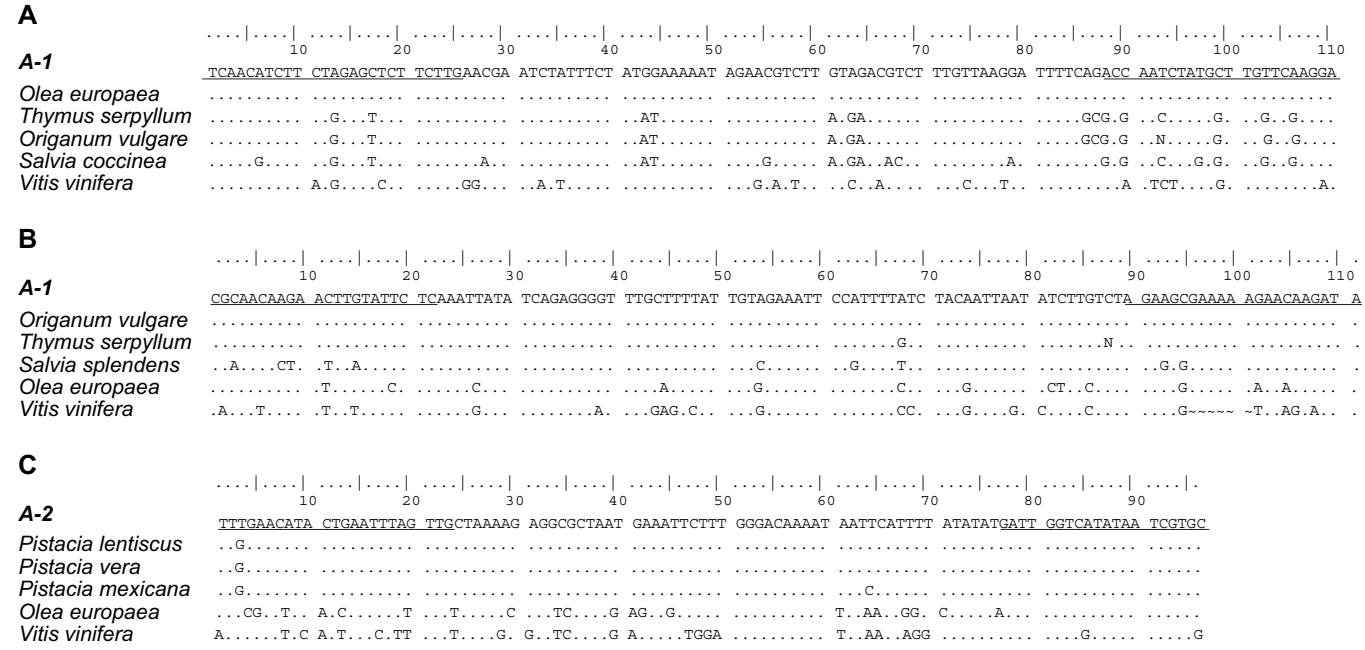

Fig. 3. Alignments with amplified fragments from *A-1* and *A-2* template. Primer sequences are underlined. Alignments were made using BioEdit program. Dashes (∼) indicate gaps introduced for optimal sequence alignment, dots (·) indicate similarity between sequences. a. Alignment of 111 bp chloroplast MatK gene fragment amplified from *A-1* template using primers Mat-K-F2 and Mat-K-R. GenBank accession numbers of the sequences included are as follows: *Origanum vulgare* AY840165; *Thymus serpyllum* AY840173; *Salvia splendens* AF477765; *Olea europaea* AJ429335; *Vitis vinifera* AJ429274. b. Alignment of 110 bp chloroplast MatK gene fragment amplified from *A-1* template using primers MatK-TO-F and MatK-TO-R. GenBank accession numbers of the sequences included in alignment are as follows: *Olea europaea* AJ429335; *Thymus serpyllum* AY840173; *Origanum vulgare* AY840165; *Salvia coccinea* AY840147; *Vitis vinifera* AJ429274. c. Alignment of 96 bp chloroplast Nadh gene fragment amplified from *A-2* using primers Nadh-M-F3 and Nadh-M-R3. GenBank accession numbers of the Nadh-gene sequences included in the alignment are as follows: *Pistacia lentiscus* (Mastic) DQ390463; *Pistacia vera* (Pistachio nut) AY677204; *Pistacia mexicana* DQ390464; *Olea europaea* AF027288; *Vitis vinifera* AJ429103.

results. Archaeologists and historians have assumed for several reasons that amphoras of this particular style from Chios usually carried wine. The Greek island of Chios was renowned in antiquity for its fine and distinctive vintages, and in the fifth and fourth centuries B.C., Chian coins depicted grapes dangling above an amphora very similar in style to our *A-1* (Fig. 4). The discovery of olive and oregano fragments in *A-1* demonstrates that Chian amphoras were not used as wine jars exclusively. Based on these results, we caution against further assumptions that unlined fourth century B.C. Chian amphoras contained wine. Additional investigations into the diversity of the island's agricultural production and trade should be considered. Since the shipwreck site contains more than 150 amphoras similar to *A-1*, a substantial part of the cargo could have contained olive products, most likely olive oil flavored with oregano and perhaps additional herbs.

A critical question is how genetic fragments from olive and oregano could have been preserved for centuries inside amphoras at the bottom of the sea. Previously performed experiments have demonstrated that when a dry amphora is filled with liquid, the amphora's interior wall can absorb as much as 1.1% of its total volume into its ceramic matrix (Pulak et al., 1987). Once this liquid has soaked into the amphora's walls, genetic material can be captured. The low water solubility of olive oil and its naturally high levels of antioxidants (e.g. hydrophilic phenols; α-tocopherol (E-vitamin); *a*, *b* chlorophylls; β-carotene (Andrikopoulos et al., 1989; Riley, 2002))

Fig. 4. An early fourth-century B.C. Chian coin with amphora and grapes in front of a seated sphinx. Image courtesy of the American Numismatic Society, 1967.152.452, Adra Newell bequest: AR tetradrachm, c. 400 BC.

could have inhibited complete degradation and lipid peroxidation. Herbal additives like oregano could have been mixed with the oil for flavor and preservation. Recent analyses have demonstrated that several different herbs inhibit oxidative processes (Martinez-Tome et al., 2001) and that oregano specifically can act as a powerful antimicrobial agent (Santoyo et al., 2006). Among the several herbs of the *Lamiaceae* family, oregano has specifically drawn the interest of many research groups as a very potent antioxidant within lipid systems. Fragments from contemporary ancient Greek texts describe olive oil, herbs and spices (such as oregano, thyme and cumin) as common ingredients in fourth century B.C. cuisine (Olson and Sens, 2000). Historians have credited the ancient Greeks with a deep knowledge of their natural resources, and it is likely that additives were combined with olive oil because of their positive flavoring and preservative properties. We conclude that under certain conditions it is possible for genetic material to remain inside the amphora walls even after >2000 years on the sea floor.

Our analyses of the second amphora, *A-2*, amplified no olive or oregano sequences but instead revealed fragments most similar to mastic (*Pistacia lentiscus*) and/or the pistachio nut (*P. vera*; Fig. 3c) when blasted to GenBank. The limited information from the short chloroplast DNA fragments obtained, a high sequence similarity between the species, and limited sequence information for *Pistacia* species in GenBank prevented us from conclusively determining which *Pistacia* species was detected. The targeted gene sequences for a third *Pistacia* species common in the area, *Pistacia terebinthu*s (terebinth) has not yet been sequenced and deposited to GenBank. It is possible that our sequences are not from mastic or pistachio but are instead from terebinth, a plant from which the ancient Greeks extracted a resin that was often used to coat the inside of amphoras before wine was added to them (Serpico, 2000).

We were unable to amplify any genetic fragments from the resin lump found at the bottom of this particular amphora. Since resin formation is a typical characteristic of both mastic and terebinth, it is not yet possible to determine conclusively what the jar once contained. The underlying reasons why DNA could not be extracted and/or amplified from the hardened lump of resin are unclear. We speculate that it is possible that the DNA may have been damaged and fragmented when the resin crystallized inside the amphora, preventing successful detection and amplification of genetic material when subjected to the analyses described here.

The *A-2* results are, however, intriguing. The southern portion of Chios island was the primary and perhaps sole source of the high-quality mastic in the ancient world, as first recorded by the Roman natural philosopher Pliny, circa 60 A.D. (Boardman, 1955, 1967). Modern scholars have hypothesized that mastic resin preserved the Chian wines and provided their distinctive flavor (Barron, 1986). If the resin in amphora *A-2* is indeed mastic, it is the first direct suggestion for mastic cultivation in the Classical era, moving the suspected origins of mastic production back in time at least 400 years. Because the source of mastic was so highly localized in the southern part of Chios Island, this suggests that this particular jar and by association the others in its class were produced either on the island itself or at a site nearby. Similarly to oregano, species in the *Pistacia* genus have been experimentally demonstrated to possess strong antioxidant and antimicrobial abilities, containing several different substances which prevent oxidation processes (Andrikopoulos et al., 2003) and microbial activity (Koutsoudaki et al., 2005). This could have facilitated preservation of the jars' ancient contents, as well as the genetic material within the amphora walls.

From our results we speculate that it is possible that *A-2* once contained either mastic or terebinth added for flavoring and preserving wine. Though we tested for grape (*Vitis vinifera*), we found no evidence for it in either amphora or in the resin template. However, grape DNA may today be undetected due to fermentation degradation, or a higher water solubility of wine compared with olive oil or resins such as mastic. In contrast, *Pistacia* DNA may persist in the amphora because the resin was in direct contact with the ceramic matrix and was retained there, or other processes. Identification of different *Pistacia* resins have in the past mainly relied on gas chromatography analyses, using the triterpenoid composition of hardened resin taken from archaeological artifacts (Stern et al., 2000, 2003; Serpico and White, 2000). We show that DNA analyses may reveal more specific details about the contents of amphoras and other archaeological materials. Because genetic fragments can be extracted from very small volumes of ceramic scrapings, the DNA analyses presented here provide archaeologists with an effective tool when template from artifacts is limited. These DNA analyses likewise are a very useful method for analyzing artifacts that cannot be damaged or destructively sampled. The presented results constitute a new step in the development of molecular markers for archaeological analyses, which may help increase understanding of early civilizations.

5. Conclusions

Our analyses are the first to demonstrate that ancient (>2000 year-old) deep water archaeological artifacts that contain no apparent physical residues can still retain genetic traces of their original contents. We show that these traces can be easily detected and amplified using modern DNA techniques. Previously, archaeologists and other scholars have relied on occasional physical residues found on land or under water to speculate on the original cargoes carried by ancient ships. Our method enables isolation and identification of genetic fragments trapped for thousands of years inside amphora walls. The results presented in this paper contribute definite evidence for Classical Greek commodity exchange and open new vistas for molecular archeological analyses. As investigations continue, these methods will provide fresh insights into the crops grown by early civilizations, the actual contents of the ancient Mediterranean shipwreck cargoes, and the functioning of protohistorical economic networks.

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