Molecular and morphological characterisation of the oldest *Cucumis melo* L. seeds found in the Western Mediterranean Basin

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Abstract

In 2008-2009, a rescue excavation uncovered an intact Late Bronze Age well in Sa Osa, Sardinia (Italy). The structure yielded a large number of waterlogged plant remains, of which a group of melon seeds (*Cucumis melo* L.) were some of the most remarkable. These seeds represent the earliest recorded remains of this taxon in the Western Mediterranean and are some of the oldest ever recorded. The plant remains were preserved in anoxic conditions and were found in a perfect state of conservation, making them ideal candidates for morphometric and molecular characterisation.

A total of 96 parameters, measured using an automatic image-analysis system, were specifically designed to evaluate the morphological features of fifteen preserved whole seeds. DNA extraction from archaeological samples followed a procedure specifically set up to avoid any kind of contamination. A 123-SNP genotyping platform that had been validated previously was used.

The morphological and molecular data of the archaeological seeds were successfully compared with those of a set of 179 accessions, including landraces, of feral and wild melons from Europe, Africa, and Asia.

Both analyses confirmed that these ancient seeds did not belong to a wild melon, but instead to a cultivated one. This primitive melon could have belonged to a group of ancestral non-sweet or semi-sweet forms of *chate*, *flexuosus*, or *ameri* varieties, showing similarities to North African and Central Asian accessions. This finding is coherent with the reportedly important role of cucumber-like melons in the species' diversification process, and with the accepted role of the *ameri* group as the ancestors of the modern sweet varieties.

Keywords

Archaeobotany; Late Bronze Age; Sardinia; melon; aDNA; morphology. SNP-platform

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Introduction

Melon (*Cucumis melo* L.), which is one of the most important cucurbits worldwide, has gone through an intense process of diversification, and today shows great morphological and physiological

variation (Naudin 1859; Munger and Robinson 1991; Stepansky et al. 1999). *C. melo* has traditionally been divided into two subspecies (*melo* and *agrestis*) according to ovary hairiness (Kirkbride 1993; Jeffrey 1980, 2005), and various infraspecific classifications have been proposed according to morphological and molecular clustering. Pitrat et al. (2000) defined 16 botanical groups or varieties: *cantalupensis* Naudin, *reticulatus* Ser. (cantaloupe, muskmelon), *inodorus* H.Jac. (winter melon, casaba melon), *adana* Pangalo, *chandalak* Gabaev, *ameri* Pangalo (Asian melons), *flexuosus* L. (snake melon), *chate* Hasselq. (cucumber melon), *chito* C.Morren (American melon), *dudaim* L. (pocket melon), and *tibish* Mohamed within subsp. *melo* (which are generally distributed in Western India, Central and Western Asia, Africa, Europe and America); and *acidulus* Naudin, *conomon* Thunb., *makuwa* Makino, *chinensis* Pangalo (pickling melons), and *momordica* Roxb. (snap melon) within subsp. *agrestis* (generally found from India to the Far East); which has been recently revised by Pitrat (2016). Burger et al. (2010) subsequently referred to these varieties as horticultural groups of cultivated and feral melons. Some of these groups, such as the African *tibish* and the feral American *chito*, have recently been reclassified into subsp. *agrestis* according to molecular studies (Esteras et al. 2012, 2013). Wild melons had previously been included in a separate tribe referred to as '*agrestis*' (Naudin 1859). However, morphological and molecular studies revealed that wild types are related to the paraphyletic *agrestis* and *melo* subspecies *sensu* Kirkbride (Pitrat 2013).

While our knowledge of the origin and diffusion of the main cultivated plants has greatly increased in the last two decades, the history of vegetable crops such as *Cucumis melo* is still incomplete (Zohary et al. 2012, Paris 2015). According to the archaeological records, North Africa and South-west Asia have traditionally been considered the centres of origin of cultivated melon (Kerje and Grum 2000; Zohary *et al.* 2012), although recent studies point to the inclusion of the Australia-Malaysia region, since the wild *Cucumis* species most closely related to melons seems to be the Australian *C. picrocarpus* F. Muell (Renner et al. 2007; Sebastian et al. 2010; Telford et al. 2011). Wild forms of *C. melo* (e.g., *C. pubescens*, *C. trigonus*, *C. turbinatus*, and *C. callosus*, now considered synonyms of *C. melo*, and other wild '*agrestis*' *sensu* Naudin melons) are distributed not only across the tropical and sub-tropical belt in Africa, but also in Asia, Australia, and around the Indian Ocean (Sebastian et al. 2010). The high level of variation found in Asian melons, especially in India, has also supported the hypothesis that melon reached Africa from there (Pitrat 2013).

89 The genome diversity data, analysed using different type of markers, suggest a polyphyletic origin
90 of melon with two or three domestication events (Bates and Robinson 1995; Blanca et al. 2012; Pitrat
91 2013; Tanaka et al. 2013): one event leading to the subspecies *agrestis* in India or Eastern Asia, another
92 leading to the subspecies *melo* in Western Asia or Africa, and a third in Africa leading to the *tibish* group.
93 The oldest known archaeological record of the genus *Cucumis*, to our knowledge, is a single seed of
94 'cucumber type' found in the Spirit Cave, Thailand, in a layer dated to 5672 ± 300 BC (Gorman 1969,
95 1972). In Asia, early findings have been reported from several sites dated to between the 3rd and 1st
96 millennium BC in China (Watson 1969; Li 1969, 1970; Chang 1973; Yu 1977; Walters 1989; Purugganan
97 and Fuller 2011; Fuller 2012), Japan (Tanaka et al. 2016), Iran (Costantini 1977; Zohary et al. 2012), and
98 India and Pakistan (Costantini 1987; Kajale 1988; Walters 1989; Weber 1991; Kajale 1996; Fuller and
99 Madella 2001). The first Mediterranean records are located in Egypt (Körber-Grohne 1994; Murray 2000;
100 Zohary et al. 2012). Desiccated melon seeds were present in predynastic *Hierakonpolis* (El Hadidi et al.
101 1996; Fahmy 2001, 2003), some doubtful non-carbonised and semi-carbonised seeds were discovered in
102 the Neolithic levels of *Maadi* 3500-3350 BC (van Zeist and Roller 1993; van Zeist et al. 2003a) and
103 further seeds were found in *Amarna*, latter half of the Eighteents-Dynasty (Renfrew 1985). The presence
104 of melon in Egypt, specifically the *chate* variety, is also corroborated by several funeral depictions and
105 sculptures since at least the Old Kingdom (Keimer 1924; Germer 1985; Manniche 1989; Janick et al.
106 2007). In Syria, one seed of *C. melo* was reported in a kitchen area in *Tell Hammam et-Turkman*, dated to
107 the Early Bronze Age IV, 2500-2000 BC (van Zeist et al. 2003b). A single pollen grain of *Cucumis* sp.
108 was present in a core in Crete, at a level dated to ca. 2300 BC (Bottema and Sarpaki 2003). Moreover, in
109 Greece, three carbonised seeds were recorded from the Late Bronze Age at *Tiryns* (Kroll 1982; Körber-
110 Grohne 1994), a few others from the Iron Age in *Kastanas* (Kroll 1983, 1984; Megaloudi 2006), and a
111 considerable amount in the Sanctuary of Hera on the island of Samos, dated to the 7th century BC (Kučan
112 1995; Zohary et al. 2012). A single melon seed was also found in a Punic channel in Carthage (van Zeist
113 et al. 2001).
114 Archaeological finds greatly increase beginning with the Roman period. In Italy, several finds
115 have been reported in the north of the peninsula (Castelletti et al. 2001; Rinaldi et al. 2013), in Pompeii
116 (Murphy et al. 2013), and in Rome, in the final phases of the harbour of Trajan (Pepe et al. 2013; Sadori
117 et al. 2014). In Central, Northern, and Western Europe, the cultivation of melon is considered
118 unimportant, and often interpreted as a sign of "Romanisation" (Körber-Grohne 1994; Livarda 2008,

2011; Bakels and Jacomet 2003; Wiethold 2003). Recently, Beneš et al. (2012) have reported the discovery of melon seeds in excavations in the area of Prague Castle and Hradčany (Czech Republic) supporting the idea of the common consumption of these fruits in the early Modern period in Central Europe. In addition to the archaeological records, in Mediterranean antiquity, *C. melo* was frequently found illustrated and mentioned by ancient authors, especially from the Roman and Byzantine periods (Janick et al. 2007; Avital and Paris 2014). The iconography, description, and representation of melon increased significantly during the Middle Ages and the Renaissance (Paris et al. 2009, 2011, 2012). In Asia, there is an even earlier written record of melon. Melon is mentioned in the Shih-Ching (Book of Songs), whose editing was attributed to Confucius (551–470 BC), which includes 305 traditional songs and poems of the Western Zhou dynasty (1046-771 BC). These poems were composed between 1000 and 500 BC, approximately (Keng 1974). Detailed information about the earliest West Eurasian *Cucumis melo* records (including archaeological finds, iconographical, and written sources) is provided in Table 1 and represented in Fig 1.

A rescue excavation carried out in 2008 and 2009 in Sa Osa, in west-central Sardinia, revealed a Nuragic settlement composed of numerous wells and pits associated with living spaces (Usai 2011). These structures were dug by local communities between the Early Copper Age and the Iron Age, mostly during the Middle and Late Bronze Age. The most remarkable structure was Well-N, dated to the Late Bronze Age (Usai 2011; Ucchesu et al. 2014). Sabato et al. studied its content, highlighting the identification of a few seeds of *Cucumis melo* that had been conserved in waterlogged conditions. A few fragments were AMS radiocarbon dated to 1310-1120 cal BC 2σ (IntCal09 calibration curve, uncalibrated radiocarbon age 2980±30 BP). This date represents the earliest known record of this taxon in the Western Mediterranean Basin and is one of the oldest in the world (Sabato et al. 2015b). The anoxic conditions of the silt and a constant temperature ensured a good state of preservation, which made these seeds the perfect candidates for morphological characterisation.

Morphometric visual evaluation is commonly used to assess the shape and size of objects in order to relate quantitative physical characteristics and qualitative aspects. However, the results of this type of evaluation are limited, since a human operator can only manage a limited number of samples and parameters. Compared to conventional seed analysis, computer-aided image analysis is exponentially faster, as well as more accurate, precise, and efficient. This technique provides a significantly broader spectrum of measurements and, at the same time, replaces subjective estimations with objective

quantifications (Bacchetta et al. 2008). Several previous works using image analysis to characterise seed collections have provided excellent classification results at infra-generic and infra-specific levels (Venora et al. 2009; Grillo et al. 2010, 2012; Bacchetta et al. 2011a, 2011b; Smykalova et al. 2011, 2013; Pinna et al. 2014). Much of this research has been focused on grape, *Vitis vinifera* L. (Lovicu et al. 2011; Orrú *et al.* 2013a; Orrú et al. 2013b; Ucchesu et al. 2015; Ucchesu et al. 2016).

Melon groups display differences in fruit and seed traits (Stepansky et al. 1999; Leida et al. 2015). Specific seed parameters, such as seed length and size, have already been correlated to genetic and geographical differentiation among melon groups, distinguishing: large-seed melons, mainly cultivated in the USA, Europe, Western and Central Asia, and Northern Africa; small-seed melons, more commonly grown in Southern Africa as well as Southern and Eastern Asia; and both large- and small-seed melons, mainly found in India (Fujishita 1983; Tanaka et al. 2013; Tanaka et al. 2016). Specific melon groups have fixed seed traits, such as Far Eastern melons, thought to have originated from the Indian gene pool, probably from small-seed Indian melons (Serres-Giardi and Dogimont 2012). Recently, Sabato et al. (2015a) performed a morpho-colourimetric analysis on melon seeds using an ample core collection. This research enabled the two melon subspecies to be separated and indicated a marked differentiation between cultivated and wild melons according to seed traits. Image analysis revealed six major seed groups within the cultivated melon that can be discriminated on the basis of specific phenotypic traits, mainly associated with seed size and morphology rather than colour. These results were in accordance with molecular data, which supports the use of seed morpho-colourimetric analysis as a complementary method to DNA molecular characterisation in the study of melon diversity. Different marker systems have been employed to study genetic diversity in the species, with the SNP collections derived from re-sequencing projects (Blanca et al. 2011, 2012) proving to be the most efficient systems, as they allow the genotyping to be automated (Esteras et al. 2013; Leida et al. 2015; Sabato et al. 2015a; Nunes et al. 2017).

The number of genetic studies on archaeological remains has increased markedly in recent years. In spite of several reviews that have tried to summarise the ample literature on this subject (Wayne et al. 1999; Gugerli et al. 2005; Willerslev and Cooper 2005; Palmer et al. 2012; Brown et al. 2015), the correct approach to the problem of ancient DNA (aDNA) extraction and sequencing is still being debated (Cooper and Poinar 2000; Rohland and Hofreiter 2007; Kistler 2012; Wales et al. 2014; Orlando et al. 2015; Druzhkova et al. 2015; Brown et al. 2015). A number of extraction techniques have been assessed

using non-charred archaeobotanical remains in an attempt to find the best protocol for obtaining a large quantity of high-quality aDNA and examining the relative amplification capabilities of different polymerases (Wales et al. 2014). Even though Wales et al. (2014) recommend avoiding commercial kits, other researchers, such as Mukherjee et al. (2008) and Li et al. (2011) have used such kits successfully. The choice between one or the other of these reviewed protocols is also influenced by the research goals and the species under examination. With the recent availability of next-generation sequencing technologies and high-throughput genotyping methods, the young field of paleogenetics has been furthered, and different strategies to try to bypass specific problems of aDNA analysis have been reported (Orlando et al. 2015; Smith et al. 2015; Brown et al. 2015).

The foremost limitations in obtaining genetic information from ancient samples are contamination from other materials, the existence of compounds, such as humic acids or polyphenols that can inhibit subsequent enzymatic reactions, and DNA damage in the form of fragmentation and altered nucleotides (Willerslev and Cooper 2005; Wales et al. 2014). Degradation is not a serious limitation with PCR-based genotyping if the amplification is designed to target small fragments (Pääbo 1989; Pääbo et al. 2004; Speirs et al. 2009; Oliveira et al. 2012). However, *post-mortem* nucleotide sequence alterations, such as the deamination of cytosines or methylated cytosines into uracils or thymines, respectively, as well as guanine to adenine transitions (reviewed by Orlando et al. 2015 and Druzhkova et al. 2015), can affect the outcome of phylogenetic and population genetic analyses, including the estimates of genetic diversity. However, the level of damage is dependent on the preservation conditions of the sample (Orlando et al. 2015), and, according to previous studies, waterlogging does not seem to be bad for aDNA preservation (Schlumbaum et al. 2008; Manen et al. 2003; Elbaum et al. 2005; Pollmann et al. 2005; Gyulai et al. 2008; Speirs et al. 2009, among others).

Based on this extensive research background, the aim of the present work is to understand the possible origin and typology of the Sardinian Bronze Age melon seeds found in Sa Osa. To achieve this goal, Sa Osa samples were analysed, both morphologically and molecularly, to compare them with modern worldwide melon landraces, both wild and feral types.

Materials and methods

Seed lot details

The archaeological seeds, sampled during the excavation works carried out in 2008 and 2009 in Sa Osa (Sardinia), were found preserved under waterlogged condition, and were stored in a sterile tube with distilled water at a temperature of +5ºC at the BG-SAR (Sardinian Germplasm Bank) facilities (Sabato et al. 2015b). Fifteen fully preserved seeds were selected for morphological analysis (Fig 2), while several others were reserved for the subsequent molecular analysis.

Apart from these seeds, a total of 179 lots representative of all melon typologies from 47 countries in Europe, Africa, and Asia, including landraces, both feral and wild melons, were considered for the present study (details reported in Online Resource 1). In order to reduce misinterpretations, modern breeding lines and American landraces were not considered, where 'modern' is defined as the patented melon breeds produced during the 20th century. Most of these accessions belonged to the melon core collection built as part of the framework of a previous project (MELRIP 2007-2010; Esteras et al. 2012, 2013) and some were initially provided by the NPGS-USDA Genebank and then multiplied at the COMAV (Instituto de Conservación y Mejora de la Agrodiversidad Valenciana). Most of these lots had been genotyped with SNP (Single Nucleotide Polymorphism) markers, and extensively phenotyped for plant and fruit traits (Leida et al. 2015). To better represent melon diversity, additional seed lots, mostly Asian *flexuosus* and *dudaim*, were provided by the COMAV collection. Nine Sardinian landraces, mostly described in Attene and Rodriguez (2008), were supplied by the Agriculture Department at the University of Sassari. Finally, one additional Sardinian *ameri* was collected from a local farmer and four seed lots from Cyprus were provided by the Cyprus Germplasm Bank.

Morphometric seed analysis

The archaeological seeds were morphologically compared to 122 lots selected from those previously described (details in Online Resource 1).

Images of both modern and archaeological seeds were acquired using a flatbed scanner with a resolution of 400 dpi, 24 bit-depth, and stored in TIFF format following the protocol described in Sabato et al. (2015a). Two images of each lot were obtained with black and white backgrounds. Ancient seeds were scanned with an eye to reducing any risk of contamination. Firstly, the image acquisition of modern and ancient seeds took place in two different laboratories. Secondly, the working area, pincer, and facilities were cleaned and bleached before the scanning. Lastly, the samples were placed on a disposable acetate sheet that never came into contact with the scanner screen. The digital images were analysed using

the KS-400 V3.0 software package (Carl Zeiss, Vision, Oberkochen, Germany). The accuracy and speed of the measurements was maximised by running an automated macro, specifically developed for seed characterisation (Venora et al. 2007; Bacchetta et al. 2008; Grillo et al. 2010).

Considering that seed colour is altered in the archaeological samples, aspects such as colour and texture were not considered in this study. A total of 18 parameters describing seed size and shape were computed (Table 2), along with 78 Elliptic Fourier Descriptors (EFD) calculated according to Hâruta (2011). Stepwise Linear Discriminant Analysis (LDA) was conducted using SPSS version 20.0.

The ancient seeds were first compared to the seeds of three groups of accessions: the cultivated melons of the two subspecies, subsp. *melo* and subsp. *agrestis*, and the wild melons. At a later stage, a more detailed analysis was performed, comparing the ancient seeds with the same reference accessions, but grouped into five main groups. These five groups were established using the six major seed groups defined in Sabato et al. (2015b), but employed only the morphological seed features, excluding the colour and texture parameters (Online Resource 1):

- **Sweet melon group** (hereinafter referred to as SWG) includes the seventy-three sweet melon lots (6,766 seeds) belonging to subsp. *melo*: *cantalupensis*, *reticulatus*, *inodorus*, *ameri*, *adana*, *chandalack*, and the indeterminate landraces of subsp. *melo*;
- **Intermediate group** (hereinafter referred to as ING) includes the eighteen non-sweet and semi-sweet melons with intermediate characteristics between the two melon subspecies (1,689 seeds): *dudaim*, *chate*, *flexuosus*, and *momordica*;
- **African *agrestis* group** (hereinafter referred to as AFG) includes the nine non-sweet African *acidulus*, *tibish*, and the two African indeterminate landraces of subsp. *agrestis* (807 seeds);
- **Conomon group** (hereinafter referred to as COG) includes the fourteen sweet, semi-sweet, and non-sweet Far East Asian melons (1,366 seeds) belonging to subsp. *agrestis*: *conomon*, *chinensis*, *makuwa*, and Asian *acidulus*;
- **Wild melon group** (hereinafter referred to as WTG) includes the eight wild and feral melons (746 seeds): *chito* and wild *agrestis sensu* Naudin.

Molecular analysis

All the DNA extractions were performed at the facilities of the COMAV Institute. Six samples of the archaeological seeds were selected: four with a single seed and two with a pool of three seeds. DNA extraction of archaeological samples followed a special procedure to avoid any possible risk of contamination. The applied procedure was as follows:

- The archaeological material was not manipulated in labs where modern cucurbits had previously been processed.
- Extractions were carried out in a sterile flow-hood chamber which had previously been bleached, sealed and UV-irradiated for 12 hours.
- Non-disposable tools, such as pliers and steel beads, were autoclaved prior to the irradiation.
- All other tools involved, such as tubes, lab coats, gloves, and blades, were disposable and were UV-irradiated within the flow-hood chamber.
- All of the reagents were factory-sealed and were only opened inside the flow-hood chamber during the process.
- The seed surfaces were flushed with distilled water and then gently cleaned with a solution of 10% $Ca(OCl)_2$ w/v for one minute.
- Ancient seeds were cleaned externally with sterilized water.
- Samples were mechanically disrupted using new steel beads.
- DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was applied to extract endogenous aDNA using manufacturer's instructions with minor changes (2 hours with the initial buffer at 65º C). A new kit was used to avoid possible contamination in the employed buffers.
- To confirm the lack of contamination during the whole process, a negative control was included (a sample that follows all extraction steps without containing any archaeological or modern tissue).
- The DNA concentration in all of the samples, as well as in the negative control, was determined using the Nanodrop ND-1000 Spectrophotometer v.3.5 and visualized in an 0.8% agarose gel.
- The samples were immediately stored at -20°C in preparation for further analysis.

Based on gel visualization and on the quantification carried out, only one sample out of the six extractions yielded the minimum amount of aDNA needed for genotyping. The negative control presented no trace of DNA. The remaining DNA extractions from the melon germplasm collection (144 accessions

296 selected to represent melon diversity, Online resource 1) were carried out afterwards in the cucurbits
297 laboratory using another DNeasy Plant Mini Kit (Qiagen).

298 With the aim of comparing the ancient sample to this large germplasm collection, we used melon–
299 specific SNP markers that had already been validated and mapped in previous studies (described below),
300 implemented in a medium throughput genotyping platform, Sequenom's iPLEX® Gold MassARRAY
301 technology. Genotyping with specific primers avoids the high DNA quantity and quality requirements of
302 ngs sequencing, as well as the drawback of sequencing exogenous DNA from bacteria or other
303 contaminating microorganisms (Smith et al. 2015). The DNeasy kit extraction method was employed not
304 only to achieve high quality standards and to avoid contamination using new controlled products, but also
305 because of the absence of inhibitors. However, the amount of extracted DNA is usually very low,
306 although it is generally enough for the genotyping procedure using the Sequenom technology. This
307 technique employs mass-modified dideoxynucleotide terminators to carry out a single base extension. The
308 primers used are designed to anneal immediately upstream of the polymorphic site in order to generate
309 different allelic products when they are extended with the different terminators. The SNP call is
310 performed by detecting the distinct mass of these allelic products using MALDI-TOF mass spectrometry
311 (Gabriel et al. 2009). The SNP genotyping was performed at the Epigenetic and Genotyping unit of the
312 University of Valencia (Unitat Central d´Investigació en Medicina (UCIM), University of Valencia,
313 Valencia, Spain).

314 The reactions were performed on a 384-well PCR machine. In order to reduce manual sample
315 handling and thus cross-contamination, a high-throughput liquid handling robot, capable of processing
316 samples from over 1000 individuals per day in preparation for genotyping, was used. This step was
317 carried out in a pre-PCR lab different from the one that was used during the following steps. After the
318 amplification, in the extension step, the use of mass modified dideoxynucleotides caused the extended
319 products to have a specific mass that is unattainable by normal oligonucleotides, which constitutes
320 another way to reduce contamination by small DNA fragments. The handling of the extended primers
321 when subjected to MALDI-TOF analysis was also performed by a Samsung robot nanodispenser in a
322 post-PCR laboratory in order to reduce the possibility of contamination. For automated allele calling,
323 Agena's SpectroTyper 4.0 software was employed, although this step was followed by a thorough review
324 by an expert technician. The analysis consistently provided genotyping calls with more than 99%
325 accuracy. Marker polymorphism and genotyping suitability were validated using a sample of genotypes

whose alleles are known from previous genotyping assays with other methods or by sequencing. This enabled us to perform genotype concordance analysis, as information on these SNP alleles is available. Apart from these positive controls, negative controls were also included at this step to test the whole procedure for contamination.

A total of 123 melon-specific SNP markers, evenly distributed throughout the genome, were selected from the SNP melon collection available in the Melogene database (http://www.melogene.net/). This database contains a collection of 38,587 SNPs that were identified *in silico* in two previous re-sequencing analyses (Blanca et al. 2011; Blanca et al. 2012). The most important of these (Blanca et al. 2012) re-sequenced 67 genotypes, grouped into eight pools that represent all the cultivated and wild melon groups. Information about the SNPs used is available in Online Resource 2, and detailed information for each SNP marker, such as sequence, allele variation, and location, is available in the Melonomics database (https://melonomics.net/) and in the new consensus melon map (Díaz et al. 2015). Most SNPs used in this study had also been employed in previous genotyping experiments of the species, and their position in the melon genetic map is known (Esteras et al. 2013; Leida et al. 2015; Sabato et al. 2015a; Nunes et al. 2017). Major allele frequency, gene diversity, heterozygosity, and polymorphism information content (PIC) for each *locus* were calculated for this melon collection using PowerMarker software (Liu and Muse 2005). The genotype of the DNA extracted from the archaeological sample was then compared to that of the 144 accessions selected to represent melon diversity, which included two other Sardinian genotypes, one *ameri* (AmITS10) and one *flexuosus* (FxITS9) (Online Resource 1).

The genetic relationships among the accessions were studied using both a Principal Coordinate Analysis (PCoA) as well as a study of the population genetic structure. GenAlEx 6.501 was used to perform the PCoA, whereas STRUCTURE v.2.3.3 software was used to analyse the genetic population structure. Twenty independent runs for each K value, ranging from 2 to 10, were performed with a burn-in length of 500,000 and 1,000,000 iterations. The optimal subpopulation was calculated from the second order rate of change of likelihood (ΔK method, Evanno et al. 2005). The parameters used for the STRUCTURE analysis were: POPDATA=0, which means that the input file doesn't contain a user-defined population of origin for each individual; ancestry model: model with admixture (NOADMIX=0); linkage model: no background LD between very tightly linked markers (LINKAGE=0); locprior model: no *a priori* models for the geographical sampling location is inferred to the model (LOCPRIOR=0);

inferalpha: most individuals are admixed (INFERALPHA=1); allele frequency: default setting: λ=1, which means that in each population allele frequencies are assumed to be independent. λ=1 is usually set as in Pritchard et al. (2000) (LAMBDA=1). The increasing number of iterations: 1,500,000, and burning: 700,000. Genotyping data were depicted using GGT 2.0, a software program designed to visualise and analyse genetic data (van Berloo 2008).

Results

Morphological analysis

An initial morphological comparison applying stepwise Linear Discriminant Analysis (LDA) was carried out between the 15 archaeological seeds, added as the unknown group, and the reference collection, which was classified into three groups: the cultivated accessions of subsp. *melo* and subsp. *agrestis* and the wild melons (*agrestis sensu* Naudin) (Table 3). Based on 11,374 seeds, the analysis resulted in the classification of 92.6% of all cases. The correct classification value indicates the percentage of cases in which the classification based on the morphometric analysis agrees with the predetermined groups (Esteras et al 2013; Leida et al 2015; Sabato et al 2015b). Misclassification between wild and cultivated melon was fairly close to zero; only 4.6% of wild seeds were classified as cultivated *agrestis*. The correct classification of subsp. *melo* was also high (98.9%), whereas subsp. *agrestis* overlapped with subsp. *melo* in 21.6% of the cases. None of the ancient seeds were classified as wild melons; most were classified as cultivated *agrestis* (80%, 12 seeds), with several being classified as cultivated *melo* (20%, three seeds).

A more detailed analysis was performed comparing the archaeological seeds, considered the unknown group, to the reference accessions classified in the five main groups described in the Materials and Methods section and specified in Online Resource 1 (Table 4). Most melon seeds were correctly classified within these five macro groups, with 81.3% overall correct classification. The lowest value of misclassification was found in the Sweet melon group (SWG), which was correctly identified in 93.4% of cases, whereas the African agrestis group (AFG) and the conomon group (COG) groups were successfully classified in 71.9% and 69.3% of cases, respectively. The intermediate group (ING) overlapped with SWG in 55.1% of cases. None of the archaeological seeds were classified as SWG or WTG, whereas nine (60.0%) were classified as ING, three (20.0%) as AFG, and another three (20.0%) as COG.

Table 5 shows a list of the 28 parameters that contributed to discrimination according to the *F-to-remove* value, which indicates the weight of a single parameter in the statistical analysis. The most important traits of discrimination were related to seed dimension: area (A), diameter value of a circle with an equivalent area (Ecd), and the minimum axis value of an ellipse with equivalent area (EA_{min}). Seed shape descriptors, such as compact grade value (Com), the ratio between minimum and maximum diameters (D_{min}/D_{max}), and several Elliptic Fourier Descriptors (EFDs), also contributed to group discrimination.

Fig 3 shows a scatter-plot graph generated from the LDA data while considering each accession as an independent group. Each seed lot is represented by the average of their coordinates (centroid). The archaeological seeds are represented both individually and by the centroid. The first three functions explain 89.5%, 6%, and 3% of total variation, respectively. According to the first function, the archaeological seeds occupy an intermediate position between subsp. *melo* and subsp. *agrestis*. This position is mostly occupied by African *acidulus* and *tibish* (AcZA98, AcZW99, AcZW100, AcSN46, AcSN45, TiSN198, and TiSN199), several similar indeterminate landraces from Africa (LaMG202, LaZA47, and LaET11), as well as ancient Eurasian types, such as *dudaim* (DuGE296) and *flexuosus* (FxIQ23). All are non-sweet and semi-sweet types. Among the sweet melons, only one Ukrainian low-sugar *ameri*, one French unclassified type, and one French cantaloupe (AmUA90, LaFR151, CaFR172) were close to the archaeological seeds. Only one seed from the archaeological sample was markedly closer to the *conomon* typology (CnKR32, MkJP188, CnCH6, CoJP136 of CoJP185).

Molecular analysis

Unfortunately, only one sample from the various extractions yielded enough DNA to be visualized in an agarose gel, although DNA fragmentation was evident due to the smear observed. Therefore, the remaining samples (with values that were 0 ng/µl or negative, as measured with the spectrophotometer) were discarded. The selected ancient DNA extraction was successful in carrying out the genotyping reactions, while the negative control failed for every marker, as was expected. The genotyping results for both the modern seed collection as well as the archaeological seeds are detailed in Online Resource 3: spreadsheets A, B, C, and D. Only 18 *loci* were not amplified in the archaeological material.

The PCoA results are shown in Fig 4. The first three coordinates explained 49.05%, 4.40%, and 3.63% of the total variation, respectively. According to the first coordinate, the archaeological sample was located in the left section of the graph, grouped together with accessions of subspecies *melo*, and separated from accessions of subspecies *agrestis*, including both cultivated *agrestis* and wild *agrestis sensu* Naudin. Three *ameri* genotypes from Central Asia and Northern Africa (AmRU42 from Russia, AmMA37 from Morocco, and AmIR26 from Iran) were close to the archaeological sample. Interestingly, the group that had the closest accessions also included several Italian *ameri* (AmITS10), *chate*, and *flexuosus* landraces (ChIT27, ChIT122, FxITS9), plus additional *flexuosus* from Spain (FxEs82) and Turkey (FxTR15). All *flexuosus* and *chate* are elongated non-sweet melons (Brix degree 4 to 6), whereas *ameri* can be considered non-sweet or low-sugar melons (Brix degree 5 to 8) (Leida et al. 2015). Furthermore, some subsp. *melo* landraces that were close to the archaeological seeds also characterised as low-sugar melons, these being from Italy, France, Algeria, and Mali (LaIT00, LaFR151, LaDZ4, LaML35). Only a few representatives of modern sweet melons (some French landraces belonging to the *cantalupensis* group and a Portuguese *inodorus*) were molecularly close to the archaeological sample (CaFR179, CaFR161, CaFR121, CaFR191, InPT40).

Analysis using STRUCTURE (following the Evanno ΔK approach to determine the number of populations) gave a maximum value of K=2, separating the accessions into the two subspecies, followed by K=8 (Fig 5), which was consistent with groupings based on geographical origin and morphotypes and with previous results obtained with a larger collection (Leida *et al*. 2015).

Resolution into two populations (K=2) placed the archaeological seed within the subsp. *melo* accessions (Fig 6). The K=8 analysis differentiated two populations in the *agrestis* group. The first (Pop. 1, dark blue in Fig 7) included all African wild *agrestis* as well as the domesticated *tibish* (subsp. *agrestis*) from Sudan. The second group (Pop. 2, red) was mostly composed of accessions of the *conomon*, *chinesis*, and *makuwa* groups from the Far East (all cultivated types of subsp. *agrestis*). Most of the other accessions of subspecies *agrestis*, African and Indian *acidulus* accessions, Indian *momordica* and wild types from India, like *C. callosus* (syn. of *C. melo*) AgIN128, were in a third group (Pop. 3, green). Some accessions of Pop. 3 had a significant degree of admixture with Pops. 1 or 2. One wild *agrestis* type from India, not clearly assigned to any of these populations (AgIN204), displayed an admixture of the three populations.

There were three main populations in the subspecies *melo*. One includes most of the *inodorus* Spanish and Portuguese landraces (Pop. 8, pink in Fig 7). The second (Pop. 7, light blue) was composed of *inodorus* accessions from Southern Europe and Northern Africa, unclassified landraces, and *ameri* from Eastern Europe and the Near and Middle East. The third population (Pop. 6, orange) includes mostly Central Asian *ameri* accessions. There is a certain degree of admixture between Pops. 8 and 7 and between Pops. 7 and 6, suggesting a continuous variation. Most of the *cantalupensis* landraces from France and Italy formed a different population (Pop. 5, turquoise) with a higher degree of admixture.

The Late Bronze Age sample from Sa osa was included in a separate population along with accessions of subsp. *melo* (Pop. 4, dark purple). Pop. 4 was composed mostly of elongated non-sweet types of the *flexuosus* group from Spain, Turkey, and Afghanistan (FxES82, FxTR86, and FxAF174), along with *chate* Italian types (ChIT27, ChIT122). The two genotyped Sardinian landraces, one *flexuosus* and one *ameri* (FxITS9, AmITS10), were also present in this population, along with some *ameri* and indeterminate African and Asian landraces (AmMA37, AmRU42, AmTN84, LaML35). A few sweet accessions are also included in this population (In PT40, InES75, CaFR172). In general, these results were consistent with those obtained in the PCoA. Fruits of some of the accessions closer to the archaeological sample in Pop. 4 are shown in Fig 8. The archaeological sample was one of the accessions of Pop. 4 that displayed the highest levels of admixture. In fact, it showed variable levels of admixture with all the populations of both subspecies. A similar, but higher *melo-agrestis* admixture was found in a set of *flexuosus* accessions from North Africa, the Middle East, and India that could not be assigned to any population (Fig 7).

The analysis of allelic diversity in the archaeological melon indicated that 70 of the 105 successfully genotyped SNPs (67%) were fixed in this sample (Online Resource 3 A and B). We generated a graphical genotype of the archaeological sample along with representatives of the various STRUCTURE populations (Fig 9), along with a second one using the complete collection in Online Resource 3 C (the homozygous *loci* in the archaeological sample are called *s* in Online Resource 3 B and are represented in blue in Fig 9 and Online Resource 3 C, whereas the homozygous genotypes for the alternative allele in these *loci* and the heterozygous genotypes are called *a* and *h*, respectively, and are represented as green and yellow in Fig 9 and Online Resource 3 C). The archaeological sample was more similar to the accessions of subspecies *melo* in these fixed genomic regions than to those of subspecies *agrestis* (Fig 9). The percentage of these *loci* with *s* genotype ranged from 17.1% to 90% in subspecies

melo and from 8.6% to 54.3% in subspecies *agrestis* (Online Resource 3 B). Far Eastern *conomon*, *chinensis*, and *makuwa* types (STRUCTURE Pop. 2) were the group that displayed the lowest percentage of the archaeological genotype in this part of the genome (from 8.6% to 40.0%), followed by wild and cultivated African and Asian *agrestis* (Pop. 1 and 3, ranging from 17.1% to 47.1%). In contrast, *inodorus*, *cantalupensis*, and *ameri* from different regions of Europe, Western and Central Asia, and Northern Africa (Pops. 5, 6, 7, and 8), displayed the highest percentages of *s* genotypes (from 57.1% to 88.6%).

Apart from these fixed regions, the most characteristic feature of the archaeological genotype is the high number of amplified *loci* that were heterozygous (35; 33%). Seventeen of these (49%) carried the C/T and G/A combinations (Online Resource 3 D). These *loci* were called *h* in the archaeological sample, while in the reference accessions they were called *m*, *a*, or *h* if homozygous for the allele of the subspecies *melo*, for the allele of the subspecies *agrestis*, or if they were heterozygous, respectively (in Online Resource 3 B), and were represented as orange, green, and yellow in Fig 9 and Online Resource 3 C. The archaeological sample turned out to have one of the highest heterozygosity levels (Online Resource 3 B, D). This is a common feature of *flexuosus*, *chate*, and *ameri* from Europe, Northern Africa, and Western and Central Asia (Fig 9 and Online Resource 3 B and C), mostly from Pops. 4 and 6 and from the admixture group; most of the remaining accessions, on the other hand, were quite homozygous. The alleles of these heterozygous *loci* often differ between subspecies *melo* and *agrestis*, suggesting that the archaeological seed represents variation found in both subspecies.

Discussion

The archaeological seeds from Sa Osa belong to the most advanced culture of prehistoric Sardinia, that of the Nuragic period. During the Late Bronze and Early Iron Ages, Sardinia played a significant role in an exchange network between the western and eastern Mediterranean (Lo Schiavo 2003; Bernardini and Perra 2012). In fact, the early presence of melon during the Late Bronze Age in Sardinia may be explained as a result of this consistent commercial contact (Sabato et al. 2015b). The integrated approach that combines morphological and molecular analyses of the melon seeds retrieved in Sa Osa represents a unique opportunity to explore the history of the spread of melon in the Mediterranean Basin and Europe.

According to morphological descriptors related to seed dimension, none of the ancient seeds were similar to the current Indian/African wild types (WTG); however, they do share similarities with cultivated melons. Within this last category, they differed from the majority of the Far Eastern melons

(COG), which have smaller seeds. On the other hand, they were mainly comparable to the non-sweet and semi-sweet African *agrestis* accessions (AFG), *acidulus* and *tibish*, and to a few Eurasian *flexuosus* and *dudaim* ones. Most accessions of the intermediate group (ING) and of the sweet melons of subsp. *melo* (SWG) used as references showed higher seed dimension values compared to the archaeological seeds. However, the distance between the ancient seeds and these modern large-seed melons can be overestimated due the occurrence of human selection. A strong positive correlation has been found in melons between seed and fruit size (Sabato et al. 2015a), and after more than three millennia of constant selection with the objective of increasing fruit size, current melon landraces are likely to produce larger seeds than the archaeological forms. The increase in seed and fruit size through human selection has already been demonstrated for cucurbits (Paris and Nerson 2003; Fuller 2012; Tanaka et al. 2016), and a similar trend has been found in other cultivated plants (Fuller 2007; Fuller 2012).

Ancient DNA analysis provided additional information of great value about the typology of these ancient melons. DNA from ancient seeds was successfully extracted using a commercial kit, as previously reported by other researchers such as Mukherjee et al. (2008). Little to no inhibitors were found within this sample as most of the markers were successfully amplified (85% successfully genotyped). Only 15% of the analysed *loci* failed to amplify in the archaeological material. This failure could be due to DNA degradation or to the occurrence of additional mutations in the flanking regions of the SNPs that hamper primer annealing. These additional polymorphisms might have disappeared in the currently analysed germplasm collection due to natural evolution or human selection. In fact, the aDNA sample is the one with the most failed SNPs within the collection. These failed SNPs seem to be concentrated in LGVI, VIII, and XII. This might reflect a differential loss of polymorphism during melon evolution/selection, as has been demonstrated recently in a melon re-sequencing assay (Sanseverino et al. 2015), or it might be the result of a more intense degradation in the aforementioned genomic regions. Likewise, the heterozygosity level in the aDNA (the highest in the analysed collection, 33%) may have been overestimated, as some of these heterozygous *loci* may be a product of *post-mortem* miscoding. About 49% of the heterozygous *loci* inspected presented the genotypes C/T (26%) or G/A (23%) (Online Resource 3 D), which might be a consequence of 5-methylcytosine to thymine and guanine to adenine transitions which can occur in ancient DNA, especially in single-stranded ends (Gilbert et al. 2007; Orlando et al. 2015). However, the fact that for all these *loci,* heterozygous genotypes can also be found

in several accessions of the melon germplasm reference collection (On line Resource 3 A) suggests that they may be true heterozygotes, although these *loci* are mostly fixed in current melon germplasm.

Ancient melon clearly differs from the currently existing forms of wild melon (*agrestis sensu* Naudin) found in Africa and India, and molecular results agree on this point with morphological analysis. Additionally, molecular data indicate that these Late Bronze Age seeds are undoubtedly more different from current cultivated melons of subspecies *agrestis* than from those of subspecies *melo*. In fact, the archaeological sample was separated from the Far Eastern *conomon*, Indian *momordica*, and African *acidulus* and *tibish* by both PCoA and STRUCTURE analyses, which, moreover, were coherent with the genetic structure of the species previously reported for the reference collection (Esteras et al 2013; Leida et al 2015). According to Serres-Giardi and Dogimont (2012) and Pitrat (2013), the horticultural groups of subspecies *agrestis* were probably domesticated in at least two independent events: one in India/Eastern Asia leading to the African and Asian cultivated forms of subsp. *agrestis*, and the other in Africa, leading to the *tibish* group. A third and independent domestication event might have occurred in Western Asia or Africa resulting in the high diversity of the subspecies *melo* (Pitrat 2013). The Late Bronze Age melon from Sa Osa could represent one of the first forms of cultivated melon derived from this latter domestication event.

PCoA and STRUCTURE results show genetic similarity between the archaeological melons and *chate* and *flexuosus* landraces as well as with the *ameri* accessions, mostly from the Mediterranean basin. Accessions of these groups are usually classified as subsp. *melo*, although they are sometimes considered intermediate types between the two subspecies due to their high levels of allelic diversity (Blanca et al. 2012). The typology of *chate* is that of a kind of cucumber-like melon (elongated, non-sweet, climacteric, and with low aroma) highly-valued in southern Italy, especially in the Apulia region, where it is known as *Carosello, Meloncella,* and *Cummarazzo* (Laghetti et al. 2008). The similarities between the archaeological seeds and the current Italian *Carosello* suggest an ancient origin for this traditional landrace. The *flexuosus* accessions (also known as snake melons, the most elongated forms of melon, which are also non-sweet, non-aromatic, and climacteric) are molecularly closer to the ancient seeds, and come from Sardinia, where they are known as *Facussa* or *Cucummaru* (Attene and Rodriguez 2008), as well as from Spain and Turkey. Most of the other *flexuosus* accessions, from the Near and Middle East and India, were less similar to the ancient seeds, supporting the high variation previously reported in this group (Yildiz et al. 2011; Soltani et al. 2010; Blanca et al. 2012; Leida et al. 2015). These data suggest

561 that the archaeological sample could be a climacteric, non-sweet and low-aroma, elongated melon type
562 consumed like a cucumber. Its molecular closeness to *flexuosus* and *chate* melons agrees with the history
563 proposed for *melo* diversification, as varieties of these two elongated melons played a central role in
564 primitive crop selection. These cucumber-like forms are thought to have been cultivated at that time in
565 North Africa and Near East (Paris 2015). In fact, they are represented in 3000-year-old Egyptian
566 depictions and were undoubtedly valued by ancient Mediterranean cultures (Janick et al. 2007, Murray
567 2000). The cultivation of these varieties, consumed unripe in salad, is often mentioned by classical
568 authors, such as *Columella* (ca. 64 AD) and *Plinius* the Elder (ca. 77 AD) (Table 1). Despite being
569 considered cucumbers for many years (*Cucumis sativus*), today they are recognised as *flexuosus* and *chate*
570 melons (Janick et al. 2007; Avital and Paris 2014).

571 Regarding the *ameri* types that are closer to the archaeological seeds, they consisted of one from
572 Sardinia and four oval-to-elongated low-sugar, medium-aroma, and white-to-light orange-fleshed
573 landraces from Morocco, Tunisia, and Russia (Leida et al 2015). All these accessions were also
574 climacteric. *Ameri* types (including *ameri*, *adana*, and *chandalack*) that share properties with the non-
575 sweet *flexuosus* and *chate*, although they are less elongated and accumulate some sugars in the fruit. This
576 group originated in Central Asia and is considered the precursor of the sweet European *inodorus* and
577 *cantalupensis*, and has been reported to be one of the most variable groups of melons, which is coherent
578 with the organisation into different subpopulations described in the present work. In addition to a certain
579 flesh sweetness (they are sweet but with a lower sugar content than modern *inodorus* and *cantalupensis*),
580 round fruit shapes are also frequent in this group (Blanca et al. 2012; Leida et al. 2015). Recent reviews
581 have reported that round and somewhat sweet melons have been grown since at least Roman times,
582 although they have been mentioned less often than snake melons (Janick et al. 2007; Paris et al. 2009,
583 2011; Avital and Paris 2014; Paris 2015). The reliable presence of sugary melon in Central Asia and the
584 Middle East has been recorded since at least the 9th century, but its introduction in Europe is supposed to
585 have occurred later, probably during the Arab domination (Paris et al. 2012). Nineteenth-century sources
586 (Jacquine 1832) reported a traditional Sardinian melon that could be morphologically associable to an
587 *ameri* that they described as “mediocre”, suggesting that these fruits were not highly sweet.

588 Some *cantalupensis* or cantaloupe-like melons closer to the archaeological seeds were landraces
589 from France. These cantaloupes are also molecularly close to some Mediterranean *ameri*, from which
590 they could have derived. In contrast, the archaeological seeds were more genetically distant to the

591 *inodorus* lots, which are far from *ameri* typologies, which may suggest that they derived from a different
592 introduction line of sugary melons.
593 As we have already discussed above, the Late Bronze Age sample was more similar to cultivated types of
594 subspecies *melo*, but according to STRUCTURE it showed a quite high degree of admixture with all
595 subpopulations of both subspecies. This high degree of admixture is also found in current *flexuosus*,
596 *ameri*, and *momordica* types (Esteras et al. 2013; Leida et al. 2015). This is in accordance with its high
597 level of heterozygosity, which it also shares with some current *flexuosus*, *chate*, and *ameri* melons. Most
598 of these heterozygous *loci* have alleles that are still frequent in current melons, but which are usually
599 alternatively fixed in accessions of each subspecies, although in some cases alleles found in these
600 heterozygous *loci* in the archaeological sample have low frequency in current melons. Most of the *loci*
601 that were fixed in the archaeological sample shared the homozygous genotype with reference melons of
602 subspecies *melo*, as can be seen in the graphical genotype with representative accessions (Fig 9, Online
603 Resource 3). Only a few *loci* shared the homozygous genotype with *agrestis* types. These were mainly
604 specific regions of LG II, III, IV, and V. In some of these regions, major QTLs related to sugar content
605 and fruit shape (regions LGII 18-23cM, LGIV 0-34cm, and LGV 0-26 cM in Díaz et al. (2011, 2015)) are
606 mapped, supporting the idea that the archaeological melon might have had a more elongated shape and a
607 lower sugar content than current *inodorus* and *cantalupensis* types. For example, Argyris et al. (2014)
608 reported a QTL in LGV (CMPSNP898- CMPSNP726) in which the presence of *conomon* alleles
609 significantly decreases sugars in fruits. The archaeological samples were heterozygous or homozygous for
610 the *conomon* allele in most of the markers analysed in this region. Other *loci* also suggest the ancient
611 melons were non-sweet. Association analysis has recently been carried out in melons (Leida et al. 2015)
612 showing several markers associated with fruit sweetness or ripening behaviour. Leida et al. (2015) found
613 that marker CMPSNP711, located in LGI, is associated with fruit sugar content. This *locus* was
614 heterozygous C/T in the archaeological material, as occurs in various *flexuosus* and *chate* references.
615 Most of the *ameri* and sugary types (*cantalupensis* and *inodorus*) were homozygous for the T allele, while
616 most of the non-sweet or low-sugar ones (a few *ameri*, *flexuosus*, *momordica*, *acidulus*, *tibish*, *dudaim*,
617 *conomon*, and wild melons) were homozygous for the C allele. Another interesting region is located in
618 LGIX (CMPSNP144-CMPSNP1035). Dai et al. (2011) demonstrated that the acid invertase 2 (AIN2), a
619 gene involved in sugar accumulation in melon fruits, maps in this region. The archaeological accession in

this region is heterozygous, as in certain *flexuosus* and *chate* melons, while the two alternative alleles were fixed in most of the sweet/non-sweet melon types, respectively.

The study of these remains from the Late Bronze Age has enabled us to throw light on the domestication, diversification, and trait selection in melons, processes that are still poorly understood (Pitrat 2013). A wide variability study, based on re-sequencing, was recently published (Sanseverino et al. 2015), identifying not only SNPs but also other kinds of structural variation, like transposon insertion polymorphisms and large deletions. Regions with very low variability have been found in chromosomes I and VI of improved cultivars (which may respond to a strong selection process), and highly variable regions have also been found in chromosomes III and VIII. Therefore, a profound study of these regions could be of great benefit to the study of the genes involved in the domestication and selection of melon during its evolution.

Conclusions

The characterisation of the Late Bronze Age melon seeds from Sa Osa based on morphometric analysis and SNP genotyping was successfully carried out and has enabled the study of a crucial period in melon diversification. Both molecular and morphological analyses suggest that this archaeological sample belonged to a cultivated melon and not to a wild type. This extinct, primitive melon was probably close to varieties of *chate, flexuosus,* and *ameri*, carrying both currently frequent as well as rare alleles. Specific genomic regions suggest non-sugar/low-sugar content for this fruit, which agrees with the idea that non-sweet cucumber-like forms of *chate* and *flexuosus* melon played a central role in early selection. These elongated types seem to have been the most consumed in ancient Mediterranean cultures according to several sources, and they continue to be locally important in this region in present times. *Ameri* types, mostly diffused in the Near East and Central Asia, are thought to be the ancestors of the modern sweet varieties, such as *inodorus* and *cantalupensis,* and they also showed a certain affinity with the ancient materials. A relationship between the archaeological seeds and African landraces has also been suggested.

Despite these remarkable conclusions, a deep study of other genomic regions in this material could be of great interest in order to analyse genes involved in the domestication and selection of melon during its evolution, with special attention to important traits, such as sweetness, shape, and climacteric behaviour.

650 **Table captions**

651 **Table 1.** West Eurasian *Cucumis melo* records in chronological order from the earliest identifications
652 until approximately the 6th century AD, including archaeological finds, artistic representations and written
653 sources. Chronology is approximate as none of the archaeological remains, apart from the records from
654 Sa Osa, have been directly radiocarbon dated.

655 **Table 2.** List of morphometric characters measured on each seed, excluding the Elliptic Fourier
656 Descriptors (EFDs), calculated according to Hâruta (2011).

657 **Table 3.** Results from the stepwise LDA comparing the archaeological seeds (archaeo), considered the
658 unknown group, cultivated melon subspecies (*C. melo* subsp. *melo* and *C. melo* subsp. *agrestis*) and wild
659 melon (*C.melo agrestis sensu* Naudin). Percentage and number of seeds are indicated for each category.

660 **Table 4.** Results from the stepwise LDA comparing the archaeological seeds (archaeo), considered the
661 unknown group, and the five groups with similar morphological characteristics considered in this study.
662 SWG (sweet melon group) includes all sweet melon lots belonging to subsp. *melo*: *ameri*, *inodorus*,
663 *cantalupensis*, *reticulatus*, and the indeterminate landraces of subsp. *melo*; ING (intermediate group)
664 includes non-sweet melons with intermediate characteristics between the two melon subspecies: *dudaim*,
665 *chate*, *flexuosus*, and *momordica*; AFG (African *agrestis* group) includes all African *acidulus*, *tibish*, and
666 the two African indeterminate landraces of subsp. *agrestis*; COG (conomon group) includes all sweet,
667 low-sugar and non-sweet Far East Asian melons belonging to subsp. *agrestis*: *conomon*, *chinensis*,
668 *makuwa*, and Asian *acidulus*; WTG (wild melon group) includes wild and feral melons: *chito* and wild
669 *agrestis sensu* Naudin. Percentage and number of seeds are indicated for each category.

670 **Table 5.** Morphological features used for discrimination among groups sorted in decreasing order of *F-to-*
671 *remove* values, which describes the power of each variable in the model. The *Tolerance* indicates the
672 proportion of a variable variance not accounted for by other independent variables in the equation. *Wilks'*
673 *lambda* is a direct measurement of the proportion of variance in the combination of dependent variables
674 that is unaccounted for by the independent variable.

675

676 **Figure captions**

677 **Fig 1.** Map of published West Eurasian *Cucumis melo* records earlier than Sa Osa. For reference numbers
678 see Table 1.

679 **Fig 2.** The Late Bronze Age waterlogged melon seeds from Sa Osa (Cabras, Sardinia).

Fig 3. LDA analysis results of morphological comparison between the archaeological melon seeds and the modern collection. Each seed lot is represented by the average of its coordinates (centroid). The archeological seeds are represented both individually and by the centroid. SWG (sweet melon group) includes all sweet melon lots belonging to subsp. *melo*: *ameri*, *inodorus*, *cantalupensis*, *reticulatus*, and the indeterminate landraces of subsp. *melo*; ING (intermediate group) includes non-sweet melons with intermediate characteristics between the two melon subspecies: *dudaim*, *chate*, *flexuosus*, and *momordica*; AFG (African *agrestis* group) includes all African *acidulus*, *tibish*, and the two African indeterminated landraces of subsp. *agrestis*; COG (conomon group) includes all sweet and semi-sweet Far East Asian melons belonging to subsp. *agrestis*: *conomon*, *chinensis*, *makuwa*, and Asian *acidulus*; WTG (wild melon group) includes wild and feral melons: *chito* and wild *agrestis sensu* Naudin.

Fig 4. PCoA analysis showing the molecular results from the archaeological seeds and the modern melon collection.

Fig 5. Estimated number of clusters obtained with STRUCTURE for K values from 2 to 9 using SNP data for the entire germplasm collection. Graphical representation of the derivative statistics of the estimated mean L (k) (ΔK) (Evanno *et al*. 2005) and estimated probabilities of K (L(K)=LnP(D)) as an average value of 20 runs for each K from K=2 to K=10.

Fig 6. Inferred population structure with best K choice (K=2) in which accessions are represented by a line with different-colored segments according to their estimated belonging to the corresponding populations. The blue line represents subsp. *agrestis* and the red one subsp. *melo*. The archaeological sample is indicated with an arrow.

Fig 7. Inferred population structure with second best K choice (K=8) in which accessions are represented by a line with different-colored segments according to their estimated belonging to the corresponding populations. The dark blue line represents African '*agrestis*' (Pop. 1), the red line represents *conomon* (Pop. 2), the green line represents *acidulus* and *momordica* (Pop. 3), the dark purple line represents *ameri* and intermediate *flexuosus-chate* types (Pop. 4), the turquoise line represents *cantaloupensis* landraces (Pop. 5), the orange line represents Central Asian *ameri* (Pop. 6), the light blue line represents *inodorus* from Southern Europe and Northern Africa and *ameri* from Eastern Europe and the Near-Middle East (Pop. 7), and the pink line represents Spanish *inodorus* (Pop. 8).

Fig 8. Fruits of landraces included in Population 4 that are close to the archaeological sample according to STRUCTURE analysis.

Fig 9. Graphical representation of genotyping results from the archaeological seeds and several representatives of the populations obtained by STRUCTURE using GGT2 software. Grey: failed SNPs; blue: homozygous SNP for allele *s* from archaeological seeds; green: homozygous SNPs for allele *a*, the most common in subsp. *agrestis*; orange: homozygous SNP for allele *m*, the most common in subsp. *melo*; yellow: heterozygous SNPs (*h*).

Online Resource captions

Online Resource 1. Seed lots detail. Code abbreviations: Ca= *cantalupensis*, In= *inodorus*, Am= *ameri*, Fx= *flexuosus*, Ch= *chate*, Du= *dudaim*, Co= *conomon*, Cn= *chinensis*, Mk= *makuwa*, Mo=*momordica*, Ct= *chito*, Ti= *tibish*, Ag= *agrestis*, La= indeterminate landraces. The third and fourth letter indicates the provenience according to ISO 3166-1 alpha-2 country code.

Online Resource 2. SNP details: Information about the 123 SNP markers employed in the genotyping assay and summary statistic results generated in genotyping analysis with PowerMarker software. In Esteras *et al.* (2013) and Leida *et al*. (2015), markers were experimentally validated and further information is available.

Online Resource 3. Genotyping results: **Spreadsheet A** Genotyping data of the entire melon collection. **Spreadsheet B** genotyping data expressed as *s*, *a*, *m*, and h for graphical genotype construction and related data such as percentages of heterozygosity or failed SNPs. **Spreadsheet C** Graphical genotypes obtained with GGT2 software for the entire collection ordered by the STRUCTURE population. **Spreadsheet D** Information about heterozygous *loci* for the archaeological sample.

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1083 **Table 1**

	Age		Country	Place	Reference
1	3800-3500 BC 3750-3300 BC	A	Egypt	Hierakonpolis	El Hadidi et al. 1996[1,2] Fahmy 2001[1], 2003
2	3500-3350 BC	A^{+}	Egypt	Maadi	van Zeist and Roller 1993[1,2], van Zeist et al. 2003a[1]
3	3000-2000 BC	A	Oman	Hili	Tengberg 2003
4	2686-2181 BC	G	Egypt	several	Keimer 1924[1,2,3,4],Germer 1985[2,3]
5	2500-2000 BC	A	Syria	Tell Hammam	van Zeist et al. 2003b[1]
6	ca. 2350 BC	P*	Greece	Kournas, Crete	Bottema and Sarpaki 2003
7	ca. 2000 BC	A	Iran	Shahr-I Sokhta	Costantini 1977[1,3]
8	1550-1300 BC	G	Egypt	Theban Necropolis	Manniche 1989[2,4]
9	1517-1192 BC	G	Egypt	Theban Necropolis	Darby et al. 1977[2,3,4]
10	1350-1330 BC	A	Egypt	Amarna	Renfrew 1985[2]
11	**1310-1120 BC**	**A**	**Italy**	**Sa Osa, Sardinia**	**Sabato et al. 2015b**
12	1200-1000 BC	A	Greece	Tirynth	Kroll 1982[1,3,5]
13	1050-900 BC	A	Greece	Kastanas	Kroll 1983[3,5], 1984
14	700-600 BC	A	Greece	Heraion, Samos	Kučan 1995[1,5]
15	ca. 350 BC	A	Tunisia	Carthage	van Zeist and van der Veen 2001
16	100 BC-500 AD	A	NW Europe	several	Livarda 2008, 2011
17	10-0 BC	A^{++}	Switzerland	Vindonissa	Jacomet et al. 2002
18	15-40 AD	A	Italy	Mutina	Rinaldi et al. 2013
19	50-200 AD	A	Egypt	Mons Porphyrites	van der Veen and Tabinor 2007
20	50-250 AD	A	C Europe	several	Bakels and Jacomet 2003
21	ca. 64 AD	W	Italy	-	Columella [4,6]
22	ca. 77 AD	W	Italy	-	Gaius Plinius Secundus[4,6]
23	ca. 79 AD	A	Italy	Pompei	Murphy et al. 2013
24	100-200 AD	A*	Egypt	Mons Claudianus	van der Veen 1996[2], 2001
25	100-300 AD	G	Tunisia	several	Balmelle 1990, Blanchard-Lemé et al. 1995, Yacoub 1995[4]
26	180-250 AD	A	France	Alesia	Wiethold 2003
27	200-300	A	France	Chalon-sur-Saône	Marinval 2000
28	220-651 AD	A	Turkmenistan	Merv Oasis	Nesbitt and O'Hara 2000
29	ca. 250 AD	G	Greece	Thessaloniki	Pazaras 1981[4]
30	ca. 260 AD	W	Italy	-	Quintus Gargilius Martialis[6]
31	300-400 AD	G	Spain	Mérida	Álvarez Martínez et al. 2000[4]
32	ca. 400 AD	W	Italy	-	Palladius[6]
33	ca. 400 AD	W	Italy	-	Apicius[6]
34	500-600 AD	G	Lebanon	-	Baratte 1978[4], Balmelle et al. 1990[4]
35	500-600 AD	A	Italy	Portus	Pepe et al. 2013, Sadori et al. 2014
36	550-600 AD	A	Tunisia	Carthage	van Zeist and van der Veen 2001

A = Archaeological finds
G = Artistic representations
W= Written sources
P = Pollen record
* identified as *Cucumis* sp.
$^{+}$ doubtful find
$^{++}$ identified as cf. *C. melo*

[1] References reported in Zohary et al. 2012
[2] References reported in Murray 2000
[3] References reported in Körber-Grohne 1994
[4] References reported in Janick et al. 2007
[5] References reported in Megaloudi 2006
[6] References reported in Paris et al. 2012

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Table 2

Shape parameters	
A	Seed area (mm^2)
P	Seed perimeter (mm)
P_{conv}	Convex perimeter of the seed (mm)
P_{Crof}	Crofton's perimeter of the seed (mm)
P_{conv}/P_{Crof}	Ratio between P_{conv} and P_{Crof}
D_{max}	Maximum diameter of the seed (mm)
D_{min}	Minimum diameter of the seed (mm)
D_{min}/D_{max}	Ratio between D_{min} and D_{max}
EA_{max}	Maximum axis of an ellipse with equivalent area (mm)
EA_{min}	Minimum axis of an ellipse with equivalent area (mm)
Sf	Seed shape descriptor: $4\pi A/P^2$ (normalized value)
Rf	Seed roundness descriptor: $4A/\pi D_{max}^2$) (normalized value)
Ecd	Diameter of a circle with equivalent area (mm)
F	Seed length along the fiber axis (mm)
C	Curl degree: ratio between D_{max} and F
Conv	Convexity degree: ratio between P_{Crof} and P
Sol	Solidity degree: ratio between A and Convex area
Com	Compactness degree: $(\sqrt{2}\ (4/\pi)\ A)/D_{max}$

1089 **Table 3**

	subsp. *melo*		subsp. *agrestis*		Wild melon		Total	
	%	n°	%	n°	%	n°	%	n°
C. melo subsp. *melo*	98.9	8072	1.2	96	-	-	100.0	8168
C. melo subsp. *agrestis*	21.1	539	71.8	1830	7.1	181	100.0	2550
Wild melon	-	-	4.6	30	95.4	625	100.0	656
Archaeo	**20.0**	**3**	**80.0**	**12**	**-**	**-**	**100.0**	**15**

- 92.6% overall classification

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Table 4

	SWG		ING		AFG		COG		WTG		Total	
	%	n°	%	n°	%	n°	%	n°	%	n°	%	n°
SWG	93.4	6317	5.2	351	1.4	94	0.1	4	-	-	100.0	6766
ING	55.1	930	44.2	747	0.6	10	0.1	2	-	-	100.0	1689
AFG	7.6	61	7.1	57	71.9	580	13.5	109	-	-	100.0	807
COG	4.4	60	2.9	39	11.3	155	69.3	947	12.1	165	100.0	1366
WTG	-	-	-	-	-	-	11.8	88	88.2	658	100.0	746
Archaeo	**-**	**-**	**60.0**	**9**	**20.0**	**3**	**20.0**	**3**	**-**	**-**	**100.0**	**15**

- 81.3% overall classification

Table 5

	Parameter	*F-to-remove*	*Tolerance*	*Wilks' lambda*
1	**A**	253.072	0.001	0.059
2	**Ecd**	193.983	0.001	0.057
3	**EA_{min}**	141.611	0.005	0.056
4	**Com**	132.240	0.003	0.055
5	**FD22**	85.535	0.593	0.054
6	**FD14**	81.101	0.200	0.054
7	**FD6**	72.134	0.011	0.054
8	**FD11**	66.003	0.144	0.053
9	**FD10**	64.820	0.337	0.053
10	**FD26**	37.425	0.663	0.052
11	**P**	35.608	0.004	0.052
12	**FD18**	31.017	0.676	0.052
13	**FD2**	21.483	0.978	0.052
14	**D_{min}/D_{max}**	21.474	0.015	0.052
15	**FD42**	17.029	0.721	0.052
16	**FD15**	15.730	0.299	0.052
17	**FD47**	13.211	0.632	0.052
18	**FD21**	7.645	0.641	0.051
19	**FD36**	7.273	0.404	0.051
20	**FD13**	6.563	0.270	0.051
21	**FD12**	5.957	0.219	0.051
22	**FD56**	5.898	0.476	0.051
23	**FD40**	5.679	0.282	0.051
24	**FD24**	5.335	0.376	0.051
25	**FD52**	5.326	0.505	0.051
26	**FD23**	4.643	0.278	0.051
27	**FD50**	4.364	0.790	0.051
28	**FD75**	4.056	0.941	0.051