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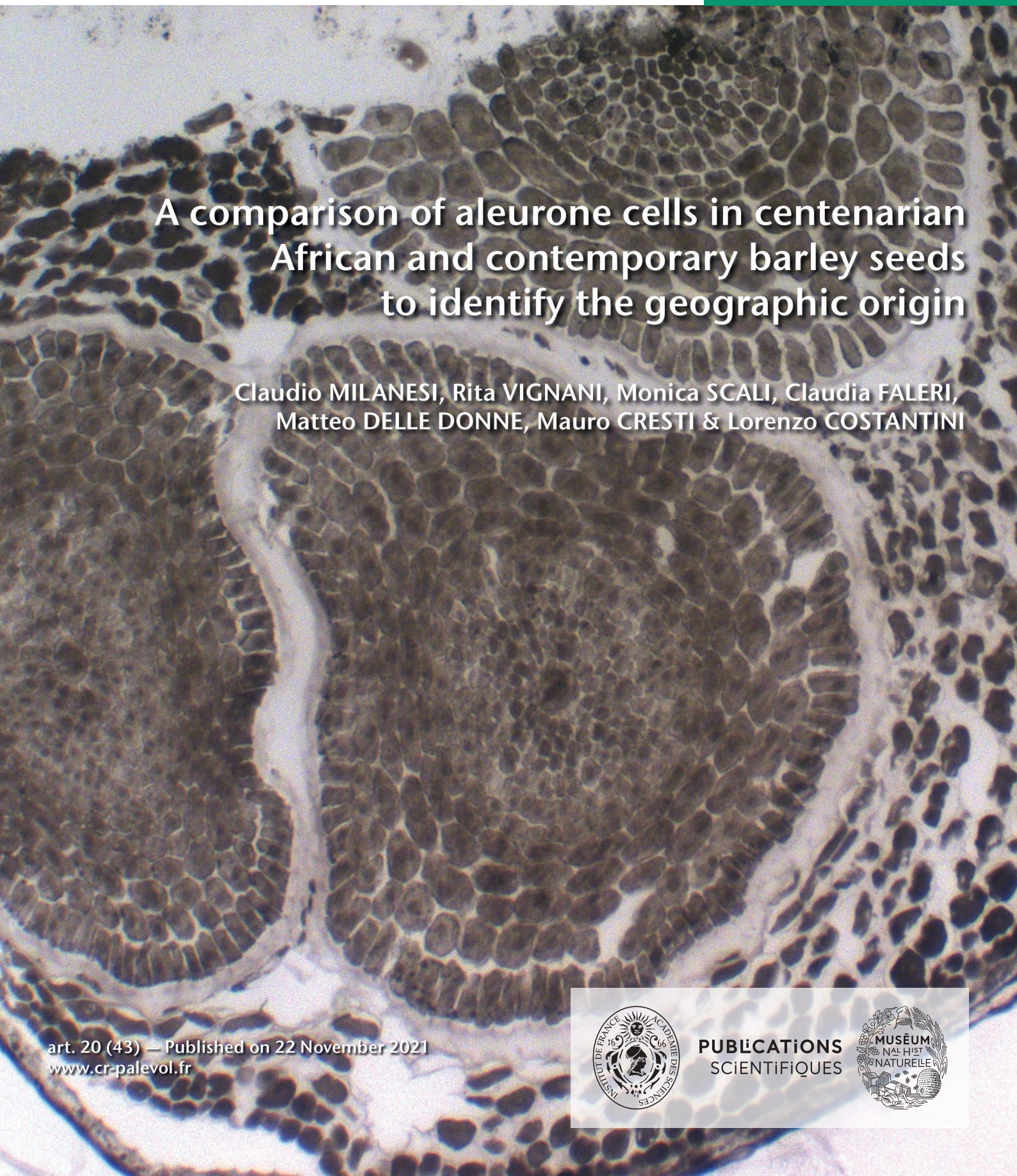

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Claudio MILANESI, Rita VIGNANI, Monica SCALI, Claudia FALERI, Matteo DELLE DONNE, Mauro CRESTI & Lorenzo COSTANTINI

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# A comparison of aleurone cells in centenarian African and contemporary barley seeds to identify the geographic origin

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## ABSTRACT
Barley (*Hordeum vulgare* L.) is one of the main domesticated cereals. For this reason, barley seeds have been found in numerous archaeological sites, and since the mid-19th century have been available in numerous natural museum collections. About a hundred years ago samples were collected in the African countries of Eritrea and Cyrenaica (now Libya), and have been preserved as ex-situ in the museum collection "L'Orientale" of the University of Naples. The varieties of contemporary barley selected for comparative analysis were grown in Tuscany and are used in the production of craft beer. To ascertain their vitality, the ancient and contemporary seeds were placed in Petri dishes to hydrate under a sterile hood at room temperature after a sterilization procedure. Morphological and ultrastructural observations performed on the aleurone cells of the ancient samples presented vital cells. The extraction and purification of DNA from seeds produced results while the genotype comparison of ancient and contemporary barley varieties enabled the construction of a dendrogram of similarity, useful in describing barley from museum genetic heritage collections and in providing a molecular imprint of extant varieties.

KEY WORDS
Aleurone cells, ancient DNA, dormancy seeds, factory beer, SSR nuclear genomic, ultrastructure.

**RÉSUMÉ**
*Une comparaison des cellules d'aleurone dans les graines d'orge centenaires et contemporaines d'Afrique pour en identifier l'origine géographique.*
L'orge (*Hordeum vulgare* L.) est l'une des principales céréales domestiquées par l'homme. C'est pour cette raison que des graines d'orge ont été retrouvées sur de nombreux sites archéologiques et que depuis la seconde moitié du XIXe siècle, ces graines sont disponibles dans de multiples collections de référence. Il y a une centaine d'années, des échantillons ont été collectés en Érythrée et en Cyrénaïque (actuelle Libye) pour être conservés *ex situ* dans la collection « L'Orientale » du musée de l'Université de Naples. Les variétés actuelles d'orge, comparées aux graines archéologiques, ont été cultivées en Toscane et utilisées pour produire de la bière artisanale. Pour s'assurer de leur vitalité, les graines archéologiques et actuelles ont été hydratées à température ambiante sur boîtes de Petri préalablement stérilisées. Les structures histologiques et morphologiques ont été observées sur l'aleurone des graines archéologiques présentant des cellules vivantes. L'extraction et la purification de l'ADN issu de grains a permis la comparaison du génotype des variétés anciennes avec celui des variétés actuelles à l'aide d'un dendrogramme de similitude. Cette étude permet de décrire et de comprendre l'héritage génétique des variétés des collections de référence et de fournir une série de traits moléculaires, propre à chaque variété actuelle.

**MOTS CLÉS**
Cellules d'aleurone, DNA ancien, graines dormantes, bière d'usine, génomique nucléaire SSR, ultrastructure.

## INTRODUCTION

Barley has been one of the main species of cereals cultivated since ancient times by sedentary populations, and has genetically adapted to various climatic environments (Pourkheirandish *et al.* 2015; Mascher *et al.* 2016; Schmid *et al.* 2018). It is adaptable to environmental stress, including quite cool climates and soil salinity (Baik & Ullrich 2008; Goyal & Ahmed 2012; Allaby 2015). Wide uses of human consumption and malt in the brewing and distilling industries make barley the fourth crop in world ranking.

The morphology of the endosperm cell in barley and other cereal caryopses can be assessed by light microscopy staining (Zhao *et al.* 2016; Matsushima & Hisano 2019). Additionally, during the greatest expansion of the electron microscopy technique, barley grain ultrastructure was studied in-depth (Gram 1982). The total number of cells in the endosperm is higher than that of wheat or rice, which is why barley grains contain more material in the cell walls, such as starch, lipids, antioxidants and fibres (Newman & Newman 1992). Most of these nutrients accumulate in the layer of aleurone cells that are located on the periphery of the endosperm, a tissue providing physical protection during seed development (Yan *et al.* 2014). The aleurone appears as a single layer of cells with a cuboidal shape and reinforced cell walls which divide to form more layers during development (Brown & Lemmon 2007). Aleurone development begins approximately five days after fertilization of the cell inside the embryonic sac (Wilkinson & Tucker 2017). During germination, the aleurone is responsible for the release of hydrolytic enzymes that degrade the polysaccharides of cell walls and starch granules a fundamental requirement for barley malt production (Aubert *et al.* 2018).

Recently, seed conservation has been given great attention due to their longevity. This characteristic has influenced many researchers to study the genetic heritage of ancient seeds and their ability to germinate even after long periods of extreme environmental conditions. However, finding intact archaeological seeds with good structural conditions is quite rare, such as in the case of palm seed germination after 2000 years (Sallon *et al.* 2008). Cultivated barley and its wild progenitor (*Hordeum vulgare* spp. *spontaneum*) are part of a wider common gene pool in which greater allelic diversity was detected in wild accessions (Nandha & Singh 2013; Ferreira *et al.* 2017). Wild and ancient barley is of great interest for breeding because its rich source of genes possibly promotes adaptation to different biotic and abiotic stresses (Nevo *et al.* 2012; Behbahanizadeh *et al.* 2016). Genetic diversity found in cultivated barley varies among different geographic areas of study (Mzid *et al.* 2016). Remarkably, genotypes from Europe showed lower genetic diversity than barley landraces from other continents (Malysheva-Otto *et al.* 2006). Despite adaptations to diverse cultivation environments, the morphology of cultivated barley did not significantly diverge after domestication.

The visible phenotype of domesticated barley did not diverge in all aspects from its wild form. Spike rachis brittleness has remained the only well-characterized domestication trait that exhibits a clear dimorphism between wild and domesticated subgroups, which are characterized by brittle and non-brittle spikes, respectively (Pourkheirandish *et al.* 2015). In this context, the study of barley spikelets is commonly used by archaeo-botanists to detect wild and domestic barley from early Holocene sites (Tanno & Willcox 2012). The model of a monophyletic barley origin has long persisted in the scientific community. Classical studies favoring a monophyletic origin of barley based on allelic frequencies at 400 AFLP, polymorphic loci were studied in 317 wild and 57 cultivated lines (Badr *et al.* 2000). However, in contrast with the analyses based on locus-specific DNA markers (such as the Simple Sequence Repeats [SSR]), genome-wide analyses based on high numbers of SNPs (Single Nucleotide Polymorphisms), or next generation sequence techniques, depict a quite complex ancestry model (Lazaridis *et al.* 2016; Pankin *et al.* 2018).

Wild barley populations collected from areas corresponding to the current areas of Israel and Jordan appeared genetically closer to cultivated barley strains (Forsberg *et al.* 2014). The first non-brittle barley spikes were discovered in the Fertile Crescent at excavation sites dating back to *c.* 10 KA BP (*c.* 8050 BC). Other secondary domestication centers are equally significant in the history of barley domestication (Mascher *et al.* 2016). For instance, the Horn of Africa, Morocco and the Tibetan plateau are now considered evolutionary "hot-spots" for this crop species (Pankin & Von Korff 2017). Early molecular evidence indicating that cultivated barley could descend from several distinct lineages came from classical studies by Morrell *et al.* (2014). According to his findings, obtained using 684 SNPs mapping in 18 genes, the population structure split 25 wild barley genotypes into two populations: "Eastern" and "Western". Interestingly, the study revealed that the ancestry assignments bisected the cultivation areas of Near Eastern and Asian barley landraces into the Western and Eastern clusters relative to the Zagros Mountains (Morrell *et al.* 2014). Sequencing the Btr1 and Btr2 genes allowed the origin of mutations underlying the spike brittleness of wild barley. Mutant Btr with truncated forms of the genes were associated with the loss of the brittleness phenotype – characteristic of cultivated barley strains (Pourkheirandish *et al.* 2015). Generally, studies of genetic characterization in barley have focused on small sets of elite germplasm collections, whereas population-based studies are mostly confined to wild barley (Schmid *et al.* 2018). Therefore, it is not unrealistic to state that even after years of research efforts, not every phylogeographic theory hypothesized on barley domestication has been proven, and perhaps little is known about within-population diversity in domesticated barley. Due to its economic importance, barley has been intensively subjected to contemporary breeding, leading to the development of genetically uniform, elite germplasm collections and gathering a large number of high-performing varieties in terms of environmental fitness and productivity (Fischbeck 2003). However, this intense and selective breeding has led to the loss of many local barley strains.

In Europe, most landraces were already replaced by contemporary cultivars in the 1920s, and the remaining autochthonous types were often organized in gene bank depositories, but these provide poor representation of the old biodiversity (Leino & Hagenblad 2010). Precious genetic resources, other than gene bank material, are historic specimens conserved in museums or in herbaria. The origin and age of the samples are not always well documented, and often lack information like origin, cultivar name, and dating (Jones *et al.* 2008). Even if herbarium or museum material is limited, this can potentially be valuable material to enrich living plant germplasms. Among the existing collections, the KSLA (Royal Swedish Academy of Agriculture and Forestry) seed collection is of exceptional importance. It contains more than 3300 seed samples representing 582 plant species collected in the late 19th and early 20th centuries. Although germination capacity is often lost, DNA can be extracted and analyzed (Leino & Hagenblad 2010).

An important component of the Museum of the Società Africana d'Italia (SAI), located at the University of Naples "L'Orientale", is a botanical collection including raw and processed materials and objects of plant origin collected between the late 19th and the early 20th centuries, mostly during SAI financed expeditions to Africa. The genetic prospection of museum collections and the biological classification of their accessions could help in widening the permanent exposition at the SAI Museum. It further represents a precious reference for the study of archaeological plant remains from Africa in order to reconstruct food habits, agriculture and the environment in pre-protohistoric times (Delle Donne 2019), and can be potentially useful in enriching actual grain germplasms. The first molecular genetic map of barley comprised RFLP markers (Graner *et al.* 1991), but with the advent of high-through put sequencing technologies, PCR-based molecular markers have become dominant. Simple Sequence Repeats (SSRs) have proven to be the markers of choice for marker-assisted selection (MAS) in breeding and genetic diversity studies of many animal and plant species. This is because they are user-friendly, genetically co-dominantly inherited, multi-allelic, highly informative, ubiquitous, and prone to establish exchangeable international genetic databanks (Ferreira *et al.* 2017). In the majority of classical studies, the SSR markers were developed after screening small insert/microsatellite-enriched genomic libraries for SSR motifs. Later, because of the wide availability of expressed sequence tags (EST) datasets and the development of new bioinformatics methods, it has been possible to identify and develop SSR markers from ESTs (Varshney *et al.* 2007) that rendered the SSR-EST more linked to the phenotype of the crop species of interest. Microsatellite markers have been developed in many crop species such as soybean, wheat, maize, barley, rice and potato. In cereals, they show a much higher level of polymorphism than other marker systems due to the extreme plasticity of the genome and to the polyploidy of many cereals, such as the wheat spp. SSRs are able to exhibit high levels of polymorphisms within and between species and populations (Ferreira *et al.* 2017). According to MAS selection programs, barley lines were characterized for possible traits involving drought tolerance (Gougerdchi *et al.* 2015).

The aims of the present study were to investigate the ultrastructure inside ancient barley seed cells and then compare this with a fresh contemporary samples control, and in addition to testing the state of the ancient cells we also wanted to investigate the feasibility of current methods of DNA analysis on ancient samples. DNA was extracted from dry and hydrated barley seeds in order to evaluate the best extraction conditions. The seeds used for comparison were modern barley seeds obtained from fields grown in Tuscany (central Italy) and centenarian and archaeological records taken from museum collections. The relative genotypes using 7 SSR marker mapping in the barley nuclear genome were confronted so that genetic characterization through SSR genotyping could establish analytical methods to correlate contemporary and ancient barley varieties in order to confirm the viability of ancient barley seed samples. The study could be a first step in the search for ancient genetic resources potentially useful for enriching contemporary germplasm collections.

TABLE 1. — Historical and contemporary barley grain samples with their accession numbers, origin and description. Chronology: late 19th early 20th century.

| Barley seeds samples | Museum accession code | Geographical origin | Annotation | Chronology | Phenotype |
|---|---|---|---|---|---|
| Eritrea | OSA-695 | Eritrea Hamasien | Orzo nudo | End of the 19th century AD | Naked/Dry seeds |
| Cirenaica | OSA-714 | Libia Cirenaica | Orzo distico | End of the 19th century AD | Distic/Dry seeds |
| Concerto | – | Siena Tuscany IT | – | Contemporary | Dry seeds |
| Traveller | – | Siena Tuscany IT | – | Contemporary | Dry seeds |
| Scarlett | – | Siena Tuscany IT | – | Contemporary | Dry seeds |

TABLE 2. — Simple Sequence Repeats (SSR) marker list. Seven SSR markers tagging different chromosomal sites in the barley genome were used for assessing genetic variability among contemporary, centenarian and archaeological barley seeds.

| SSR | CH | FORWARD | REVERSE | REPEAT | AVERAGE SIZE (bp) |
|---|---|---|---|---|---|
| Bmac0134 | 2H | CCAACTGAGTCGATCTCG | CTTCGTTGCTTCTCTACCTT | (AC)28 | 148 |
| Bmac0156 | 7H | AACCGAATGTATTCCTCTGTA | GCCAAACAACTATCGTGTAC | (AC)22(AT)5 | 139 |
| Bmac0181 | 4H | TAGATCACCAAGTGAACCAC | GGTTATCACTGAGGCAAATAC | (AC)20 | 177 |
| Bmac0713 | 7H | GGTAAAACATTTCCCTCGT | TAGAGATCACTCTCTTCTGTGC | Not available | 168 |
| Bmag0131 | 3H | TTTCAGAAACGGAGTTTTG | CCTCCACACAAAAAATCC | (AG)16G(AG)15 | 149 |
| Bmag0135 | 7H | ACGAAAGAGTTACAACGGATA | GTTTACCACAGATCTACAGGTG | (AG)10GG(AG)12 | 161 |
| Bmag0211 | 1H | ATTCATCGATCTTGTATTAGTCC | ACATCATGTCGATCAAAGC | (CT)16 | 174 |

## MATERIAL AND METHOD

### Seed selection and sterilization

The ancient seeds (Eritrea and Cyrenaica) were taken from the botanical collection of the SAI, housed by the University of Naples "L'Orientale", which includes objects and derivatives of vegetable origin collected between the end of the 19th century and the beginning of the 20th century, mostly during Italian scientific study campaigns in Africa. The morphology and genetic characterization of three commercial contemporary varieties of barley (Concerto, Traveler and Scarlet), chosen based on preliminary morphological similarity, was done in collaboration with a local brewery which provided the samples from their cultivated fields located around Siena. More details on each sample, including the original annotation, can be found in Table 1.

Twenty-five seeds for each ancient and contemporary sample were selected and placed under a biohazardous laminar flow hood irradiated with UV for 72 h in sterile conditions. Ten samples for each sterilized seed category were taken and used for molecular analysis while the remaining per all samples were subjected to the rehydration procedure. The seeds were put in 50 mL Falcon tubes at room temperature. With the help of a pair of forceps the seeds were put into a tube with 25 mL 50% NaCl (v/v) of sterilization solution, and then turned upside down every ten minutes for a total of five times to ensure the seeds and the NaCl (v/v) solution uniformly mixed. The seeds were then rinsed twice with 50 mL of sterile mQ water, each time letting the seeds pellet in the tubes for 10'. The seeds hydrated in 100 mm × 20 mm Petri dishes that were prepared with sterile moist absorbent Whatman® Cellulose Filter Paper (Sigma-Aldrich). The seeds were then gently transferred from the Petri dish to sterile, moist, absorbent filter paper with a pair of forceps and kept in the dark for 72 h at room temperature. The seeds were later kept for five days in the light to mature, and then subjected to ultrastructure investigations and molecular analysis.

### Light and transmission electron microscopy

For morphological observations five ancient and five fresh seed samples which had previously been subjected to a sterilization and rehydration procedure, were cut in half with a microscopy blade and observed with a stereomicroscope.

To examine the original morphological ultrastructure of the non-hydrated seeds, ten ancient dried Eritrea and Cyrenaica seeds were added to the ten ancient and fresh samples previously subjected to the sterilization and rehydration procedure. Then the embryo, the starchy endosperm and the aleurone layer of all the samples were selected from the apex of each seed (see Fig. 1 Hatch box) and put in 10 ml Falcon capsules in a 3% glutaraldehyde diluted in 0.066M cacodylate buffer for 60' and washed in the same buffer, then post fixed in osmium tetroxide at 1% dissolved in a 0.066M cacodylate buffer for 60', rinsed three times in the same buffer and one in H2O, dehydrated gradually in ethanol, then slowly embedded in epoxy resin and polymerized at 70° C for 8 h. The samples were finally sectioned with ultramicrotomes LKB III and a diamond knife and then collected in slides and viewed with an optical microscope Zeiss Axiophot for morphological observations; for ultrastructural investigations they were collected in 3 mm diameter greed stained with uranyl acetate 1% for 20' and lead citrate for 5' and then observed with a Philips Morgagni 268D transmission electron microscope.

## Molecular study

### *DNA extraction*

Genomic DNA was extracted from dry and hydrated barley seeds in order to evaluate the best conditions for extraction. Current commercial kit protocols were used for both the modern and ancient seeds. One of the goals of this work was to test the feasibility of current commercial protocols on aDNA-rich samples. We used the Qiagen DNeasy Plant Mini kit for 20 mg of dry seeds and 50 mg of hydrated seeds. DNA integrity was checked by the incorporation of Ethidium Bromide (EtBr) on a standard 1% agarose gel run at a constant 80 Volts for 30 min in a TAE 1x buffer. 250 ng of Lambda DNA cut with Hind III was used as ladder.

### *PCR amplification at SSR loci of the barley genome*

The DNAs extracted from the barley varieties and types were genotyped using seven SSR (Table 2) markers mapping in the nuclear barley genome: Bmac0134, Bmac0156, Bmac0181, Bmac0713, Bmag0131, Bmag0135, Bmag0211 (Ramsay *et al.* 2000). Each accession was amplified in double technical replicas. The PCR reaction consisted in a total volume of 12.5 µl containing: 2.5 µl DNA, 0.25 mM of dNTPs, 0.25 µM of each primer, 1× Green Go Taq Reaction buffer containing 1.5 mM MgCl2, 0.1 U of Taq DNA Polymerase (Promega). PCR Conditions: Denaturation step at 95° C for 5'; then 39 Cycles of: 30 s at 95° C and 30 sec. at 55° C 1' at 72° C, Final extension of 10' at 72° C.

### *Agarose gel electrophoresis to check SSR amplification*

After SSR marker amplification a gel electrophoresis was prepared. A solution of TAE 1× containing 1% (w/v) agarose was prepared. Five µl of each DNA (either barley total DNA, or PCR obtained from SSR amplifications) were loaded in the wells. 250 ng of lambda/HindIII or 1Kb ladder were generally used as a reference for the alleles generated by nuclear marker amplification, and uncut lambda DNA was the reference for the genomic barley. The running parameters used were 100 V, 200 mA, 45'. At run completion DNA quality was estimated on gel by Ethidium Bromide incorporation upon analysis on a UV transilluminator. Total DNA concentrations were estimated by spectrophotometry.

### *Genotyping*

The polymorphism of microsatellites was examined by sizing the alleles using capillary electrophoresis, which works on the principle of electrokinetic energy; ensuring the migration of the DNA towards the positive electrode from the sample kit. The genotyping was done on an ABI 3130 DNA sequencer and fragment analyzer (Applied Biosystems). 1 µl of PCR product(s) was added to 10 µl of highly deionized Formamide (Sigma Aldrich) and 0.2 µl of the internal standard; GeneScan 400 HD labelled with ROX fluorescent dye (Thermo Fisher Scientific). The samples were denatured at 95° C for 5' and later kept on ice for 2' before loading onto the DNA sequencer at a temperature of 60° C. Evaluation of allele size after comparison with the internal standard was done by the use of GeneMarker free software (SoftGenetics, ver. 1.2). Cluster analysis of the barley varieties was performed by the UPGMA (Unweighted Pair Group Arithmetic Means Average) method using the SAHN subprogram of NTSYS 2.0 Software (Exeter Software, East Setauket, NY). A dendrogram of similarity was generated.

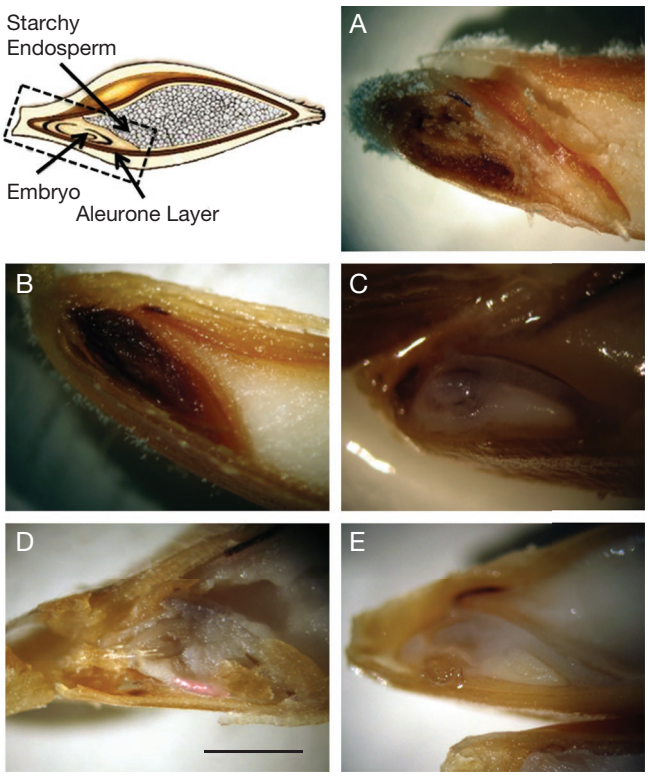


Fig. 1. — Embryo graph selected (hatch box) for stereomicroscope experiment observations of ancient (**A**, **B**) and fresh (**C**, **D**, **F**) embryo seeds. Scale bar : 0.8 mm.

## RESULTS AND DISCUSSION

All types of ancient and contemporary barley seeds described in Table 1 underwent sterilization and rehydration procedures. The goal was to not introduce external foreign materials that could have possibly altered their natural and physiological state, and to exclude any possible contamination in the subsequent phases of morphological and molecular investigations.

Internally the barley seeds are made up of the embryo, the endosperm, the layer of aleurone cells and the external integument (Fig. 1) and each confers multiple biological functions during maturation. In particular, barley contains more aleurone cells than wheat and other cereals. These strengthen the seed wall (Brown & Lemmon 2007) and after about five days of hydration of a quiescent seed they proliferate and increase in number (Wilkinson & Tucker 2017); Aubert and colleagues (2018) have further added that in the first stage the aleurone cells appear as a single layer and subsequently form multiple layers.

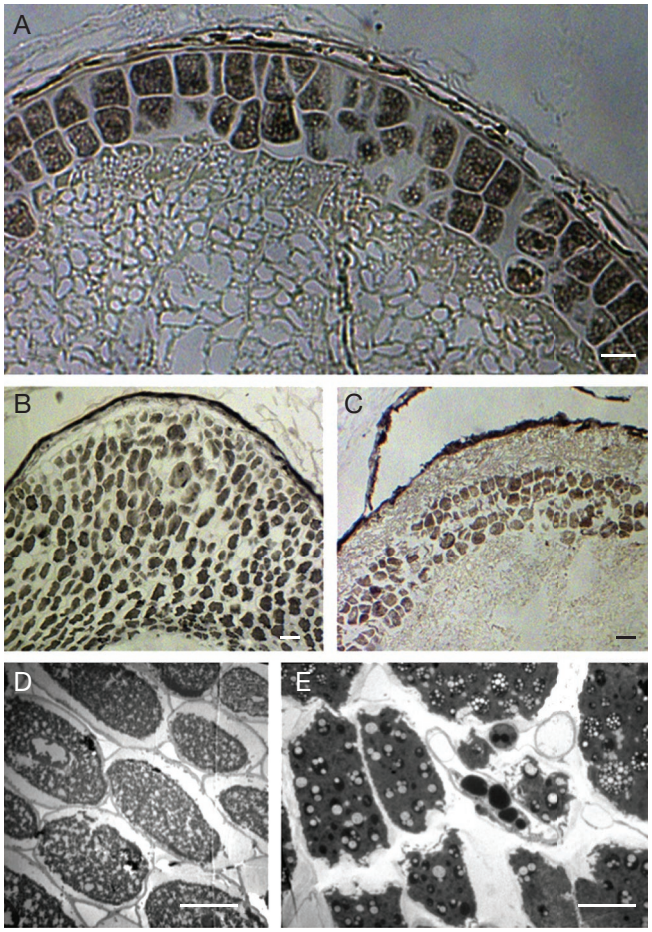


FIG. 2. — Light microscopy and transmission electron microscopy morphological observation of ancient and fresh seeds: **A**, light microscopy original ancient dried with the thickness of the aleurone cell wall; **B**, light microscopy revitalized contemporary showing the increase of the number of the cell wall; **C**, light microscopy revitalized ancient showing the increase of the number of cell wall; **D**, transmission electron microscopy well preserved contemporary aleurone cell; **E**, Transmission electron microscopy well preserved ancient aleurone cell. Scale bars: A-C, 20 µm; D, E, 10 µm.

Our samples were hydrated and irradiated by light for five days and the result found under the stereomicroscope of the longitudinal section showed that ancient seeds (Fig. 1A, B) are very similar to contemporary seeds (Fig. 1C-E).

The morphological investigation was performed on all samples described in Table 1, but this research was aimed at monitoring the physiological changes of the ancient seeds after hydration and not to present a panoramic detailed morphological investigation of all the samples. Thus, in this paper we show morphological and ultrastructural data relating to the ancient dried and hydrated seeds of Cyrenaica compared with some hydrated seeds of the contemporary variety, Scarlet.

Optical microscopic observations of the morphology of the aleurone cells of the Cyrenaica dried seeds confirmed that this layer was composed of two cubic aleurone cells (Fig. 2A). The hydrated Scarlet contemporary seeds instead showed an exponential increase in the number of cells of aleurone layers (Fig. 2B), and surprisingly the hydrated ancient Cyrenaica showed increasing aleurone cells which also formed a double layer (Fig. 2C).

By ultrastructural transmission electron microscopy investigations observed that in contemporary seeds about 92% of these cells were well-preserved while in ancient seeds only 40% showed an excellent ultrastructure while the others were degenerated and empty. In particular, for the well-preserved samples the contemporary aleurone cells (Fig. 2D) were very similar to the ancient ones (Fig. 2E), both showed electron-dense cytoplasmic materials with an abundance of lipids, starch, vacuole and ribosomes. The surprising result of the increase in the number of aleurone cells and their revitalization shows that a metabolic mechanism was reactivated, which is not a guarantee to promote seed germination but facilitated molecular investigations with total aDNA extraction.

The beta-amylase gene is the most abundant in wheat development and accumulates in aleurone cells during the early stages, filling cereals with a link between molecular pathways, which influence the early development of aleurone (Aubert *et al.* 2018). Further, the aleurone cells are enriched in 60% starch providing energy for germination. Barley seeds also contain 2-3% of lipids (Newman & Newman 1992). During germination, the embryo releases gibberellic acid, which translocate to the aleurone where it induces the transcription of genes encoding hydrolytic enzymes (Fath *et al.* 2000). Enzymes, such as 1,3; 1,4-β-glucanase (β-glucanase), α-amylase and β-amylase, are released to catalyse the breakdown of cell wall polysaccharides and starchy energy reserves that are essential for germination and the production of malt for brewing (Betts *et al.* 2017). Seed dormancy, an adaptive trait that favours plant sexual reproduction and the adaptation of plants to diverse environmental conditions was recently revealed to be a complex phenomenon regulated by a decision-making centre localized within the dormant seeds, which perceives temperature transitions to produce hormone responses (Tophama *et al.* 2017).

Genomic DNA was extracted from dry and hydrated barley seeds in order to evaluate the best conditions for extraction. The DNA extraction from dry seeds (data not shown) was more efficient in terms of DNA yield compared to hydrated seeds, probably due to the presence of a greater quantity of polysaccharides, proteins, tannins and polyphenols in the hydrated seeds, deriving from the metabolic re-activation of the seed cells (Abdel-Latif & Osman 2017). Total DNA was extracted from museum and contemporary hydrated seeds, and the museum degraded DNA was detected on agarose gel (Fig. 3A). Total DNA concentration and its degree of purity were estimated by spectrophotometry (Table 3). After SSR amplification a gel electrophoresis was prepared. Results from the museum barley genome, amplification at two SSR loci: Bmac0156 and Bmac0134 were shown after gel analysis on a UV transilluminator (Fig. 4). For all the samples genotype information was obtained. The genotypes were obtained using 6 out of 7 SSR markers, since the Bmag0131 was not always able to produce detectable amplification results.

Degraded DNA is defined as ancient DNA or aDNA (Wales *et al.* 2014; Mikić 2015). The major issues in aDNA studies we can list are: chemical degradation, mutations or the presence of chemical compounds that can impair downstream molecu-

TABLE 3. — DNA quantifications. DNA Spectrophotometer readings extracted from museum and contemporary seed samples.

| Barley Seed Samples | Total DNA (ng/µL) | 260/280 ratio | 260/230 ratio |
|---|---|---|---|
| Eritrea 3 | 11.84 | 1.34 | 1.45 |
| Cirenaica 5 | 9.43 | 1.43 | 1.79 |
| Concerto | 82.11 | 1.75 | 1.88 |
| Scarlett | 52.27 | 1.68 | 1.71 |
| Traveller | 30.44 | 1.62 | 1.87 |

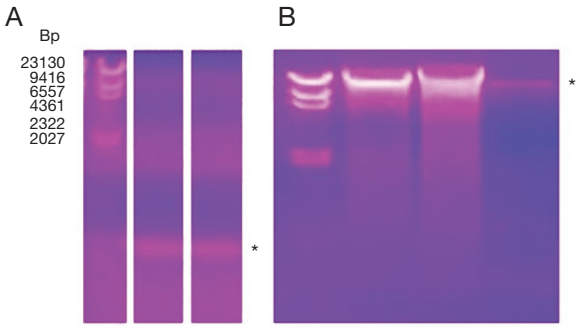


FIG. 3. — Total DNA purification. Ethidium-Bromide staining of 1% Agarose gel to check DNA extraction procedures: **A**, first left lane molecular weight standard of 250 ng Lambda/Hind III Ladder, second and third line in order 5 µL of purified DNA from Eritrea and Cyrenaica museum barley seeds, DNA appears degraded and migrating in the lower part of the gel (see *); **B**, first left lane molecular weight standard of 250 ng Lambda/Hind III Ladder and then from left 5 µL of purified DNA of contemporary barley varieties in order Concerto, Scarlett, Traveller (DNA see *).

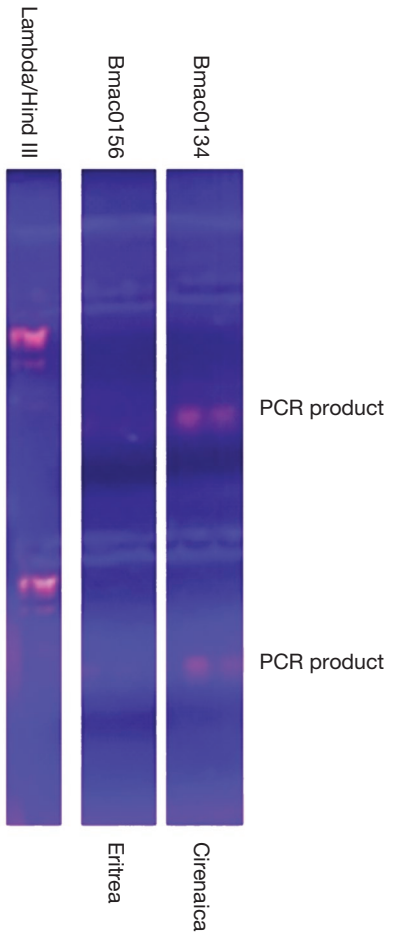


FIG. 4. — SSR amplifications checked on agarose gel. Museum barley genome amplifications at two SSR loci: Bmac0156 and Bmac0134. From left: Lambda/Hind III was used as a ladder. The PCR products from Eritrea and Cyrenaica were loaded in double.

lar biology application. However, the study of aDNA may be crucial for crop history if integrated with "ancient genomics", as well as other inputs including research done by historical linguistics, both general and relating to agriculture (Brown *et al.* 2015). It has long been debated that aDNA is unable to produce reliable results. Recent work (Fernández *et al.* 2013; Wales *et al.* 2013; Schiermeier 2015; Ucchesu *et al.* 2016) has demonstrated that contamination problems associated with the analysis of archaeological remains, including charred barley and wheat seeds, can be managed by the implementation of protocols in commercial kits. In our study, despite that no DNA was apparently detectable by conventional analytical methods (nanospectrophotometer, fluorimeter or agarose gel), our results demonstrate that even the ancient barley seed contained some genuine nuclear DNA and that this could be amplified by conventional PCR leading to a partial genotype. Deepening molecular genomics of barley aDNA may provide taxonomists with the answers to diverse questions related to the phylogeny of this important grain and crop species. The similarity dendrogram of the contemporary and ancient barley accessions was based on the observed genotype obtained at 6 SSR markers. Based on the number of observed alleles, the genotype diversity of ancient barley appears to be significantly higher than in contemporary barley.

Interestingly, the museum accession Cyrenaica (Fig. 5) branches in the same cluster as the Concerto, Scarlett and Traveller. According to the preliminary data obtained, SSR analysis seems to be able to discriminate samples in the barley population according to the respective geographical origin. As a matter of fact, Cyrenaica, one of the two Italian colonies in Libya (Northern Africa), is located in the Eastern region of Libya, while Eritrea takes its name from the nearby *Erythræan Sea* (Red Sea, Eastern Africa). In the first part of the 20th century, Italian botanists improved our knowledge about the different varieties of barley from this African region (Chiovenda 1912; Ciferri 1942a, 1942b). As highlighted, the degree of genetic diversity appears higher in museum barley than in contemporary varieties. This could easily be the result of a tendency of loss of genetic biodiversity, a trend characterizing many crop species since the advent of the green revolution in highly industrialized countries where industry prefers standard material to the detriment of loss of biodiversity (Hunter *et al.* 2017).

This study has further proven that even commercial kits optimized for complex matrixes efficiently purify DNA out of difficult biological specimens. The data obtained from the

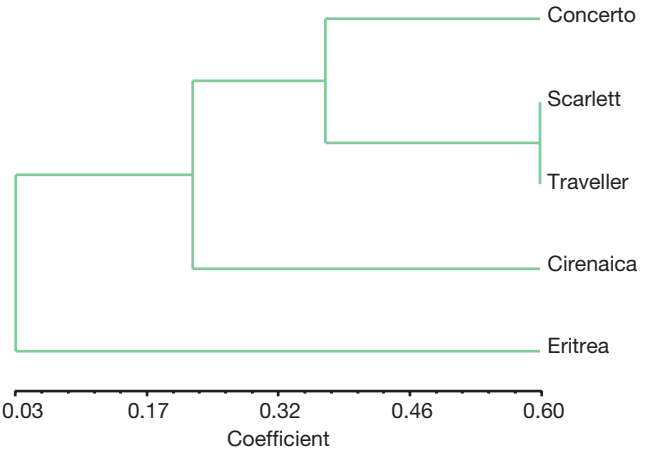


Fig. 5. — Cluster dendrogram of barley accessions. Barley accessions show a high degree of genetic diversity. Scarlett and Traveller are only 60% genetically similar. Cyrenaica branches together with contemporary barley ccessions, sharing only 20% of genetic similarity. Based on observed alleles the genotype diversity of ancient barley appears to be significantly higher than in contemporary barley.

museum sample is promising in terms of possible future studies, where a wider panel of similar samples could be analyzed by current genomic approaches such as Next Generation Sequencing (NGS) technologies, which could shed light on the phylogeny of barley. Regarding immediate application of genetic characterization as it was carried out, one can say that more in-depth knowledge on the identity of the many plant samples sitting in private collections or conserved in museums and herbaria could be gained. This might help in improving museum material management, in exploring neglected plant germplasm accession and seeds found to be alive still might be the first step in developing new plant varieties/strains (https://www.kamut.com/en/discover/the-story) distinguishable from the standard, commercial ones. Finally this approach tends to provide local products, such as artisan beers, with not only territorial rooting but also a historical perspective, putting actual barley varieties in correlation with antique or even archaeological ones. Furthermore, based on current technologies it would be extremely easy to authenticate the value of local products produced with particular barley strains via DNA testing. Molecular authentication of local products based not only on documental traceability but on voluntary certifications using DNA testing would add great value to territorial products, rendering them identifiable and defensible on international markets.

## CONCLUSIONS

The morphological study of the in vitro hydrated ancient barley seeds showed about 40% starch and lipid-rich aleurone cells, confirming a reactivation of the metabolic mechanism. This reactivation was not a guarantee to confirm seed germination but facilitated with total aDNA extraction. Genomic DNA was extracted from the seeds and demonstrated that commercial kits are reliable for complex matrices and capable of purifying DNA from biologically difficult samples. Genetic analysis of barley seeds recovered from private collections and kept in museums and herbariums can provide key insights into many important archaeological research issues, including barley phylogeny, exploration of neglected germplasm plants, domestication processes and human interactions with the environment. For commercial interests the study was expanded due to the participation of a brewery interested in craft beers looking for links between the varieties of barley in use and historical varieties whose cultivation was interrupted years ago. In this case, the centenary variety Cyrenaica shows small genetic homologies with some varieties of commercial barley, chosen based on preliminary morphological similarity. Molecular prospection of local genetic resources gives local products, such as artisan beers, not only territorial rooting but also historical perspective as the actual barley varieties are compared to antique or even archaeological ones. Furthermore, based on current technologies, it would be quite straightforward to provide molecular identification tags on local products. Analytical authentication of local products based not only on documental traceability, but on voluntary certifications using aDNA testing, would add great value to territorial economy, rendering products identifiable and defensible on international markets.

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