Journal of Archaeological Science 39 (2012) 389–398

Contents lists available at SciVerse ScienceDirect

Journal of Archaeological Science

journal homepage: http://www.elsevier.com/locate/jas

Aspects of ancient Greek trade re-evaluated with amphora DNA evidence

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ARTICLE INFO

Article history:
Received 29 March 2011
Received in revised form
19 September 2011
Accepted 23 September 2011

Keywords:
Greece
Amphora
Ancient DNA
Shipwreck
Olive
Wine
Trade

ABSTRACT

Ancient DNA trapped in the matrices of ceramic transport jars from Mediterranean shipwrecks can reveal the goods traded in the earliest markets. Scholars generally assume that the amphora cargoes of 5th–3rd century B.C. Greek shipwrecks contained wine, or to a much lesser extent olive oil. Remnant DNA inside empty amphoras allows us to test that assumption. We show that short ~100 nucleotides of ancient DNA can be isolated and analyzed from inside the empty jars from either small amounts of physical scrapings or material captured with non-destructive swabs. Our study material is previously inaccessible Classical/Hellenistic Greek shipwreck amphoras archived at the Ministry of Culture and Tourism Ephorate of Underwater Antiquities in Athens, Greece. Collected DNA samples reveal various combinations of olive, grape, *Lamiaceae* herbs (mint, rosemary, thyme, oregano, sage), juniper, and terebinth/mastic (genus *Pistacia*). General DNA targeting analyses also reveal the presence of pine (*Pinus*), and DNA from *Fabaceae* (Legume family); *Zingiberaceae* (Ginger family); and *Juglandaceae* (Walnut family). Our results demonstrate that amphoras were much more than wine containers. DNA shows that these transport jars contained a wide range of goods, bringing into question long-standing assumptions about amphora use in ancient Greece. Ancient DNA investigations open new research avenues, and will allow accurate reconstruction of ancient diet, medicinal compounds, value-added products, goods brought to market, and food preservation methods.

1. Introduction

The remains of shipwreck cargoes represent a major and underutilized source of information about early Mediterranean cultures and their economies. Often wrecks are marked by piles of amphoras, the ceramic jars used throughout antiquity for maritime transport of trade goods. Archaeologists and historians of Classical and Hellenistic Greece generally have assumed that amphoras carried wine, but they have had little direct evidence to support this contention. That situation is changing. Identification of amphora contents is now possible to the species level, even when the jars are empty, through trace DNA amplification. As molecular methods are accepted and more frequently applied to archaeological artifacts, it will be possible to reconstruct in minute detail not only the wine trade, but ancient agricultural production, diet, food preservation techniques, and medicines.

Amphoras from Classical and Hellenistic Greece have long been recognized as important sources of economic information, but their exact contents are seldom known (Garlan, 1983). Classical studies and numismatics offer only limited assistance, as extant ancient written references to amphora contents are few and general (Demosthenes, 1936; Yardeni, 1994) and iconography is circumstantial and difficult to interpret. Archaeological analyses of amphoras provide some additional hints. Exterior graffiti, dipitni, or stamps sometimes directly indicate the goods in the jar, but they appear on perhaps less than 10% of amphoras (Lang, 1976; Lawall, 2000, 2001). The interiors of some amphoras hold stains or small deposits that can be analyzed with chemical methods, but the information obtained is often inconclusive and always highly speculative (McGovern et al., 2009). In rare situations, amphoras excavated from archaeological sites occasionally contain identifiable remains: fish scales, bones, grape seeds, grain kernels, or resins (Carlson, 2003; Maniatis et al., 1984; Pulak et al., 1987; Ward Haldane, 1990). Apart from these special cases, the vast majority of amphoras appear to be completely empty when recovered, providing no physical residues of their contents. This lack of direct

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doi:10.1016/j.jas.2011.09.025

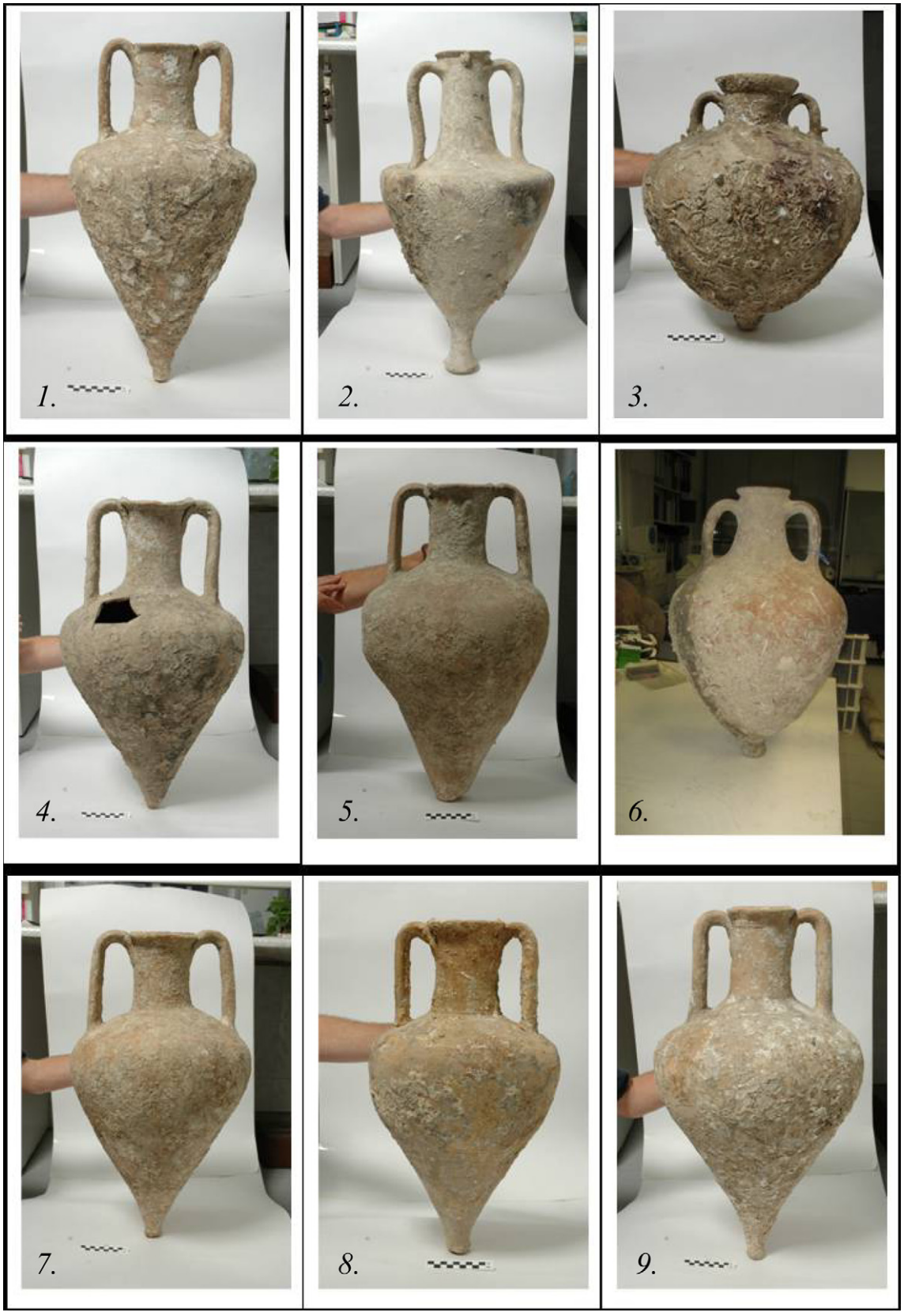

Fig. 1. Pictures of the amphoras used for the study (1–9). Names, ages and origins are listed in Table 1.

evidence of amphora contents has forced continued debate about ancient agricultural production and consumption, amphora use and reuse, and the scale of trade in wine, olive oil, and other commodities. As a result, scholars commonly assume that 5th–3rd century BC Greek amphoras carried wine, or to a lesser extent olive oil.[1]

To determine the original contents of ancient amphoras, we have developed sensitive molecular methods to extract and identify genetic material trapped in amphoras' ceramic matrices. We focus our inquiries on artifacts recovered from shipwrecks because they are regularly encountered intact, have been protected from DNA-destroying ultraviolet (UV) light while underwater, and are less likely to be contaminated with land plant DNA than those excavated from terrestrial contexts.

In our preliminary experiments, interior scrapings from two ~2400-year old shipwreck jars delivered ancient DNA from olive (*Olea*), oregano (*Origanum*), and *Pistacia* (Foley et al., 2009; Hansson and Foley, 2008). Recently we identified the *Pistacia* species in the unattributed jar from the Chios-Oinousses shipwreck; it is *Pistacia terebinthus*, terebinth. The DNA result of olive and oregano from the 4th century B.C. Chian amphora from that wreck is noteworthy, as Chian jars are assumed to have carried wine (Boardman, 1963; Boulter, 1953; Demesticha, 2011; Maniatis et al., 1984; Zimmerman Munn, 2003). These initial successes and the questions they raised prompted us to undertake further investigations of ancient DNA in amphoras.

The aim of this study is to determine prevalence primarily of olive and grape DNA in a random sample of Greek ancient amphoras. We run general searches for DNA inside amphoras and also search specifically for DNA from terebinth/mastic (*Pistacia*), Juniper (*Juniperus*), and species within the *Lamiaceae* family.

2. Materials and methods

For this study, the Hellenic Ministry of Culture and Tourism Ephorate of Underwater Antiquities granted access to its collection of amphoras in Athens, Greece. The study group is a random sample, not associated with any excavated site because the jars were inadvertently recovered from the sea floor in commercial fishing nets. Greek fishermen remitted the amphoras to the Ephorate at various times since 1993. The jars were not subjected to any conservation procedures, and have been archived away from UV light sources in windowless storage rooms.

From the Ephorate storerooms, we selected nine intact amphoras (see Fig. 1 and Table 1 for details) dating to the 5th–3rd centuries B.C. Like the vast majority of amphoras encountered, these jars bear no stamps or graffiti and appear completely empty. Resin linings were not apparent in any of them, though it is possible that such a lining once existed and dissolved away during centuries underwater. Seven of the amphoras were probably manufactured in ancient Corcyra (Göransson, 2007; Preka-Alexandri, 1992) on the island of Corfu (see Fig. 2 for detailed artifact illustration), are generally referred to as type 'Corinthian B',[2] and are invariably described as wine jars in the archaeological literature (Koehler, 1981; Kourkoumelis, 1998; Papadopoulos and Paspalas, 1999; Zimmerman Munn, 2003). The eighth amphora is from 4th century B.C. Mende, and the ninth jar is of an indeterminate Northern Aegean origin, but closely resembles 5th century B.C. jars from Mende. Except for ten Mendean amphoras recovered from the Tektas Burnu wreck containing pitch and beef bones, amphoras from that region are generally assumed to have carried wine (Carlson, 2003; Hadjidaki, 1996; Papadopoulos and Paspalas, 1999). To test the assumption that 5th–3rd century B.C. amphoras from Mende and Corcyra generally carried wine, we designed primers to amplify the DNA of grape and several other land plant species believed to be part of the ancient Greek diet.

Table 1
The specifics of the amphoras analyzed in the study.

Number	Ephorate-assigned artifact number	Origin	Approx. age	Received by ephorate
1	BE 93-161	Corcyra (*Corfu*)	½ 3rd c. B.C	1993
2	BE 95-46-9	Mende (*N. Aegean, Greek Mainland*)	½ 4th c. B.C.	1995
3	BE 96-17-5	Corcyra	½ 5th c. B.C.	1996
4	BE 96-17-1	Corcyra	½ 3rd c. B.C.	1996
5	BE 96-17-7	Corcyra	½ 3rd c. B.C.	1996
6	BE 95-7-1	Mende?	5th c. B.C.	1995
7	BE 96-17-6	Corcyra	½ 3rd c. B.C.	1996
8	BE 93-58	Corcyra	½ 3rd c. B.C.	1993
9	BE 94-27	Corcyra	½ 3rd c. B.C.	1994

2.1. Sample collection

We collected several samples from each amphora's interior surfaces. Using a sharp steel tool we scraped ceramic samples (~1 cm^3) directly into test tubes. To test non-destructive DNA collection methods, we also took samples using swabs (Copan Italia) saturated in lysis buffer (0.1 M Tris, 0.005 M EDTA, 0.2% SDS, 0.2 M NaCl, with pH 8.5). The swabs were brushed against the jars' inside walls adjacent to the scraped loci, and also in other areas of the jars. Swab samples were easier to collect from more areas than scrapings because less force needed to be applied to the sampling head, allowing more loci within the jar to be effectively sampled. Due to the nature of each method, swabbed samples were drawn from a larger surface area than the scraped samples. Upon use, the swabs were encapsulated in plastic tubes until analyzed at the molecular laboratory facilities at Lund University, Sweden. After collecting swabbed and scraped samples from the dry amphoras, we rinsed the amphoras with fresh water. We then repeated the sampling procedures to determine if DNA could be retrieved from water-saturated ceramic (Table 2).

[1] A literature review of journal articles concerning 5th–3rd century B.C. Greek amphora contents reveal that in the absence of physical remains within the jars (seeds, pits, nuts, resins, bones, etc.), archaeologists generally assume wine as contents. In 27 peer-reviewed articles drawn from 10 journals over the period 1946–2011, contents of 5860 amphoras are discussed. Of these amphoras, 5549 (95%) are stated to have carried wine. In the majority of these article the authors do not specify the number of amphoras under study, instead using terms such as "many", or "several tons" of sherds; the contents of these jars are described as "wine" or "chiefly wine", or occasionally "oil". See Supplementary Information. Occasionally, archaeologists refrain from guessing at contents. For instance, see Lawall, M.L. 1999. Studies in Hellenistic Ilion: The Lower City. The transport amphoras, *StTroica* 9, 187–224; and Garlan, Y. 2000. Amphores et timbres amphoriques grecs. Entre érudition et idéologie, *MémAcInscr* s. XXI. Paris.

[2] The most useful recent discussion of Corinthian B amphoras and their use is Göransson, K. 2007. *The Transport Amphorae from Euesperides: The Maritime Trade of a Cyrenaican City 400–250 BC*. Lund University.

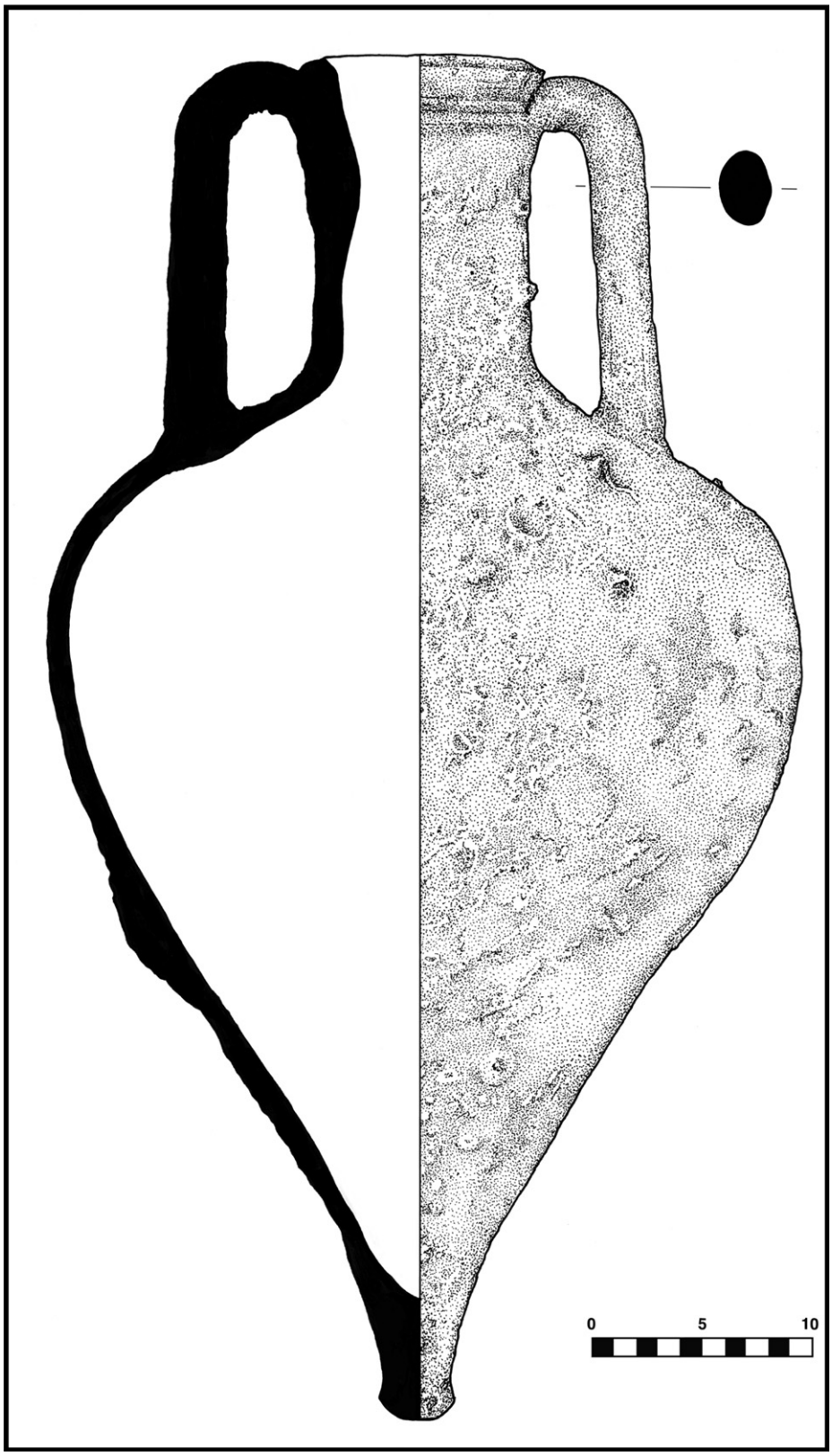

Fig. 2. Detailed drawing of Corcyran amphora BE 94-27 (Amphora nr 9). Black and white scale is 10 cm.

Table 2
Samples taken from analyzed amphoras: name; if the sample was from scrapings (SC) or a swab (SW) soaked in lysis buffer; if the sample was collected before (BR) or after (AR) the amphora was rinsed with water; where the sampling took place (Sides: S; Bottom: B) inside the amphora.

Amphora	Our numbering	Scraping/ swab	Before/after rinse	Side/base
1. BE 93-161	1.1	SC	BR	S
	1.2	SC	AR	S
	1.3	SW	BR	B
	1.4	SW	BR	S
	1.5	SW	AR	S
2. BE 95-46-9	2.1	SC	BR	B
	2.2	SC	AR	S
	2.3	SW	BR	S
	2.4	SW	BR	B
	2.5	SW	AR	B
3. BE 96-17-5	3.1	SC	BR	S
	3.2	SC	AR	S
	3.3	SW	BR	S
	3.4	SW	BR	B
	3.5	SW	AR	S
4. BE 96-17-1	4.1	SC	BR	S
	4.2	SC	AR	S
	4.3	SW	BR	S
	4.4	SW	BR	B
	4.5	SW	AR	S
5. BE 96-17-7	5.1	SC	BR	S
	5.2	SC	AR	S
	5.3	SW	BR	S
	5.4	SW	BR	B
	5.5	SW	AR	S
6. BE 95-7-1	6.1	SC	BR	S
	6.2	SC	AR	S
	6.3	SW	BR	S
	6.4	SW	BR	B
	6.5	SW	AR	S
7. BE 96-17-6	7.1	SC	BR	S
	7.2	SC	AR	S
	7.3	SW	BR	S
	7.4	SW	BR	B
	7.5	SW	AR	S
8. BE 93-58	8.1	SC	BR	S
	8.2	SC	AR	S
	8.3	SW	BR	S
	8.4	SW	BR	B
	8.5	SW	AR	S
9. BE 94-27	9.1	SC	BR	S
	9.2	SC	AR	S
	9.3	SW	BR	S
	9.4	SW	BR	B
	9.5	SW	AR	S

2.2. Molecular DNA analyses

We performed DNA extraction at the Department of Biology, Lund University, Sweden. To minimize risk of DNA contamination from the surroundings, we treated all lab benches with 99% ethanol and UV light before handling samples, and used new pipettors and filter tips. We added 800 μ lysis buffer to the test tubes containing scrapings. For the swabbed samples, we cut off the tips of the swab sticks with sterilized, UV-treated scissors and placed the untouched tips into 1.5 mL test tubes containing 800 μ of lysis buffer. We added Proteinase K (Fermentas, Sweden) to all tubes and placed them in a 56 °C waterbath for three hours. We then performed extraction steps as described in Hansson and Foley (2008). We dissolved all samples in 50 μL 1×Tris-EDTA, pH 8.0.

2.2.1. Modern DNA contamination addressed

We avoided signal interference from marine algae by designing primers specific only for land plant chloroplast DNA. The chloroplast is a cellular organelle that contains its own separate nucleus with DNA. Chloroplasts are found in multiple copies inside plant cells and other eukaryotic organisms that conduct photosynthesis. Initially we designed primers to target fragments from 96 to ~500 nucleotides. Test runs showed that no amplifications took place with primers targeting DNA fragments longer than 200 nucleotides. Consequently, we used primers targeting sequences with fewer than 200 nucleotides for all subsequent analyses.

Because our test runs amplified no long nucleotide fragments, we concluded that modern DNA contamination in these amphoras was unlikely. Furthermore, we believe the mechanism for DNA entrapment in the ceramic matrix relies on liquid or semi-liquid introduction into a dry jar, with subsequent absorption of DNA-carrying liquid into the walls of the jar. This situation was not presented to the study amphoras after their recovery from the sea floor until we saturated the jars for our final DNA retrieval tests, reducing the probability of modern DNA contamination.

2.2.2. Primer design and limitations

Primers were designed to target areas of high variability between species, while taking into account the length limitations presented by degraded ancient DNA: typically short strands of <200 base pairs. Primers also had to have matching melting points (as this increases specificity), while avoiding primer–dimer formation and secondary structures. All these factors greatly limit sites where primers can be placed in order to perform useful ancient DNA analyses.

2.2.3. Methods

We designed several species/genus specific primers (Table 3) from various land plant chloroplast DNA sequences publicly available through the Entrez Nucleotide database (www.ncbi.nlm.nih.gov). The targets for this study were species/genera expected to be part of early Greek diet and trade: olive, juniper, mastic/terebinth and species within *Lamiaceae*. The *Lamiaceae* primers target DNA from species belonging to *Salvia* (sage), *Thymus* (thyme), *Mentha* (mint), *Origanum* (oregano), and *Rosmarinus* (rosemary). We made use of four chloroplast genes Maturase K, Nadh, the tRNA-Leu and trnL-trnF intergenic spacer (*trnL-trnF*), and the ribulose-1,5-bisphosphate carboxylase large subunit gene (*rbcL*). We also designed generic land plant chloroplast DNA primer pairs in the trnL-trnF and rbcL genes.

We ran polymerase chain reactions (PCR) with Titanium Taq polymerase (Clontech, Takara Bio Europe) in 25 μl reactions. PCR conditions were as follows: 95 °C for 1 min; 45 cycles of: 95 °C for 10 s.; primer specific annealing temperature (see Table 1) for 10 s.; 68 °C for 20 s, ending with a 3 min step at 68 °C and 5 min at 72 °C. We included a blank containing no template in all PCRs to control for contamination. PCR amplification success was analyzed by electrophoresis on a GelRed (Biotium Inc.) stained 3.5% agarose (Invitrogen, Life Technologies) gels together with a 1 kb DNA ladder (Invitrogen) for sizing the length of the fragments.

Two PCR products showing amplification of the correct sequence size on the gels were directly sequenced in both directions. The sequencing reaction was performed with an ABI PRISM BigDye® Terminator V3.1 sequencing kit (Applied Biosystems), followed by analysis on an ABI3100 instrument (Applied Biosystems). Each BigDye reaction contained 2 μL purified PCR product, 1 μL BigDye terminator, 1.5 μL 5× sequencing buffer and 1 μL of 4 mM primer in a final volume of 10 μL, which was adjusted with MilliQ water (Millipore). The following cycling conditions were used in the BigDye sequencing reaction (run on PCR

Table 3
The plant genera and genes targeted in the PCRs; length of fragments amplified; the primer sequences and the annealing temperatures used in PCR analyses.

Targeted plant genus/genera/family	Targeted gene	Fragment size (bp)	Primer names	Primer sequences (5′ → 3′)	PCR annealing temp. (°C)
Land plants, general	trnL-trnF	Variable (~100)	trnL-F trnL-R	GCAATCCTGAGCCAAATCCT TGTTAGAACAGCTTCCATTGAGTC	62
Land plants, general	rbcL	122	rbcL-F rbcL-R	ATGCATGCAGTTATTGAYAGACA CAAGTTTACCTACTACRGTACC	62
Pistacia	ndhF	96	Ndh-F Ndh-R	TTGGAACATACTGAATTTAGTTG GCACGATTATATGACCAATC	57
Thymus, Origanum	MaturaseK	111	MatKTOF MatKTOR	CGCAACAAGACTTGTATTCTC TATCTTGTTCTTTTTCGCTTCT	56
Thymus	trnL-trnF	188	trnL-Th-F trnL-Th-R	AATCTTTCCATCGAAACTTTAT CGGATTATGGAGTTAATGAGT	57
Origanum	trnL-trnF	181	trnL-Or-F trnL-Or-R	CCATGGAAACTTTCTAAAGG TCGGACTATGGAGTTAATAAGTT	57
Olea	MaturaseK	114	MatKOF MatKR5	TCACATTTAAATTTTGTGTTAGAT TCGTAATAAATGCAAAGAAGAG	58
Vitis	trnL-trnF	128	V-trnL-F V-trnL-R	GAGTTGACTATGTTGCGTTGTTA AGATTCGAGCCATCATTAATCAT	60
Lamiaceae	trnL-trnF	118	L-trnL-F L-trnL-R	TCCGATAGATCTTTTAAAGAACT CGACGGATTTTCCTCTTAC	56
Juniperus	rbcL	126	J-rbcL-F J-rbcL-R	ATGGTCGTCCTTTGTTGGG TCACGTTTTCATCATCCTTGG	62

machine): 1 min at 96 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. After the BigDye reaction, the products were purified with NaAc/EDTA/ethanol (BigDye Terminator Cycle Sequencing Kit v.3.0 manual). After the ABI3100 instrument sequencing reading confirmed that the correct product had been amplified, we assumed that amplified products of the same size from the remaining amphora templates had amplified the same product. If a product was amplified and showed a distinct band on a gel after electrophoresis, we concluded that the amphora contained DNA from the plant targeted by the primers. All species-specific PCRs were run twice to ensure accuracy of the amplification.

PCR with general primers (trnL-Fw/trnL-Rv and rbcL-Fw/rbcL-Rv) amplified products from all samples (confirmed by agarose gel analysis). We pooled products amplified from scrapings and swabs taken from the same amphora into one test tube and used the combination in cloning reactions (TOPO TA, Invitrogen). Several clones were sequenced from each of the PCRs using the cloning vector M13 Forward primer (5′-GTAAAACGACGGCCAG-3′). Sequences were submitted to NCBI Blast (http://blast.ncbi.nlm.nih.gov) and assigned to a plant species/genus /family.

3. Results

Results from the PCRs with general primers are listed in Table 4. The general primer pairs were good indicators of plant families, but in many cases the sequences were too short to allow identification of the genus or species. The high variability of the tRNA-Leu and trnL-trnF intergenic spacer in the chloroplast, a region where both species and in particular genera differ in length, allowed several identifications to genus level. By comparison, the *rbcL* gene is less variable and has no length polymorphism. Accordingly, those sequences could rarely be identified beyond the family level. Another limiting factor was that some plant families still have few species represented in the Entrez Nucleotide database. This prevented identification of several sequences. Since most common food species are represented in the database already, it suggests that the unknown DNA sequences identified with general plant primers did not originate from families or genera of anticipated food products. The general primers were designed to target a very conserved region in plants. It is possible that DNA fragments of high-interest species may be lost/not identified during the amplification process if they were present only in low copies (e.g. overwhelmed by DNA from high-copy DNA). Accordingly, general primer analysis is useful for quick screening purposes but should be followed by species-specific primer analyses.

Results from the PCR amplification with the species/genera-specific primers are listed in Table 5. More PCR products were amplified from the swabs (43 DNA "hits" from 27 swabbed samples) than from the ceramic scrapings (16 DNA "hits" from 18 scraped samples); the non-destructive sample collection method is superior to the invasive method. Some differences exist in results among samples collected from different loci within the same amphora, suggesting that DNA distribution can vary within an artifact. The species-specific identification from swabs and scrapings is robust, shown by consistent PCR amplification of products when analyses were repeated. No amplifications took place in the blanks (no template control), indicating that no contamination occurred in the laboratory.

4. Discussion

Our results show that ancient DNA from original cargo contents can be collected non-destructively from empty amphoras retrieved from the sea floor. We further demonstrate that amphoras contained a multitude of products, especially olive products (possibly oil) and herbal additives. Olive DNA is present in six of nine jars (nos. 1,2,5,6,7,9) (67%). Grape DNA appears in five of nine amphoras (nos. 1,5,7,8,9) (55%), and only weakly in three of those five (nos. 5,8,9) (Fig. 3). This is a surprising result given assumptions about the ancient wine trade. The genus most often detected in these amphoras is *Juniperus*, appearing in eight of the nine jars (nos. 1,2,3,4,5,6,7,9) (89%) (Fig. 4). The mixtures of species trapped in the amphora walls may indicate initial one-time transport of diverse food products in amphoras, *and/or* reuse of these transport jars to carry various commodities.

Table 4
Identification by NCBI Blast (Basic Local Alignment Search Tool) of DNA sequences isolated and amplified from analyzed amphoras. The sequences were amplified by PCR using primer pairs that were designed to be general for most land plants (targeting the tRNA-Leu and trnL-trnF intergenic spacer as well as the ribulose-1,5-bisphosphate carboxylase large subunit gene – *rbcL* – in the chloroplast). Resulting PCR products were separated by cloning and individual clones were sequenced to identify a species, genus or family. From the trnL-Fw/trnL-Rv PCR we analyzed 10–20 sequences in clones; from the rbcL-Fw/rbcL-Rv PCR we analyzed 5–11 clones (number of clones analyzed listed in parenthesis). If a clone (sequence) matched more than one genus, the family is listed. *Spp* = species.

Amphora	DNA fragments (~100 bp) amplified with general plant primers *trnL-Fw/trnL-Rv*	DNA fragments (122 bp) amplified with general plant primers *rbcL-Fw/rbcL-Rv*
1. BE 93-161	- *Solanaceae (6)* - *Juglandaceae (Walnut family) (2)* - *Oleaceae (Olive family) (1)* - *Platanaceae (Plane-tree family) (1)* - *No exact match/unknown sequences (3)*	- *Lamiaceae: Thymus or Salvia (1)* - *Fabaceae (Legume family) (1)* - *Salicaceae (1)* - *Sequences similar to multiple families (3)*
2. BE 95-46-9	- *Fabaceae (Legume family) (5)* - *Zingiberaceae (Ginger family) (5)* - *Rutaceae (Citrus family) (1)* - *No exact match/unknown sequences (6)*	- *Cupressaceae (Cypress family) (1)* - *Fabaceae (Legume family) (1)* - *Fagaceae (Beech family) (1)* - *Sequences similar to multiple families (8)*
3. BE 96-17-5	- *Plantago spp. (3)* - *Zingiberaceae (Ginger family) (11)* - *No exact match/unknown sequences (3)*	- *Pinus spp. (2)* - *Lamiaceae (Mint family) (3)* - *Sequence similar to multiple families (1)*
4. BE 96-17-1	- *Fagaceae (Beech family) (7)* - *Rutaceae (Citrus family) (4)* - *No exact match/unknown sequences (4)*	- *Pinus spp. (2)* - *Cupressaceae (Cypress family) (2)* - *Sequences similar to multiple families (4)*
5. BE 96-17-7	- *Quercus spp. (7)* - *Gesneriaceae (6)* - *Fabaceae (Legume family) (1)* - *No exact match/unknown sequences (5)*	- *Cupressaceae (Cypress family) (2)* - *Sequences similar to multiple families (3)*
6. BE 95-7-1	- *Musaceae (10)* - *Betulaeae (Birch family) (7)* - *Zingiberaceae (Ginger family) (4)* - *Apiaceae (3)* - *Gesneriaceae (3)*	- *Pinus spp. (6)* - *Sequences similar to multiple families (8)*
7. BE 96-17-6	- *Pinus spp. (10)* - *Solanaceae family (10)*	- *Sequences similar to multiple families (13)*
8. BE 93-58	- *Fabaceae (Legume family) (5)* - *Fagaceae (Beech family) (5)*	- *Sequences similar to multiple families (8)*
9. BE 94-27	- *Pistacia spp. (1)* - *Solanaceae (8)* - *Platanaceae (Plane-tree family) (3)* - *Apiaceae (1)* - *Brassicaceae (Cabbage/mustard family) (1)* - *Fagaceae (Beech family) (1)*	- *Sequences similar to multiple families (14)*

Even after decades above-water, enough ancient DNA persists in the ceramic matrices of >2000 year old shipwreck amphoras to allow identification of their original contents. All the DNA fragments identified in the study amphoras were very short, about 100 base pairs (bp), suggesting an ancient origin. If the DNA fragments originated from modern plants as "contamination", amplifications of chloroplast gene fragments would have been longer than 200 bp.

We improved our DNA capture techniques compared to our first study by applying a minimally invasive swabbing method. This was first suggested to us by the Massachusetts State Police Crime Lab's Technical Manager for Forensic Biology, based on forensic methods for crime scene trace DNA collection. We gathered from each of the nine test amphoras two physical scrapings as well as three or four samples from swabs saturated with lysis buffer. The physical scrapings were necessary for accurate comparisons of DNA results between the two methods. Each of the samples was obtained from different loci within each jar to maximize chances of finding and capturing ancient DNA. Our results show that rubbing swabs soaked in lysis buffer across the inner amphora surfaces traps enough ancient DNA fragments for molecular analyses. Delicate artifacts can be tested for DNA without any harm to the objects.

We also tested if wet ceramic would relinquish DNA when swabbed. While more DNA was detected in swabs from dry rather than wet jars, some positive results swabbing wet jars (5 of 9 swabs (55%) taken from wet jars delivered 9 DNA "hits") indicate that DNA samples may be collected from amphoras immediately upon retrieval from underwater sites. A direction for future research will be to test the possibility of collecting DNA samples from amphoras in situ, without recovering the artifacts.

We conducted the molecular PCR analyses in two stages: first, with general land plant chloroplast primers; and second, with species-specific primers targeting a single species or genus. Analyses from general primers (trnL-trnF and rbcL genes) and sequencing of random clones revealed DNA from several high-interest plant families: *Fabaceae* (Legume family); *Zingiberaceae* (Ginger family); *Juglandaceae* (Walnut family). With the general primers it was not possible to determine the exact species represented by this DNA, but legumes, ginger, and walnuts or walnut oil probably were transported across the Mediterranean. We speculate that these ingredients and other species yet undiscovered by DNA tests (meat or fish, for instance) were perhaps suspended and preserved in olive oil with herbal and resin additives.

Amplifications and sequencing of clones containing sequences from the rbcL-general primer PCRs in most cases did not conclusively identify the products' families; the sequence variation was too limited in the short fragments. However, one clone from Amphora 1 specifically matched the DNA of either thyme or sage. Pine (*Pinus*) DNA sequences found in Amphoras 3, 4, 6 indicate that these jars once contained pine products: for example, pine resin. We did not screen amphoras specifically for pine DNA due to

Table 5
Listing of PCR amplification of DNA using species/genera-specific primers. If a fragment was amplified from a sample it is represented by *x* in the table. Amphora sample numbers represent if they were taken as physical scrapings (starting with 1.1 going up to 9.2) or as swabs soaked in lysis buffer (10.1 up to 18.3).

AMPHORA	SAMPLE	*Olea*	*Vitis*	*Pistacia*	*Juniperus*	*Lamiaceae*	*Thymus/ Origanum/ Mentha*	*Thymus*	*Origanum*
1. BE-93-161	1.1	x			x				
	1.2								
	1.3	x	x		x			x	
	1.4	x	x	x	x	x	x		
	1.5	x			x				
2. BE-95-46-9	2.1	x			x				
	2.2								
	2.3	x			x				
	2.4	x		x	x				
	2.5								
3. BE 96-17-5	3.1			x					
	3.2								
	3.3								
	3.4				x	x			
	3.5								
4. BE 96-17-1	4.1								
	4.2								
	4.3				x				
	4.4				x		x		
	4.5								x
5. BE-96-17-7	5.1		x			x			
	5.2	x							
	5.3				x		x		
	5.4	x			x		x		
	5.5				x				
6. BE 95-7-1	6.1	x			x				
	6.2								
	6.3								
	6.4	x							
	6.5								
7. BE 96-17-6	7.1		x						
	7.2		x	x					
	7.3								
	7.4				x				
	7.5	x		x					
8. BE 93-58	8.1								
	8.2								
	8.3								
	8.4		x	x					
	8.5								
9. BE 94-27	9.1	x			x				
	9.2	x							
	9.3						x		
	9.4	x		x		x	x		
	9.5	x	x				x		
Targeted Chloroplast Gene		*MaturaseK*	*trnL*	*Nadh*	*rbcL*	*MaturaseK*	*MaturaseK*	*trnL*	*trnL*
Length of DNA Fragment (Nucleotides)		*114*	*128*	*96*	*126*	*118*	*111*	*188*	*181*

difficulties with designing species-specific primers, but it is likely that pine resin was commonly used in ancient Greece for sealing and lining amphoras.

Results from species-specific analyses strongly suggest that olive, possibly olive oil, was a major component in the contents of at least five, possibly six, of the nine amphoras studied. We detected olive DNA in swabs and scrapings from Amphoras 1,2,5,6,9. In all of these jars, olive DNA appears together with DNA from several other plant species and genera. We detected grape (*Vitis*) DNA in five amphoras: 1,5,7,8,9; juniper DNA in eight amphoras: 1,2,3,4,5,6,7,9; mastic/terebinth (*Pistacia*) DNA from six amphoras: 1,2,3,7,8,9; and finally *Lamiaceae* species (*Salvia*, sage; *Thymus*, thyme; *Mentha*, mint; *Origanum*, oregano; *Rosmarinus*, rosemary) from five amphoras: 1,3,4,5,9 (Table 5 and Figs. 3 and 4).

Ancient DNA consists of short fragments, and this restricts our ability to identify some sequences to the species level. For instance, chloroplast sequences resulting from PCR using the primers specific for terebinth/mastic were identical to each other. The same problem exists for sequences from thyme/oregano/mint. In another case, only amphora 4 tested positive when we targeted an oregano DNA fragment of 181 bp (the trnL gene) (Table 5). Similarly, only samples from amphora 1 amplified a 188 bp thyme DNA fragment (trnL gene). By comparison, the 111 bp fragment specific for thyme/oregano/mint (MaturaseK gene) readily amplified DNA from amphoras 1,4,5,9. Overall, we are satisfied with genus-level and even family-level (*Lamiaceae*) DNA identifications for the purposes of this study. We discovered that in many cases it was impossible to design primers specific for only one species while keeping the

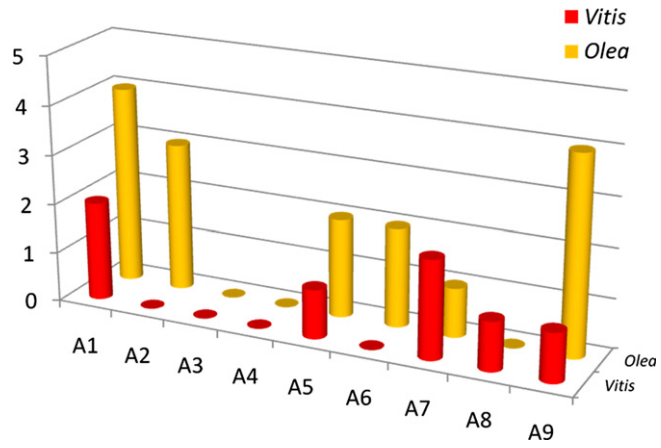

Fig. 3. Prevalence of *Olea* and *Vitis* in samples taken from the nine study amphoras.

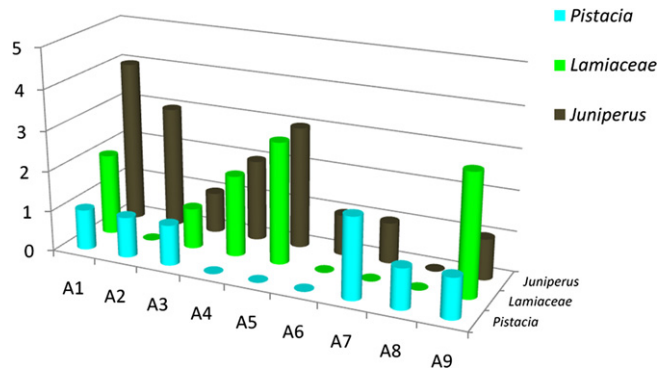

Fig. 4. Prevalence of *Pistacia*, *Lamiacea*, and *Juniperus* in samples taken from the nine study amphoras.

fragment lengths to ~100 bp (primers could e.g. amplify multiple unspecific fragments). This is mostly attributable to the very few nucleotide differences in closely related species' DNA sequences. However, we can state with certainty that the amphoras contained one or another of each of the similar herbal species. In future studies of artifacts from significant wreck sites, additional resources will be directed toward developing precise species-level identifications.

For unknown reasons the DNA signals from Amphoras 3, 4, and 8 were weak compared to the other jars in the study. It is possible that the sizeable hole in the shoulder of Amphora 4 (see Fig. 1) may have allowed ultraviolet light penetration into the interior of the jar, resulting in DNA degradation.[3] Amphora 8, a 3rd century B.C. jar from Corcyra, delivered single DNA signals for only two plants: grape and mastic/terebinth. If ancient wine was composed of fermented juice from grapes preserved with resin, then among this study group the contents of amphora 8 as revealed by DNA correspond most closely to wine.

Not all species/genera/families of the specifically targeted species were detected with the first round of general primers. That is not unexpected. Multiple simultaneous amplification of DNA within the same PCR leaves much lower copies of some fragments than others. This directly and negatively affects probability of insertion into a cloning vector and then isolation-sequencing. The dominant fragments in the reaction are more likely to be identified during sequencing. It is also likely that the number of isolated ancient DNA fragments is very low in the starting template. This could explain why the same products are not amplified in all samples taken from the same amphora.

We conclude that general primers are useful for a first-order screening, but are better used in combination in PCRs with species/genus-specific primers. Also, cloning is expensive and time consuming compared to the direct analysis approach where the specific PCRs give instant results from agarose gels with size markers. With this method only single PCR products need to be sequenced for verification purposes.

[3] Factors affecting DNA survival and distribution within an amphora may include the orientation of the jar on the sea floor, its degree of burial in sea floor sediments, the depth of water at the wreck site, sustained flow of water through the artifact while on the sea floor, and differential interior exposure to ultraviolet light after recovery. In addition, we speculate that conservation practices such as desalination may wash DNA from the matrix. While the jars in this study were not subjected to desalination, it is a concern that we will address in future experiments.

5. Conclusion

The amphoras analyzed here originate from Greece in the 5th–3rd century B.C. This was an era of increasing economic prosperity across the Hellenic world, partly due to flourishing maritime trade throughout the Mediterranean and Black Seas (Burke, 1992). This trade contributed to rising nutritional standards in mainland Greece and its colonies, which in turn stimulated population growth (Bitros and Karayiannis, 2010; Keenleyside et al., 2006; Scheidel, 2010; Waterlow, 1989). Reconstructing ancient diets would provide insights into the development of the economy through trade. However, archaeological evidence is scant due to the lack of physical remains of ancient foodstuffs (Dalby, 1996). Scholars instead have largely relied upon written sources. For example, Plato (5th–4th century BC) describes olive oil, cereals, legumes, fruits, meat, fish, wine in his texts as common food products, while fragments from Archestratos' 4th century B.C. poem describe various sea food preparations (Olson and Sens, 2003; Skiadas and Lascaratos, 2001). DNA investigations of amphoras provide a bounty of hard data to reconstruct ancient diet and trade.

All analyzed jars in this study contain products from more than one plant species, indicating contents beyond unadulterated wine or pure olive oil. This raises several possibilities relating to amphora use in 5th–3rd century Greece. One possibility is that in their first use, these jars carried the single-species commodity olive oil or dual-species resinated wine (grape and *Pistacia* such as amphora 8, or grape and some other resin-producing species such as those in the *Pinus* genus), but later the amphoras were re-used for shipping different goods.

The second possibility is that the jars were one-time-use carriers for olive oil or wine, but that these products were more complex than previously imagined. If these jars contained oil, it was not a single-species commodity (i.e.: olive DNA only), but was mixed with other species. Herbal additives would have improved flavor and promoted preservation. Likewise, if the five jars containing grape DNA were used one time only to carry wine, then it appears that it was flavored and preserved with herbal additives prior to shipping (amphora 8 is an exception, containing only grape and *Pistacia* and no other species). Spices such as mint, thyme, oregano, rosemary, sage and juniper have strong antioxidant, antibacterial, and antifungal properties and would have protected amphora contents from spoilage during transit and storage. As an added hidden benefit to consumers, plant cells also contain ubiquitous polyphenolic phytochemicals, a diverse class of secondary metabolites, which provide antioxidant protection against cell-damaging free radicals (Stevenson and Hurst, 2007). A problem

that remains for DNA studies of amphora contents is disambiguating first-use and secondary-use of the jars.

A third possibility is that Greek amphoras transported a very wide range of goods that can now be brought into view through remnant DNA studies. Grape DNA may reflect wine, or it could indicate whole grapes or vinegar as ingredients in multi-species recipes.

A combination of these scenarios is probable, with amphoras carrying complex mixes of ingredients and being re-used on multiple trading voyages. Assuming that the jars selected for this study are not anomalous among the millions of amphoras that circulated in the ancient Mediterranean, these results may encourage broader thinking about trade in agricultural products in Classical and Hellenistic Greece. Archaeologists should not assume that amphoras from that culture were merely wine jars. Instead, amphoras appear to have been general-purpose containers for diverse foodstuffs and value-added products. The picture emerging from amphora DNA studies is of an early market featuring a wide range of complex value-added agricultural products.

Acknowledgments

We thank The Royal Physiographic Society in Lund, Sweden; program benefactor the Swordspoint Foundation; program sponsors Jane and James Orr, and Joan and Joseph Patton for providing funding for this project. We thank Joanne Sgueglia, Technical Manager for Forensic Biology at the Massachusetts State Police Crime Laboratory for suggesting the swab method and sharing her knowledge of trace DNA capture and investigation. We acknowledge the kind permission to study the Ministry of Culture and Tourism amphora collection granted by Ephorate of Underwater Antiquities Directors Calliopi Preka-Alexandri and Aggeliki Simossi.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jas.2011.09.025.

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