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ORIGINAL ARTICLE

Exploring prehistoric plant use by molecular analyses of Neolithic grave goods

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Abstract

At the site of Grotta Mora Cavorso (Lazio, Italy), an unusual archaeological find, made of two coarse pottery vessels, was recovered from burial levels radiocarbon dated to 6,405–6,275 BP. These artefacts were analysed using several methods, for interpretation of the cultural practices of the earliest inhabitants in central Italy. This first molecular evidence about the potential processing and storage of poppy-based products in Neolithic pottery was obtained by detecting ancient DNA (aDNA) and chemical compounds. This study represents the second evidence from the Mediterranean area of the use of *Papaver* L. (poppies), although the actual use(s) of these plants then, for example as sedatives, drugs, or food, remains uncertain. Also, the employment of *Olea europaea* L. (olive) derivatives in foods or for other purposes was suggested, in agreement with the recovery of fruit stones at the site. The results of the present archaeobotanical investigation show the environmental knowledge of the first prehistoric communities living in central Italy, who might have shared their ethnobotanical practices.

Keywords Pottery · Ancient DNA · DNA barcoding · *Olea europaea* · *Papaver* sp. · Prehistory · Secondary metabolites

Introduction

The arrival of farming in the central and western Mediterranean area and the possible medicinal or ritual uses of plants are key issues of European prehistory; however, these topics are still relatively poorly understood, especially the level of interaction between different groups of people and diversity in the subsistence economy. In this context, ancient pottery surely represents an interesting source of data, and finding a way to successfully analyse this archaeological record allows scientists to provide important insights about past cultural traditions.

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Shape, archaeological context and association with other items are the main elements to suggest the uses of pottery (Evershed 1993; Eerkens 2007; Radford 2019). However, in the last few decades, new analytical approaches such as the use of chromatography for studying the organic residues preserved in archaeological artefacts have provided data on human activities as quantitative and qualitative results (Evershed 1993; Eerkens 2007; McGovern and Hall 2016; Dunne et al. 2017; Luong et al. 2017, 2018; Roffet-Salque et al. 2017; Smith et al. 2018; Radford 2019; Demirci et al. 2020; Pecci et al. 2020). One of the most critical issues in such types of investigation is the degradation process which affects biomolecules (Whelton et al. 2021). Chemical and physical factors of the depositional environment may affect the organic residues in different ways. Among all organic compounds, lipids are the most common to be found, due to their great resistance to deterioration. However, the study of fatty acids provides a limited amount of information and their level of identification is usually restricted (Evershed 1993; Eerkens 2007; Radford 2019).

The potential of genetic analyses for solving long-standing questions in archaeology is increasingly being used. Ancient DNA (henceforth aDNA) has been found to be well preserved in ancient remains and on artefacts, including stone tools and surfaces of unglazed pottery (Pääbo 1989;

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Hofreiter et al. 2001; Shanks et al. 2005; McGovern and Hall 2016). Pottery, in particular, can absorb compounds and then protect them from outside contamination (Evershed 1993; Eerkens 2007; Hansson and Foley 2008; Foley et al. 2012; Robinson et al. 2017; Radford 2019).

To date, no research has been done on plant aDNA extracted from early Neolithic pottery assemblages from the Mediterranean area, in contrast to more recent periods (Hansson and Foley 2008; Foley et al. 2012). On the other hand, several studies have been carried out on other ancient biomolecules, mainly lipids and proteins, isolated from similar materials (Dunne et al. 2017; Chowdhury et al. 2021; Drieu et al. 2021; Tanasi et al. 2021). The present contribution attempts to address this gap by investigating the prehistoric pottery recovered from Grotta Mora Cavorso (Jenne, central Italy). This archaeological site, at UTM coordinates (ED50) 33 T UG (03)48570(46)38010 and 715 m a.s.l., is a multi-tunnel karst cave system, located above the upper valley of the river Aniene in south-eastern Lazio. The complex stratigraphy of the site spans from the Late Pleistocene up to the present (Rolfo et al. 2009, 2016; Achino et al. 2016). The multidisciplinary data obtained from Grotta Mora Cavorso have been published in Silvestri et al. (2020), for overall information about this fascinating cave. It was on an important route between the Adriatic and Tyrrhenian coasts of Italy and has one of the most important early Neolithic burial deposits in Mediterranean Europe (Rolfo et al. 2016). The cave was considered as a sacred environment connected with the underworld; indeed, in the inner chambers, human skeletons have been found together with animal bones and pottery, which have been interpreted as grave goods (Fig. 1; Silvestri et al. 2020). Among the latter, fragments of an ovoid vessel and a hemispherical bowl were selected and analysed to obtain any clues about the plants used by the people living around the cave (Fig. 1c). This research succeeded in isolating plant aDNA from the powdered pottery, while the chromatography results support this genetic evidence.

The data obtained from these potsherds provides some information about the cultural practices and living environments of the Neolithic people in central Italy, knowledge not previously obtained from archaeobotanical or anthropological analyses (Gismondi et al. 2012; Scorrano et al. 2019; D'Agostino et al. 2022).

Materials and methods

Archaeological artefacts and sampling procedures

The archaeological pottery investigated in this study was dated to the early Neolithic (Table 1). It was found in a layer below an upper flowstone (a sheet of carbonaceous mineral deposited by flowing water), on the surface of the burial together with bones, charcoal and ash, above a lower flowstone. One of the ancient potsherds, named vessel W, was found in the upper chamber of the cave nearby articulated human bones, probably an intentional burial, and a polished sub-triangular green stone axe significantly next to the right forearm of a skeleton. Vessel W was an ovoid pot, decorated with thin engraved triangles filled with small impressed points, made up of a semi coarse impasto (thickly applied), and with brownish internal and external surfaces. The second vessel, V, was a hemispherical bowl in coarse impasto, with reddish brown internal and external surfaces. It was discovered in the lower chamber of the cave, broken and chaotically piled together with hundreds of human bone fragments. The potsherds were not washed before sampling, to avoid the introduction of any contaminant from outside or the loss of any constituents of the absorbed residues. After choosing several sampling points on each potsherd, such as base, body and rim (Fig. 1c), the corresponding internal surfaces were mechanically cleaned with an electric drill equipped with sterile single-use tips (about 2 mm) and then ground to powder (~1 g per each sampling point), for genetic and gas chromatographic mass spectrometry (GC–MS) analyses. Two samples were also taken from the external surfaces of each potsherd, to test the possibility of contamination and to obtain a background signal as a control.

aDNA analysis

The aDNA analysis was done according to Gismondi et al. (2012, 2016), rigorously following the guidelines for ancient DNA studies (Ottoni et al. 2009) and working in the dedicated laboratories at the Centre of Molecular Anthropology for the study of ancient DNA at the University of Rome Tor Vergata ("Villa Mondragone", Monteporzio Catone, Italy). This consists of rooms with restricted access and protected from contamination by ultraviolet lights, HEPA filters, a positive pressure ventilation system and instruments sterilized by autoclave, and/or UV light and sodium hypochlorite. In summary, aDNA was extracted from each sampling point on the potsherd by resuspending 500 mg of the powder sample from it at 37 °C for 1 h with 1.5 mL of extraction buffer (10 mM Tris–HCl pH 8; 100 mM EDTA pH 8; 0.5% SDS; 20 mg/mL ribonuclease A, Sigma-Aldrich RNase,) and with 100 mg/mL Sigma-Aldrich proteinase K, for another 3 h. Three consequent extractions were done with a certain volume of a cold mixture of phenol and chloroform (1:1, pH 8), alternating with agitation at room temperature for 1 h in a half volume of CTAB (cetrimonium bromide) solution (35 mM cetyl trimethyl ammonium bromide; 100 mM NaCl). The aqueous phase from the extraction was recovered, mixed with 200 µL 2 M NaCl and 2 mL cold 2-propanol, frozen at − 80 °C and centrifuged at 11,000 g for

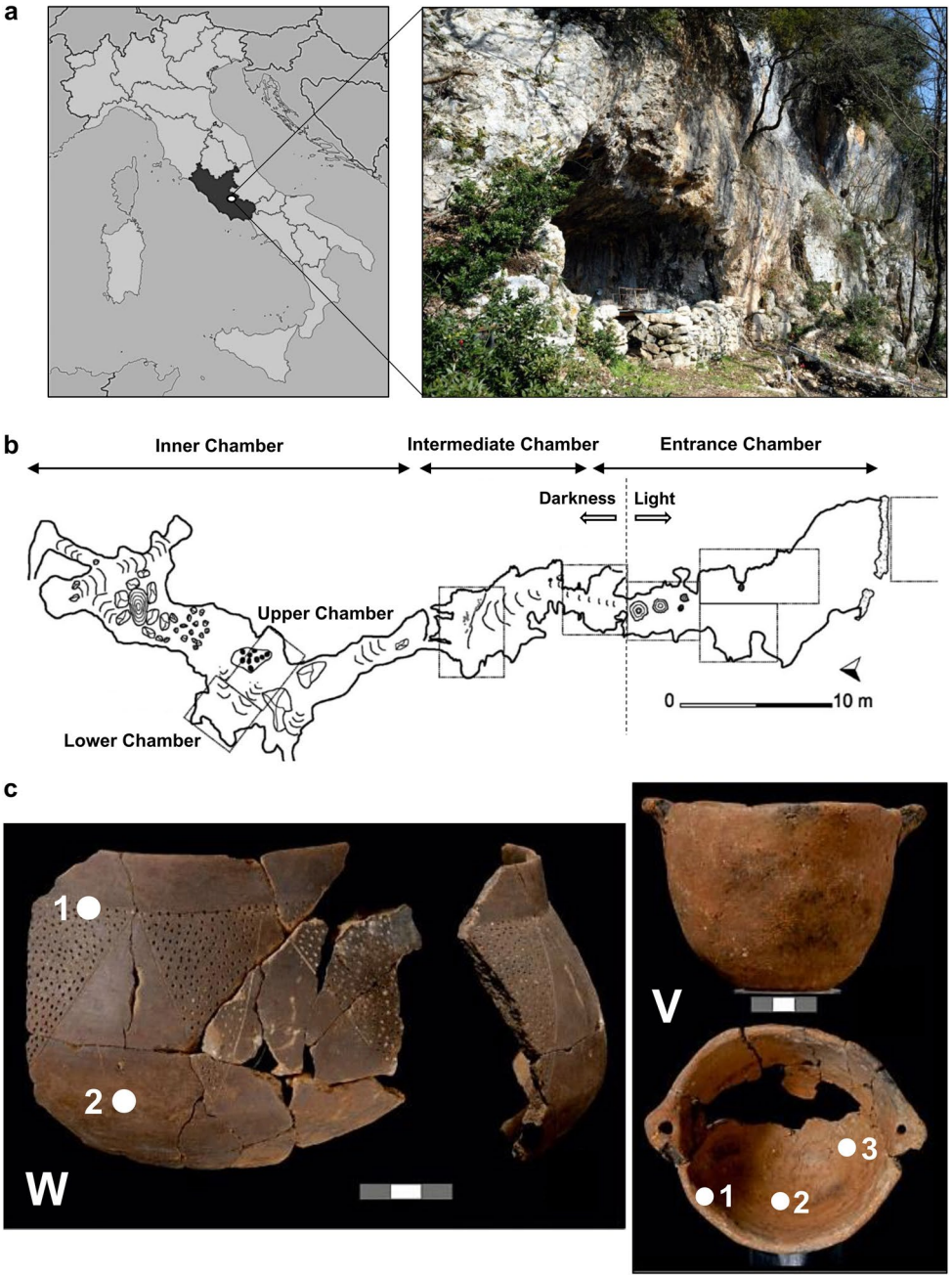

Fig. 1 Grotta Mora Cavorso and Neolithic pottery; **a,** left, map showing location of the site (Jenne, Lazio, central Italy); right, photo of the cave entrance; **b,** plan of the cave system showing excavated areas; **c** Neolithic pottery found in the inner chambers, on left, vessel W with sampling points 1 and 2; on right, vessel V with sampling points 1, 2 and 3. The sampling was done on the inner surfaces of the potsherds. Scale = 1 cm. Photos by MF Rolfo, plans redrawn from Achino et al. (2016)

Table 1 Radiocarbon dates and archaeological vessels

Lab code	Level/chamber	Sample	AMS^{14}C date (BP)	Cal. age (BCE)	Pottery
Lyon-5202	L3/UC	Charcoal	6,275 ± 45	5333–5205	Fragmented ovoid vessel, W
Lyon-3504	L3/LC	Human bone	6,405 ± 35	5474–5314	Hemispherical bowl, V

Calibration by OxCal v. 4.4.4 (Bronk Ramsey 2021), using the Int Cal20 curve (Reimer et al. 2020)

30 min at 4 °C. The pellet was resuspended in 50 µL of TE buffer (10 mM Tris–HCl, pH 8; 1 mM EDTA, pH 8) and treated with 40 µg RNase A for 20 min at 37 °C. Because of the natural time-related degradation process of DNA, the samples were treated with BioLabs USER Enzyme, Thermo Fischer Fast DNA End Repair Kit, and Macherey–Nagel NucleoSpin Gel and PCR Clean-up. All treatments were done according to the manufacturers' guidelines, except

where stated otherwise. After quantification by a Thermo Scientific NanoDrop Lite Spectrophotometer, the aDNA was used as a template for PCR chain reactions in order to amplify the three standardized plastidial barcoding genes used for the taxonomic identification, which are RuBisCO large subunit (rbcL), maturase K (matK) and the intergenic spacer between tRNAHisGUG and photosystem II thylakoid membrane protein of Mr 32,000 gene (trnH-psbA). Three different overlapping or consecutive short regions of each gene (100–250 bp) were amplified, using primer sequences from Gismondi et al. (2012) (ESM 1). The PCR reaction mixture contained 15 ng template aDNA; 0.6 U of KAPA Taq DNA polymerase; Kapa 1X Taq Polymerase buffer; 20 mM of each Sigma-Aldrich primer; 0.2 mM each Sigma-Aldrich dNTP; 3 mM MgCl2; 5% DMSO. Amplifications were done with a Bio-Rad IQ5 thermocycler following Gismondi et al. (2012). After verification on 1.5% agarose gel, cloning was done with an Invitrogen TOPO TA kit. Further PCR products were treated with Afflymetrix ExoSAP-IT) for 15 min at 37 °C and with Applied Biosystems BigDye reaction kit, using the forward or reverse primer. DNA was isolated by precipitation with 50 µL 95% ethanol and 2 µL 3 M sodium acetate pH 5.2 and centrifugation for 30 min at 1,300 g. The aDNA was washed twice with 70% ethanol, resuspended in 20 µL 100% formamide and sequenced using a Hitachi 3130 Avant Genetic Analyzer. The editing of the ancient sequence used BioEdit v. 7.2 and Nucleotide Basic Local Alignment Search Tool (BLAST) (BLAST 2022, with settings: *blastn* suite, *megablast* algorithm, for aligning the nucleotide sequences with known examples registered in the GenBank nucleotide sequence database. Intra-laboratory independent replications were done in the Laboratory of Archaeobotany DAPHNE (Diet, Ancient DNA, Plant-Human Nexus and Environment) at the Department of Biology and the Botanical Gardens of Rome Tor Vergata. Moreover, positive controls were carried out using modern template DNA of *Quercus ilex* L., separately from the ancient samples to prevent contamination risk, while negative ones were always done in parallel with them (ESM 2). The authentication criteria used in this study follow the recommendations of Mann et al. (2020).

Biochemical profiling

Organic molecules were extracted from samples of about 500 mg following the methods in Smith et al. (2018), with some modifications which were necessary to allow alkaloid analysis by GC–MS (gas chromatography-mass spectrometry). All solvents and reagents were analytical grade, typically > 98% purity. To extract lipids, half (250 mg) of the powdered pottery sample was resuspended with 2 mL of dichloromethane (henceforth DCM) with ultrasonic agitation for 6 h. After centrifugation for 8 min at 5,000 rpm, the supernatant was decanted and dried at room temperature with an Eppendorf Concentrator Plus. The centrifuge pellet was resuspended in 100 µL DCM and derivatized with 40 µL Thermo Scientific Methyl-8 reagent. To extract alkaloids, 2 mL 0.1 M hydrochloric acid were applied to the remaining powder of each sample (250 mg). After ultrasonic agitation for 15 min, lipids were eliminated using 1 mL hexane. The aqueous fraction was recovered, dried, resuspended in 100 µL methanol and derivatized as described above. Derivatized extracts were analysed using a Shimadzu QP2010 gas chromatograph mass spectrometer. The separation was carried out with a Phenomenex DB-5MS fused-silica capillary gas chromatography column (length 30 m×diameter 0.25 mm×thickness 0.25 µm). Aliquots of each sample (2 µL) were injected, in triplicate, for the analysis. Chromatographic and mass spectrometric conditions were adapted from D'Agostino et al. (2021). The temperature programming was set at 50 °C for 2 min, 200 °C for 2 min (reached at a rate of 3 °C min^{-1}) and 300 °C for 5 min (reached at a rate of 4 °C min^{-1}). Helium was used as the carrier gas, at a constant flow of 1 mL min^{-1}. An electron impact of 70 eV (scanning from m/z 100 to 700) was used for the ionization (ion source temperature 280 °C; interface temperature 300 °C; solvent cut-off time 6 min). Peaks were identified by comparison with mass spectra in the NIST 14 library and online support (NIST 2017). A compound was detected with an identical retention time and mass spectrum to an authentic reticuline external standard from Santa Cruz Biotechnology.

Results

Genetic investigation

The first aim of the present study was to check for the presence of aDNA in the Neolithic potsherds and to investigate specific plant barcode genes which would enable taxon identification. In detail, two short regions of plastid markers were successfully amplified from the aDNA samples, matK in W2 and rbcL in V3 (Table 2; ESM 2) and their nucleotide sequences are given in Table 3. The ancient sequences were compared with those in the GenBank database, and the high-quality matches produced by the BLAST alignment system for each of them are listed in Table 4. The percentage of query coverage was 100% for both genes. MatK amplicon had a 100% identity only with *Olea europaea* L. (olive) accessions, while the rbcL sequence had 98.44% identity to five different *Papaver* (poppy) species, *P. somniferum* L. (opium poppy), *P. somniferum* ssp. *setigerum* (DC.) Arcang., *P. dubium* L., *P. heterophyllum* (Benth.) Greene and *P. rhoeas* L. The genetic distance between the ancient rbcL sequence and those in GenBank consisted of two mutations (Table 3), the deletion of an adenine at the 8th nucleotide of the modern

Table 2 Overview of the results obtained by the multi-approach analysis

Pottery	Sampling points	DNA barcoding									GC–MS	
		rbcL			matK			trnH-psbA				
		1	2	(1+2)	1	2	(1+2)	1	2	(1+2)	Lipid extract.	Alkaloid extract.
W	W1	–	–	–	–	–	–	–	–	–	+	–
	W2	–	–	–	–	+	–	–	–	–	–	–
V	V1	–	–	–	–	–	–	–	–	–	–	–
	V2	–	–	–	–	–	–	–	–	–	–	–
	V3	–	–	+	–	–	–	–	–	–	+	+

Positive results achieved from the different sampling points on the Neolithic pottery (see Fig. 1, panel C) in DNA barcoding and gas chromatography mass spectrometry (GC–MS) analyses were indicated with+. In particular, for the aDNA study, the positive outcomes of PCR reactions for region 1, 2 and (1+2) of the selected plant genes (rbcL, matK and trnH-psbA). Details in Gismondi et al. (2012) and ESM 1

Table 3 Nucleotide sequences of the plant aDNAs

Pottery	Barcode gene	Ancient sequence (5'–3')
W	matK	AGATCCGCTATGATAATGAGAAAGATTTCTGCATATACGCCCAAATCGATCAATAATATCATAATCTGAGA
V	rbcL	ACTCCTGAACTATGACACCAAGGATACTGATATCTTGGCAGCATTCCGAGTTACTCCTCAACCTGGAGT TCCACCTGAGGAAGCAGGGGCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAA

Nucleotide polymorphism detected between ancient and modern sequences are marked in grey

accessions and a transversion (C → A) at the 16th base of the sequence. No plant DNA was detected in the samples from the outer surfaces of the vessels or the negative controls.

Analysis of metabolites

Considering the results obtained from the study of the aDNA, the potsherds were also analysed for their content of fatty acids and secondary metabolites. Only one sampling point per vessel showed the presence of organic compounds (Table 2). The lipid extract from W1 was characterized by a mixture of saturated long-chain fatty acids (tridecanoic, hexadecanoic and octadecanoic acids), a monounsaturated omega-9 fatty acid (9-octadecanoic acid), a bicyclic monoterpene ketone (pinocamphone) and *n*-alkanes in the carbon chain range C_6–C_{25} (Fig. 2). The chromatographic profiles of the sampling point V3, on the other hand, revealed the presence of molecular markers from both the applied methods. The original version of the extraction protocol for liquid chromatography of alkaloids was modified for gas chromatography in the present work (Smith et al. 2018). Our modification allowed GC–MS to reveal the presence of reticuline (benzylisoquinoline alkaloid) (Fig. 2). *N*-alkanes (carbon chain range C_6–C_{25}), citronellol (acyclic monoterpenoid) and tetradecanoic, hexadecanoic, and octadecanoic acids were highlighted with the lipid isolation procedure. *N*-alkanes were found only in one external sample from W2.

Discussion

In this study, the analysis of two Neolithic potsherds is discussed, which are an exceptional prehistoric cultural find in central Italy. A larger set of samples would have provided more meaningful information, but the application of a combined methodology allowed a greater reliability of data interpretation.

Ensuring the efficiency of the extraction and sampling methods is integral for carrying out correct analytical procedures and obtaining trusted results which are properly explained, especially for compounds derived from plants, which require careful and critical examination to be unambiguously validated. It is also important to remember that a "valid" biomarker does not constitute unassailable proof of the use of a plant if it also occurs elsewhere (Whelton et al. 2021).

Although a monoterpenoid and some monoterpene derivatives were detected in the potsherds, they do not provide evidence for the use of fragrance substances, such as resins and natural waxes.

The survival of *n*-alkanes is as expected in the archaeological record, due to their resistance to time-related degradation and chemical or physical agents. Nevertheless, they are generally widespread molecules, not taxonomically specific and also derived from the alteration of fat- and oil-derived acyl lipids and higher plant waxes (Luong et al. 2018; D'Agostino et al. 2021; Whelton et al. 2021). For this reason, it is not possible to infer their original source.

Table 4 BLAST matching

Species	Accession code
matK region (aDNA) vs GenBank sequences	
Olea europaea ssp. *europaea*	MG372118.1
O. europaea ssp. *europaea*	MG372117.1
O. europaea	KX783724.1
O. europaea ssp. *cuspidata*	MG372116.1
O. europaea ssp. *laperrinei*	MG372121.1
O. europaea ssp. *guanchica*	MG372120.1
rbcL region (aDNA) vs GenBank sequences	
Papaver somniferum	MN167245.1
P. somniferum ssp. *setigerum*	MK820043.1
P. somniferum	MF973030.1
P. somniferum	MF973029.1
P. somniferum	MF973028.1
P. somniferum	MK526291.1
P. somniferum	MG769001.1
P. somniferum	MG769000.1
P. dubium	MG768995.1
P. dubium	MG768994.1
P. somniferum	XM_026533548.1
P. somniferum	XM_026533547.1
P. somniferum	MH287278.1
P. somniferum	MG248990.1
P. heterophyllum	MF963351.1
P. somniferum	KU204905.1
P. somniferum	JN114830.1
P. somniferum	MT712162.1
P. somniferum	JN893507.1
P. somniferum	JN892662.1
P. dubium	JN890645.1
P. somniferum ssp. *setigerum*	JN584654.1
P. somniferum ssp. *setigerum*	HM850231.1
P. rhoeas	HM850230.1
P. dubium	HM850229.1
P. dubium	AB517147.1
P. somniferum	DQ912894.1

Scientific name (species) and GenBank accession codes of the nucleotide sequences that highly matched with the matK and rbcL genes of ancient samples. In all cases the percentage of query coverage was 100%, the percentage of identity was 100% for *Olea europaea* and 98.44% for *Papaver* sp., while the E value (significance of the result, max value is 0.0) was $4e^{-27}$ for matK and $1e^{-54}$ for rbcL

DNA barcoding and GC–MS analyses together suggested that the content of vessel W was mainly consistent with a plant-based oil. A genetic approach was applied with stringent authentication criteria (Mann et al. 2020) and revealed a fragment of plant aDNA attributable to *O. europaea*. The presence of olive at this site is already known from macrobotanical remains dated to the Neolithic period and probably representing food or funeral offerings (Gismondi et al. 2012). Oleic acid, detected in the chromatographic profile from W1, is often specifically associated with olive oil, being its major component. However, as reported in Whelton et al. (2021), the recognition of oleic acid as a biomarker for olive oil is an example of misinterpretation, as it is also a constituent of other plant oils and fats (Villa-Rodríguez et al. 2011; Liu et al. 2015; D'Agostino et al. 2020). Double bonds of monounsaturated fatty acids rarely persist in archaeological contexts; however, some ancient materials and storage conditions can allow their survival (Copley et al. 2001; Craig-Atkins et al. 2020; D'Agostino et al. 2020), as in this specific case.

Regarding fatty acids, the ratio between palmitic and stearic acids (C16:0/C18:0) has been used in literature to distinguish the original source of lipids (Kimpe et al. 2002; Eerkens 2005; Manzano et al. 2016; Łucejko et al. 2018; Guðmundsson et al. 2021). Unfortunately, in our research, the ratio has not been evaluated, as it would be too risky because the molecular profile can vary due to several highly likely causes, such as time-related degradation, absorption capacity of the pottery and original lipid materials, which make it impossible to draw a simple conclusion.

Even though the data from vessel W come from two different sampling points, the genetic ones from the bottom of the potsherd and the molecular markers from the top, it is not possible to declare that the pot only contained olive oil. Indeed, we cannot exclude that this pottery could have been used before its deposition in the cave (maybe for ritual purposes) and that various substances such as plant oils and/or animal fats had been processed and/or stored in the pot during its life.

For vessel V, the discovery of plant aDNA attributable to *Papaver* and the detection of reticuline, an alkaloid, from the same sampling point was peculiar. After the identification of the aDNA fragment, a crucial question is whether the hemispherical bowl held poppy products of some kind and, more specifically, whether it could prove to have been a receptacle of opiates with therapeutic and/or symbolic importance. In Italy, the only evidence of Neolithic charred poppy capsules has been from La Marmotta (Lago di Bracciano, Roma) and dated, indirectly, between 5538 and 5290 cal BCE (Fugazzola et al. 2010). Recently, the same archaeobotanical samples have been dated to 5620–5480 cal BCE (Salavert et al. 2020), thus slightly earlier than vessel V from Mora Cavorso (Neolithic layer; Table 1).

In this work, gas chromatography and mass spectrometry (GC–MS) analysis has revealed the presence of reticuline, a direct comparison of which with a pure standard confirmed the identification (Fig. 2). Reticuline is a chemical "chameleon" in the biosynthesis of isoquinoline alkaloids, which can lead to remarkably different compounds, including papaverine, morphine and thebaine (paramorphine) (Singh et al. 2000). It is important to consider whether reticuline is sufficiently significant for a valid interpretation, as it is the precursor of a vast number of alkaloids in plants (Fujii et al. 2007). In addition to opium alkaloids, other molecules

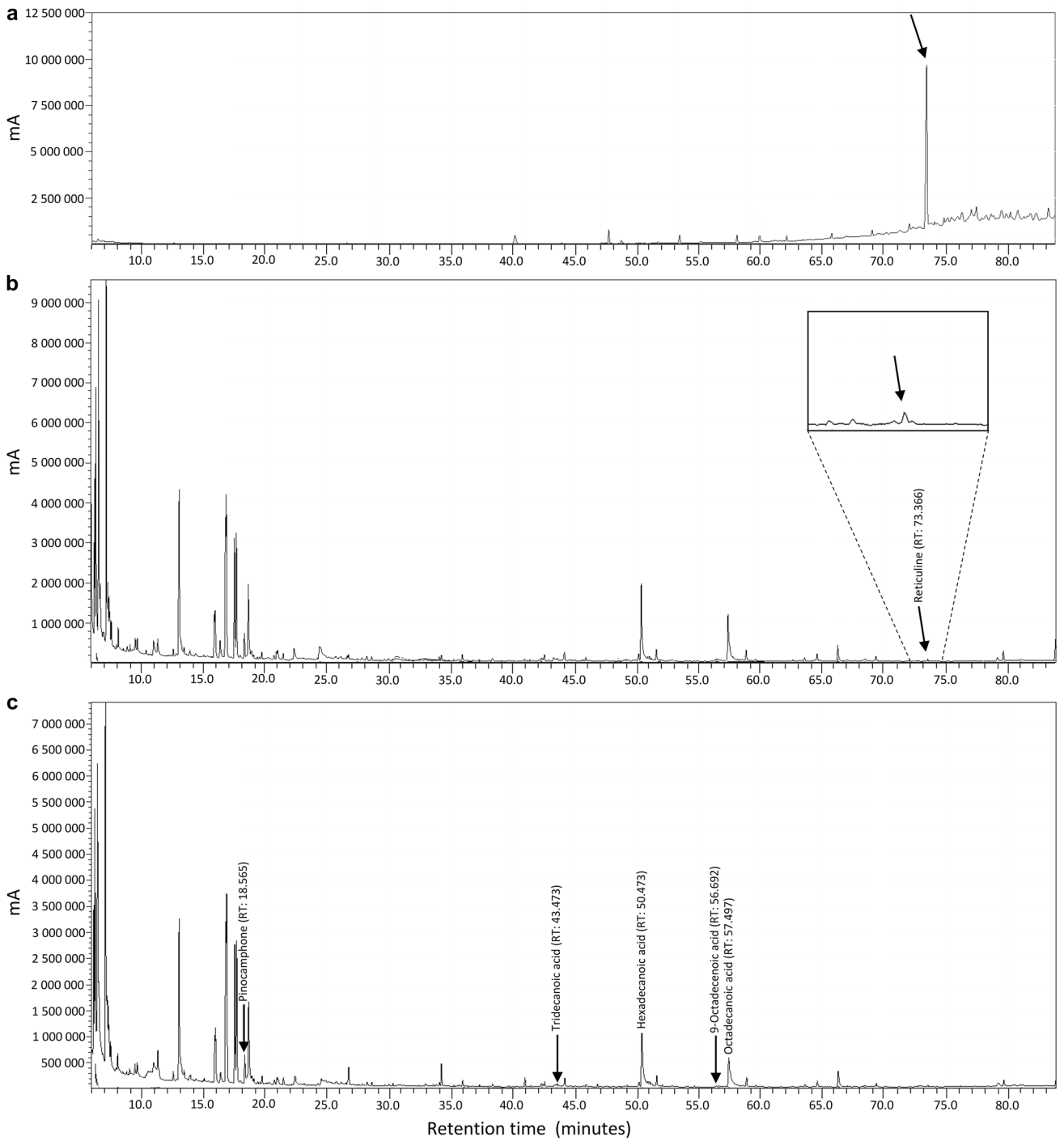

Fig. 2 Gas chromatography-mass spectrometry (GC–MS) chromatograms; **a,** reference GC-MS profile of the reticuline standard; **b,** of the ancient potsherd V; **c,** potsherd W. International Union of Pure and Applied Chemistry (IUPAC) names and retention times (RT) in minutes of the identified molecules are shown

closely related to reticuline are synthesized by shrubby and herbaceous plants which are widespread in temperate regions of Europe and parts of the Middle East and are usually used for food and medicines. Some examples are berberine, contained in the root, rhizome, stem and bark of *Berberis* L. (barberry), and protopine and corydaline, alkaloids occurring in seeds and tubers of Papaveraceae, such as *Fumaria officinalis* L. (common fumitory) and *Corydalis* spp. (Imenshahidi and Hosseinzadeh 2019; Al-Snafi 2020; Jeong et al. 2021). However, even in forensic science, reticuline is considered to be a marker of opium use (Al-Amri et al. 2004). In this regard, the genetic analysis of vessel

V narrows down the field of discussion to species of the genus *Papaver*. The inability to detect other specific poppy alkaloids in our sample is not so unexpected because, most probably, they are prone to degradation, which is one of several factors involved in the survival of molecules in different depositional environments. Consequently, the detection of rbcL amplicon from vessel V is significant, as this is the first genetic evidence in support of a link between Neolithic pottery from Lazio and poppy derivatives.

Opium is generally consumed as a narcotic, through ingestion or smoking (Smith et al. 2018). Therefore, our results would suggest that the ancient vessel had contained poppy seed oil or opium latex from green pods. Another intriguing version could be the use of poppies as ingredients of a complex mixture of scented oils. In fact, there is no way to find out whether different products were processed together or separately. No interpretation can be ruled out, not even the possible reuse of the bowl.

In the ancient lake settlement of La Marmotta, the use and potential cultivation of *P. somniferum* has been suggested, based on the numbers and ubiquity of botanical finds (Rottoli 1993), although it is possible that wild forms of *P. setigerum* had grown as weeds in the region (Jesus et al. 2021). *P. setigerum* is an annual herb closely related to the opium poppy, but not likely to have been its wild ancestor (Liu et al. 2020). Both species grow in grasslands and cultivated fields and are known for their medicinal properties, namely both for short and long-term pain control and for their psychoactive properties (Labanca et al. 2018; Liu et al. 2020). Due to the similarity in their morphological characteristics such as shape of flowers and capsules, colour and their production of isoquinoline alkaloids, the discrimination between *P. somniferum* and *P. setigerum* is still quite difficult, although recent progress in morphometric analysis has been made (Jesus et al. 2021). Among genetic markers, trnH-psbA is the best region for distinguishing them (Liu et al. 2020) but, unfortunately, we were unable to obtain amplification of this intergenic spacer.

Apart from *P. somniferum* and *P. setigerum*, three other *Papaver* species were suggested, *P. heterophyllum, P. rhoeas* and *P. dubium*. The first one, endemic in western North America, was excluded for incompatibility due to its native geographical distribution. For the other two species, there is rare evidence of their seeds in Mediterranean Neolithic sites (Fahmy 2001; Martin 2015). Although these poppies synthesize only small amounts of alkaloids, they may still have been useful as food or for human health (Grauso et al. 2021).

From the recent literature, it is known that *P. somniferum* ssp. *somniferum* was part of the broad range of Neolithic crops in the Mediterranean, where it grew wild (Salavert et al. 2020; Jesus et al. 2021). Thus, considering the evidence from La Marmotta (Salavert et al. 2020), the surroundings of Grotta Mora Cavorso could have been within the natural distribution area of the opium poppy. Here, the pioneer Neolithic farmers might have used it as a food source and/or medicinal plant. Unfortunately, the lack of seeds, pollen, capsules or stigmatic disks at Grotta Mora Cavorso (Gismondi et al. 2012; D'Agostino et al. 2022), does not help in clarifying the presence and role of poppies in this area of central Italy during the prehistoric period. Hence, it is not possible to state whether the people who used the cave for their burials already cultivated *Papaver* species, including *P. somniferum*, or just collected them as wild plants.

Some of the archaeological remains found in Grotta Mora Cavorso have been shown to have come there from a long distance, suggesting that this part of the Apennines had a key role in the social and cultural dynamics of both the main east–west and north–south prehistoric routes across the Italian peninsula (Silvestri et al. 2020). This suggestion might also imply that knowledge about the uses of plants was exchanged between the first Neolithic communities in Lazio, including those of La Marmotta, in this case about *Papaver* species for cult and/or therapeutic purposes.

All data we obtained from the prehistoric pottery from Grotta Mora Cavorso are in line with the archaeobotanical investigations mentioned above and provide food for thought about the Neolithic practices related to plants and funerary contexts.

Conclusions

This article discusses the archaeobotanical evidence obtained from two potsherds found at the prehistoric site of Grotta Mora Cavorso, central Italy. The biochemical and DNA markers detected in this ancient pottery could represent direct proof of a single utilisation or an accumulation of multiple events over the time when these vessels were in use.

In the literature, genetic analyses on archaeological materials are very rare, but extremely sensitive, powerful and innovative, making the data from this contribution valuable to the debate on the role of ancient containers.

Obviously, investigating a larger number of vessels would have provided more data but, considering the unusual nature of the archaeological site, attention towards the sampling methods and the application of two different molecular techniques in parallel, we can declare that our results raise significant issues about the uses of pots in prehistoric funerary contexts and the Neolithic relationship between people and plants.

The likelihood of the survival of organic compounds over archaeological time scales has been questioned; however, since the detected aDNA fragments were very short (as expected for ancient samples) and the identification of plant chemicals supported the genetic data, it is reasonable to believe that our evidence is authentic and may demonstrate the knowledge of the early Neolithic inhabitants in central Italy about the processing and use of olive and poppy

products, in addition to the previous records from the site of La Marmotta. However, it is still difficult to estimate the importance of *Olea europaea* and *Papaver* spp. in the overall diet and/or customs of these Neolithic people.

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Declarations

Competing interest The authors declare that they have no conflict of interest.

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