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Identification of Plant Remains in Underwater Archaeological Areas by Morphological Analysis and DNA Barcoding

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DNA barcode technique has only recently been applied to archaeobotanical studies. In fact, in association with morphological, scanning electron and optical microscopic analyses, these specific methods allow researcher to scientifically classify antique flora samples. Therefore, this project wants to improve, to encourage and spread further use of this protocol and to highlight the potentialities of the molecular biology and microscopy related to botanical fossils. In conclusion, ancient *Olea europaea* L. and *Crataegus monogyna* Jacq. seeds, a *Pinus* sp. pollen cone, a *Quercus petraea* (Mattuschka) Liebl. acorn, animal fibers and gymnosperm woody fragments, found in a 1st Century BC sunken Dressel 1B amphora, have clearly been identified, in order to enhance knowledge about Central Italy past human activity and environment. This research has also demonstrated the applicability of this scientific approach on specimens derived from underwater archaeological site.

Keywords: Barcoding; Optical Microscopy; SEM Analysis; Archaeobotany; Plant Remains

Introduction

Sea level variations, due to the effects of post-glacial ages and earth's structure modifications, caused the submersion, or the sinking, of prehistoric and archaeological sites in water (Bailey & Flemming, 2008). The detection of these areas and their great historical interest induced the development of the "underwater archaeology". On the other hand, this discipline was also encouraged by the discovery of thousands of sunken wrecks and their content. In literature, only a few works are presented about this science, although it produces useful elements to clarify, suppose and predict different aspects of human ancestors' activities and ecosystem (Willcox, 1977; Edge & Gibbins, 1988; Gorham & Bryant, 2001; Hansson & Foley, 2008; Claesson, 2011). Since the beginning of the last century, flora ancient remains have captured the attention of scientists because of the large amount of new information they could provide about the past (Banning, 2002). A lot of vegetal remains were found in archaeological sites; in fact, flowers, leaves, fruits and woods were regularly used as foods, drugs or funeral offers by ancient populations (Grasso & Fiorentino, 2009). The detection of botanical elements in caves, archaeological areas and hypogean structures and their taxonomic identification allowed scientists to increase knowledge on plant evolution, ethnobotany and reconstruction of past environments (Marota et al., 2002; Liepelt et al., 2006). Morphological observation is the principal approach applied by archaeobotanists to classify plant remains; however, this method sometimes showed wrong and contradictory results (Gismondi et al., 2012). On the other hand, the introduction and the application of molecular analyses on ancient remains, especially after the development of next-generation sequencing techniques, allowed researchers to obtain more accurate data, even in presence of scarce amounts of template (Manen et al., 2003). In particular, DNA barcoding, a method that uses standard nucleotide sequence analysis for species classification (Kress & Erickson, 2008), was recently and successfully associated to archaeobotany (Gould et al., 2010; Gismondi et al., 2012). In 1994, at seven meters underwater, a Dressel 1B amphora, dated back to the 1st Century BC, was found on the sandy seabed of the Pyrgi seaport-canal archaeological site (Santa Severa, Rome) (Enei, 2008). Aim of this work was the botanical identification of flora remains, found in Pyrgi archaeological find, applying, but also encouraging and improving, the combined use of innovative and classical scientific approaches, in order to increase knowledge about past human populations and ecology.

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Materials and Methods

Detection and Cleaning of Plant Remains

Vegetal remains were collected from a sealed amphora (type Dressel B1) found underwater in Pyrgi seaport-canal (Santa Severa, Rome) (Enei, 2008). Samples were preserved waterlogged until the analyses. Then, they were washed in H_2Odd, dried at room temperature for 48 hours, decontaminated by UV light for 30 minutes and stored at −20˚C to reduce further ancient DNA (aDNA) degradation (Poinar, 2002; Pruvost et al., 2007).

Morphological Analysis

Samples were documented by a Fujifilm Finepix S1000fd camera. Morphological observations were performed by a scanning electron microscope (SEM) Leika/LEO stereoscan 440 (25-1000X enlargement) and a light microscope (LM) Nikon Eclipse E100 (10-40X enlargement). Woody remain sections were manually carried out by a scalpel.

aDNA Extraction and Contamination Prevention

aDNA was extracted according to Gismondi et al. (2012) method. Flora remains were grinded up by a domestic grinder and then further with pestle, mortar and liquid nitrogen. Samples were resuspended in 0.8 mL of extraction buffer (10 mM Tris-HCl pH 8; 100 mM EDTA pH 8; 0.5% SDS; 20 µg/mL Ribonuclease A, RNase Sigma-Aldrich, Italy) and incubated at 37˚C for 1 hour. Samples were kept at 37˚C for other 3 hours, after addition of proteinase K (100 µg/mL, Sigma-Aldrich). A volume of cold phenol:chloroform (1:1, pH 8) was added to each sample; then, tubes were centrifuged at 12.000 rpm/min for 5 minutes (min) at 4˚C. 0.4 mL of CTAB solution (35 mM cetyl trimethylammonium bromide; 100 mM NaCl) was added to supernatants that were also incubated at room temperature for 1 hour in agitation. Two phenol:chloroform (1:1; pH 8) extractions were performed further. 100 µL of 2 M NaCl and 1 mL of cold 2-propanol were used to precipitate aDNA collected in the aqueous phase of each sample. After incubation at −80˚C over night, samples were centrifuged at 12.000 rpm/min for 30 min at 4˚C. Pellets were resuspended in 100 µL of TE buffer (10 mM Tris-HCl pH 8; 1mM EDTA pH 8). A sample incubation, of 20 min at 37˚C, was finally performed with 40 µg RNase A. All samples were stored at −20˚C. Naturally, all precautions necessary for preventing sample contaminations during the analyses (DNA extraction and PCR reactions) were taken, as recommended by Willerslev and Cooper (2004), Yang and Watt (2005) and Gismondi et al. (2012). In particular: aDNA was extracted in a specific laboratory where modern DNA was never processed; PCR amplifications were performed in a physically separated areas; each PCR amplification and sequencing analysis was repeated, in triplicate, using different PCR thermocyclers positioned in different laboratories; positive controls weren't performed in order to avoid contamination risk, instead of negative ones that were always carried out; instruments were always cleaned and decontaminated by UV, after their use.

PCR Amplifications and Target Genes

Barcode gene amplifications were carried out in a final volume of 50 µL. Each one contained: 1 - 2 µL (of the total extraction volume) aDNA, 1.5 U RBC Taq DNA Polymerase (RBC Bioscience), 20 µM of each primers (described in detail in **Table 1**), 0.2 mM of each dNTP (Sigma-Aldrich), 1X RBC Taq DNA Polymerase buffer (RBC Bioscience), 3 mM $MgCl_2$ and 5% DMSO. Amplification were performed in a IQ5 thermocycler (Biorad, Italy), using the following cycling profile: 4 min at 95˚C; 50 cycles of 30 sec at 95˚C, 30 sec at annealing temperature (**Table 1**) and 1 min at 72˚C; 15 min at 72˚C. In this study, following target genes were chosen: ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCO large subunit, rbcL) gene + maturase K (matK) gene + intragenic spacer between tRNAHisGUG gene and photosystem II thylakoid membrane protein of Mr 32,000 gene (trnH-psbA) (Chase et al., 2007; Kress & Erickson, 2007; Group CPW, 2009; Seberg & Petersen, 2009). For each barcode gene, more regions were amplified, using different primer pairs (F1-R1, F2-R2, F1-R2, F1-R3 or F3-R3, as reported in **Table 1**), according to Schlumbaum et al. (2008) and Gismondi et al. (2012).

Agarose Gel Electrophoresis

PCR products were separated on 1.5% agarose gel containing 10 mg/mL ethidium bromide. The electrophoresis was performed using 1X TAE buffer (40 mM Tris; 1 mM EDTA; 20 mM acetic acid; pH 8.5) and DNA fragments were visualized under UV light (VersaDoc 4000 MP, BIO-RAD).

Sequencing and Data Analysis

3 µL of PCR products were subjected to ExoSAP-IT (Affymetrix) treatment for 15 min at 37˚C. Then, samples were subjected to PCR (25 cycles of denaturation at 96˚C for 10 sec, annealing 50˚C for 5 sec and final extension at 64˚C for 4 min), after addition of 1.5 µL of BigDye (Applied Biosystems) and 1.5 µL of forward (or reverse) primer. Amplicons were precipitated by classical ethanol precipitation method (Gismondi et al., 2012). DNA was sequenced after resuspension in 20 µL of formamide (100%). Sequencing analysis was performed using 3130 Avant Genetic Analyzer (HITACHI, Applied Biosystems). Botanical identification of ancient remains was performed according to Gismondi et al. (2012). Briefly, high-quality (clearly

Table 1.
Primer sequences.

PRIMER PAIR NAME	SEQUENCE (5'-3')*	Tm (˚C)	Amplicon (bp)
rbcL F1 rbcL R1	ATGTCACCACAAACAGAGACT GAATGCTGCCAAGATATCAGT	57.6	120
rbcL F2 rbcL R2	ATTATACTCCTGAATACGAAA TGTCCATGTACCAGTAGAAGA	54	130
rbcL F1 rbcL R2	ATGTCACCACAAACAGAGACT TGTCCATGTACCAGTAGAAGA	52	250
rbcL F1 rbcL R3	ATGTCACCACAAACAGAGACT CTTCTGCTACAAATAAGAATCGAT	57.7	670
matK F1 matK R1	GTTCTAGCACAAGAAAGTCGA CTCAGATTATGATATTATTGA	52.2	130
matK F2 matK R2	TGCATATACGCCCAAATCGAT CCAATTATTCCTCTGATTGGA	54	130
matK F1 matK R2	GTTCTAGCACAAGAAAGTCGA CCAATTATTCCTCTGATTGGA	56.4	260
matK F1 matK R3	GTTCTAGCACAAGAAAGTCGA CCTATCCATCTGGAAATCTTAG	56.8	834
trnH-psbA F1 trnH-psbA R1	ATAAAGGAGCAATAACGCCCT CGAAGTTCCATCTACAAATGG	60	120
trnH-psbA F2 trnH-psbA R2	GGTAATGTAACGAATAAAAGT GAGCAATAAACTCTTTCTTGT	52	120
trnH-psbA F1 trnH-psbA R2	ATAAAGGAGCAATAACGCCCT GAGCAATAAACTCTTTCTTGT	60	240
trnH-psbA F3 trnH-psbA R3	CGCGCATGGTGGATTCACAATCC GTTATGCATGAACGTAATGCTC	55.5	525

Primer pair names (F: forward; R: reverse), their sequences (5'-3'), the melting temperature (˚C) used in PCR amplifications and amplicon lengths in basepairs (bp). *References: Kress et al., 2005; Kress and Erickson, 2007; Gismondi et al., 2012.

readable) aDNA regions were aligned and compared with sequences registered in scientific databases (GenBank/EMBL/nucleotide) by Nucleotide Basic Local Alignment Search Tool algorithm (BLAST, blastn, http://blast.ncbi.nlm.nih.gov/Blast), with a relaxed E-value cutoff. Resulting species list obtained for each sample was simplified according to morphological comparison of ancient remains with putative modern ones (dimension, shape and surface), possible geographical distribution, scientific/historical literature data, maximum values of nucleotide sequence max identity between subject and query and, in particular, by the intersection of result lists obtained from the same sample for different barcode regions, as performed in Gismondi et al. (2012).

Combustion Test and Dry Distillation Assay

Combustion test and dry distillation assay were performed on fiber samples, according to Goodway (1987) and Quaglierini (2012). Briefly, in the combustion test, specimens were drawn up to fire: a rapid combustion and the smell of burnt paper are typical of vegetal samples, with respect to animal fibers that are characterized by a slow combustion time and a smell of burnt hairs. In the dry distillation assay, fibers were placed in a well-closed flask with a litmus paper, previously dipped in H_2Odd. Then, the flask was put on fire and fumes, released from dissolved fibers, permeated the litmus paper that changed its color (slightly acid pH for samples of flora origin and slightly basic pH for animal fibers).

Results

Flora ancient remains, found in a sunken amphora in the archaeological site of Pyrgi (Santa Severa, Rome), were scientifically analyzed in order to taxonomically identify them. To facilitate this study, samples were named with alphabetic letters (from A to F).

Sample A

Probably because of time degradation, atmospheric agents or animal or human action, this sample wasn't conserved in one piece. Nevertheless, it appeared as an elongated seed of 0.77 cm in length (**Figure 1(A)**). SEM observations showed that seed coat was wrinkled and characterized by compact and woody isodiametric cells (**Figure 2(A)**) (Barthlott, 1981). Tegument depth constantly measured about 400 μm and it also reported a loose matrix, due to the presence of collapsed parenchyma cells (**Figure 2(A2)**). Morphological analysis couldn't determine a taxonomic identification of the specimen; so, the genetic approach was fundamental. aDNA was extracted from the vegetal remain and specific genes were amplified (as reported in "Materials and Methods"). It wasn't possible to amplify all chosen barcode regions but, with respect to the other specimens, this sample showed the best conserved template. Positive PCR products (rbcL F1-R1, rbcL F2-R2, matK F1-R3, trnH-psbA F1-R1, trnH-psbA F2-R2, trnH-psbA F3-R3) were visualized on agarose gel, under UV-light, and verified in size, with respect to the standard molecular weight (MW) (**Figure 3**). Each amplicon was sequenced and compared with the scientific nucleotide databases: this process generated species lists that respectively matched with every analyzed barcode sequence (Supplemental Data 1-Sample A). Incompatible species, according to the present morphological study, natural geographical distribution and scientific/historical literature data (Liphschitz et al., 1991; Carrión et al., 2010; Milanesi et al., 2011), were eliminated from the resulting lists. The intersection of these outcomes allowed us to scientifically reveal the botanical origin of the sample A: *Olea europaea* L.

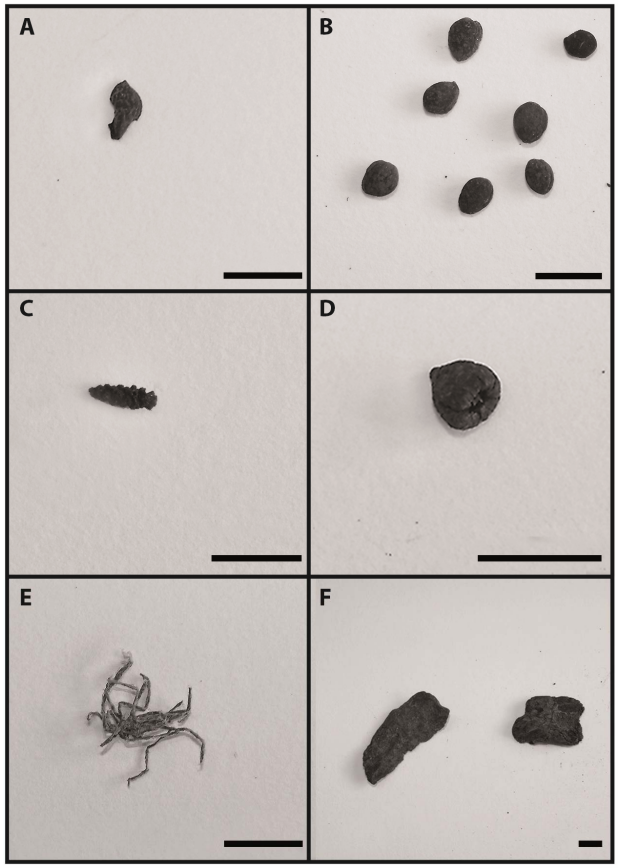

Figure 1.
Archaeological remains have been documented with photos for morphological studies: A) sample A; B) sample B; C) sample C; D) sample D; E) sample E; F) sample F. Black bars indicate 1 cm.

Sample B

In the archaeological amphora twenty-two generally round seeds, of 0.3 - 0.6 cm in diameter, were found (**Figure 1(B)**). The outside of these samples was quite regular though slightly rough and presenting some little dips (**Figure 2(B)**). No other particular characteristics were evidenced by microscopic analysis. The genetic approach was essential for the recognition of these flora remains: unfortunately, it was possible to amplify only one region of the maturase K barcode gene (matK F1-R2) (**Figure 4**). The relative nucleotide sequence showed a maximum identity and E-value (89%, 7e-66) with *Crataegus* genus species (Supplemental Data 2-Sample B). In particular, according to morphological results (here reported and published also in other works) (Dietsch, 1996; Groningen Institute of Archaeology and the Deutsches Archäologisches Institut, 2006), we concluded that samples B were *Crataegus monogyna* Jacq. seeds.

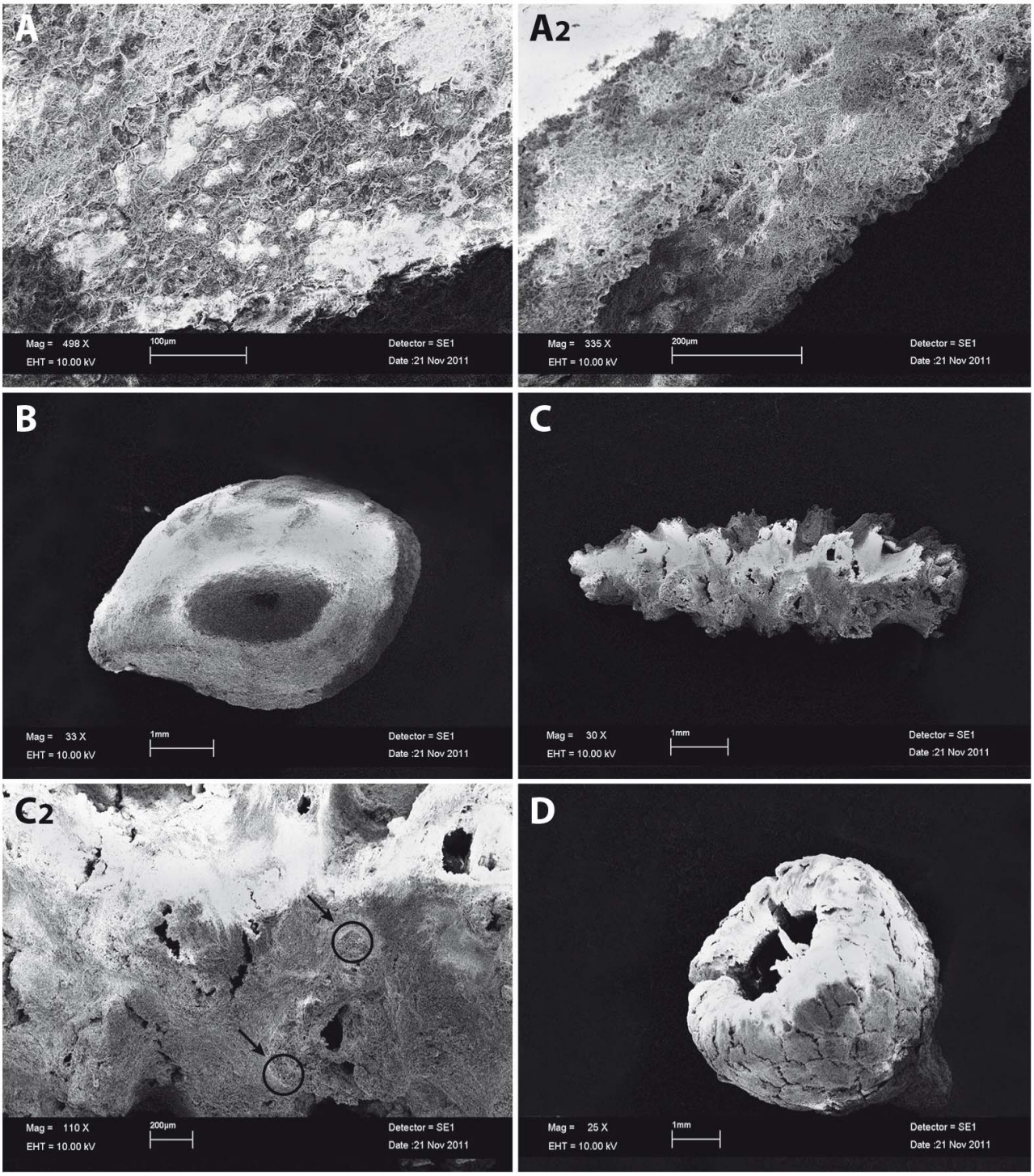

Figure 2.
SEM observations of botanical remains: A) sample A surface; A2) sample A tegument deep; B) sample B; C) sample C; C2) particular of sample C; D) sample D. Enlargement and unit bars are reported in each image.

Sample C

This specimen, already at first sight, looked like the axis of a microstrobilus. Its dimensions were 0.72 cm in length and 0.18 cm in width (**Figure 1(C)**). SEM analysis evidenced a very irregular surface and structure of the botanical sample (**Figure 2(C)**). In fact, a lot of prominent protrusions could easily be individuated: these extroflexions represented the sites where microsporophylls were attached and held on the pollen cone central axis. In **Figure 2(C2)** (inside circles), it was also possible to distinguish some vascular tissue remains, characterized by series of tracheids. aDNA was successfully extracted from

the remain but, maybe because of its excessive degradation, only one PCR amplification (trnH-psbA barcode gene, region F3-R3) was obtained (**Figure 4**). Results, reported in Supplemental Data 3-Sample C, confirmed that sample was the fossilized remains of a *Pinus* sp. pollen cone.

Sample D

Last seed remain, found in the underwater archaeological site, was clearly an acorn. The pericarp was very reduced and completely protected by a thick cupule. The whole structure was rounded, sub-spherical and measured about 0.4 cm (**Figure 1(D)**). Cupule scales were lanceolated and embricated (**Figure 2(D)**). Molecular study was performed on the oak nut and the single obtained amplicon (rbcL F1-R2) was fractionized on agarose gel (**Figure 4**). Positive PCR product was analyzed and matched up with nucleotide sequence databases. Outcomes, reported in Supplemental Data 4-Sample D, showed high sequence identity (95%) of the sample with *Quercus* genus. In particular, according to cupule specific morphological features (Pignatti, 1982; Heinz and Barbaza, 1998), this specimen was identified as *Quercus petraea* (Mattuschka) Liebl. fruit.

Sample E

No aDNA trace was detected in sample E. Therefore, for its taxonomic identification, our research was essentially based on morphological and microscopic observations. This specimen presented a filamentous structure (**Figure 1(E)**). At first, we considered the possibility that it could be a vegetal fiber (cotton-*Gossypium* sp. L., linen-*Linum usitatissimum* L., hemp-*Cannabis sativa* L., jute-*Corchorus* sp. L. or ramie-*Boehmeria nivea* L.). Light microscope image (**Figure 5(E1)**) showed that, in reality, the sample was made up of several united filaments. Each of these filaments appeared quite uniform, internally full and not characterized, on its surface, by the presence of cell walls (**Figures 5(E2)**, **(E3)**, **(E4)** and **(E5)**). According to these preliminary results, the ancient remain could be neither fossil cotton nor linen remains: in fact, *Gossipium* sp. L. product is

Figure 3.
PCR amplifications of sample A aDNA have been detected by UV-light after migration on agarose gel 1.5%. MW (molecular weight; 100, 500 and 800 base pairs are indicated); lane 1 (rbcL barcode gene, region F1-R1, 130bp); lane 2 (rbcL barcode gene, region F2-R2, 130 bp); lane 3 (matK barcode gene, region F1-R3, 834bp); lane 4 (trnH-psbA barcode gene, region F1-R1, 120bp); lane 5 (trnH-psbA barcode gene, region F2-R2, 120bp); lane 6 (trnH-psbA barcode gene, region F3-R3, 525bp).

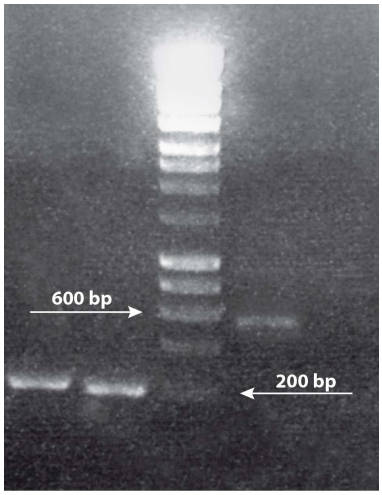

Figure 4.
Positive PCR products visualized on agarose gel 1% by UV-light. MW (Molecular weight; 200 and 600 base pairs are indicated), lane D (sample D aDNA, rbcL barcode gene, region F1-R2, 201bp), lane B (sample B aDNA, matK barcode gene, region F1-R2, 200bp); lane C (sample C aDNA, trnH-psbA barcode gene, region F3-R3, 525bp).

flat, ribbon-like and helix packaged (Han et al., 1998; Krakhmalev & Paiziev, 2006) whilst *L. usitatissimum* L. fiber is hollow and characterized by horizontal rings that regularly recur along the filament (Florian et al., 1990). Hemp, jute and ramie filaments were also observed by optical microscopy (**Figures 5(H)**, **(J)** and **(R)**), in order to discover some similarities between them and the ancient specimen. Unfortunately, all these fibers appeared very different with respect to the archaeological sample. Ramie fiber only presented a remote likeness with the sample E. So, to clarify this doubt, SEM analysis was performed on the ancient remain (**Figures 6(E1)**, **(E2)** and **(E3)**) and ramie filaments (**Figures 6(R1)**, **(R2)** and **(R3)**). Both fibers measured about 30 - 35 µm in thickness but, outwardly, they were very dissimilar: sample E showed a uniform and quite smooth surface with respect to ramie one that was characterized by filamentous micro-subunits. All these data suggested that sample E was not a vegetal fiber. Thus, we have wanted to demonstrate that it had an animal origin. Apart from the slow burning time of the sample and the smell of burnt hairs that was produced during the empiric combustion test, we also observed that, in the dry distillation assay, litmus paper slightly changing its color towards basic pH (**Figure 7**), confirming the animal nature of the ancient remain. Probably it was raw silk, according to Goodway (1987) observations.

Sample F

Last samples were represented by woody fragments (**Figure 1(F)**). DNA barcoding technique wasn't applicable, in this case, because of the absence or too low levels of aDNA. Consequently, only microscopic approach allowed us to obtain some information about them. Remain sections clearly showed the

presence of vascular bundles between intrafascicular parenchyma (**Figure 8(F1)**). By the enlargement of these fascicles (**Figures 8(F2)** and **(F3)**), lignified cell walls of xylem tissue were easily detectable. In particular, as tracheids but not vessel elements could be distinguished in the section, we concluded that ancient woods belonged to gymnosperm plants (Minnis, 1987; Yaman, 2011).

Discussion

Ancient DNA (aDNA) is the most important and informative biological component that scientists can find in archaeological areas (Gugerli et al., 2005). In fact, its analysis allowed researchers to explain, understand and know a lot of sides of human past and habitat. The presence of aDNA was also detected in underwater conditions and sunken ancient structures (Coolen & Gibson, 2009; Schlumbaum et al., 2012). These data encouraged the development of this project whose object was the taxonomic classification of botanical remains found in the underwater archaeological site of Pyrgi (Santa Marinella, Rome). Moreover, this study also shot for both promote further the use of DNA barcode scientific method, only lately applied to archaeobotany (Gould et al., 2010; Gismondi et al., 2012), and reassess the application of different microscopic techniques, in the cases where aDNA was too much damaged or not informative. In particular, in our previous work (Gismondi et al., 2012), we demonstrated that morphological observations of macroremains, also if well preserved, could sometimes induce to sample misclassifications. For this fact, the present work wants to underline the importance of the synergy of different techniques to scientifically identify past plant species. At first, as genetic investigation would have required specimen destruction, all flora ancient remains (6 different typologies, named from A to F) were photographed for macroscopic and metric documentation (**Figure 1**). Each sample was analyzed, by using molecular and observational approaches, in order to carefully recognize it. In particular, for the genetic characterization, the best

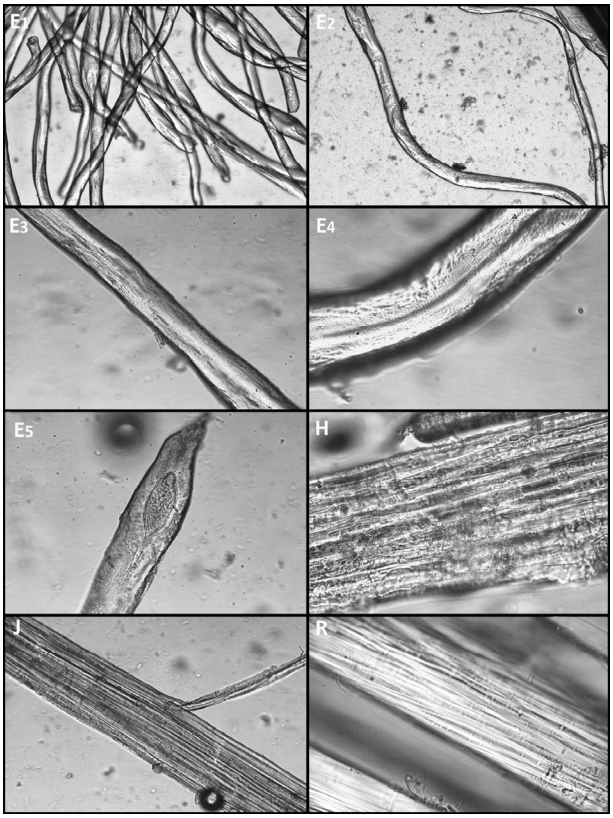

Figure 5.
Light microscope images. E1, E2, E3, E4 and E5) Sample E at different enlargement (4X-25X), H) hemp (25X), J) jute (10X); R) ramie (25X).

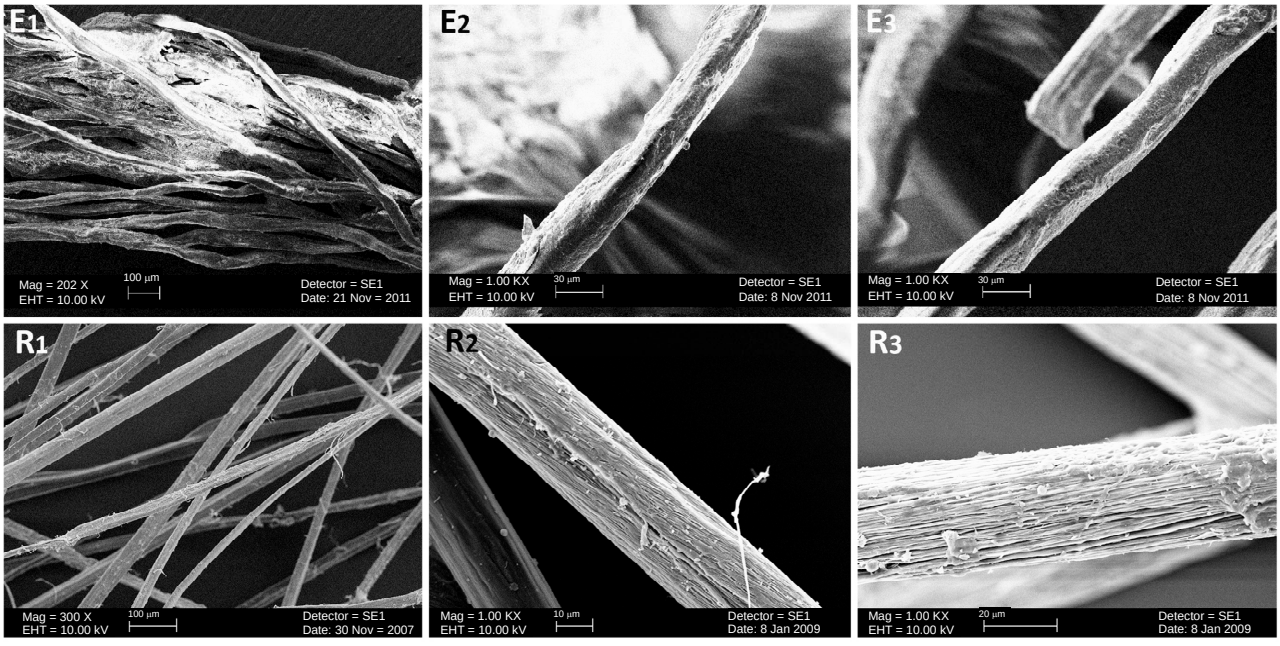

Figure 6.
SEM images of sample E (E1, E2, E3) and ramie (R1, R2, R3). Enlargement and unit bars are reported in each image.

Figure 7.
Litmus paper (on the right), used in the dry distillation assay, slightly changing its color towards basic pH with respect to control litmus paper (on the left), only imbued with H2Odd.

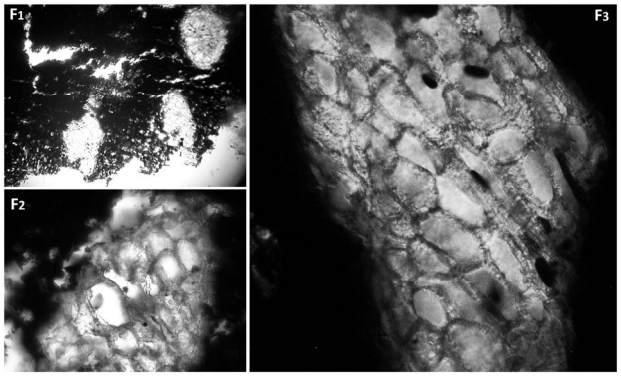

Figure 8.
Light microscope images of sample F sections. Enlargement: 10× for F1, 40× for F2 and F3.

plant barcode genes, reported in literature (Kress and Erickson, 2008), were chosen. As aDNA could easily present double strand breaks, to obtain at least one positive amplification, for each target gene, we tried to amplify more regions. So, primers were designed to produce very short (80 - 300 bp) and overlapping or consequent amplicons, according to Schlumbaum et al. (2008) and Gismondi et al. (2012). However, we also subjected aDNA to PCR amplifications of longer sequences (>500 bp) in order to determine if the template hadn't an excessive degradation level. Optimal preservation conditions of the samples can represent, in archaeogenetics, a crucial element: undamaged aDNAs, usually, are extracted from remains that were found frozen, desiccated or conserved in oxygen-free environments (Palmer et al., 2012). Probably because Pyrgi flora remains were preserved in a hermetically sealed amphora, although submerged, no external contamination or excessive physical and chemical degradation was produced on samples. In fact, in spite of the adverse environmental situation, specimen morphological and genetic studies were successfully completed. In particular, with great surprise and fortunately, aDNA extracted from A and C samples allow us to amplify even long nucleotide sequences (**Figures 3** line A3 and A6, **Figure 4** lane C), demonstrating a good preservation degree of ancient nucleic acids. In addition to anoxic conditions, in fact, remains were maybe highly preserved from the degradation by the presence in the archaeological vessel of vegetables rich in antioxidant molecules (Hansson and Foley, 2008). However, the production of long amplicons starting from ancient or degraded DNA is not so uncommon (Willerslev & Cooper, 2004; Yoccoz et al., 2012). Specimen aDNA amplifications (**Figures 3** and **4**), their sequencing and matching with nucleotide databases (Supplementary information files), in association with microscopic analyses (**Figure 2**), accurately allowed the identification of the botanical origin of sample A, B, C and D. In particular, for sample B classification the genetic analysis was essential. Therefore, aDNA techniques, even if time-consuming and expensive methods, often represent an accurate and rigorous approach able to support and balance morphological analysis lacks. Unfortunately, aDNA wasn't detectable from E and F ancient remains. In these cases, light microscopy (**Figures 5** and **8**), SEM analysis (**Figure 6**) and other experimental assays (**Figure 7**) were necessary.

Since the archaeological amphora was identified by Enei et al. (2008) as an ancient replaced of garbage can, vegetal remains, identified and classified in the present research (*Olea europaea* L. and *Crataegus monogyna* Jacq. seeds, a *Pinus* sp. pollen cone, a *Quercus petraea* (Mattuschka) Liebl. acorn, animal fibers and gymnosperm woody fragments), surely could be considered as food leftovers or remainders of antique tools. Therefore, this work increased insights about Central Italy past human activity and habitat of the 1st Century BC: in fact, it indicated the possible vegetation history and the plant species that were used, grown or traded by people of that period. On the other hand, we improved and encouraged further the complementary application of microscopic techniques and DNA barcoding to archaeobotany, underlining the innovation of its relevance also on samples derived from underwater archeological areas.

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Supplemental data files (1, 2, 3 and 4) are available on demand contacting the corresponding author (canini@uniroma2.it).