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**Archaeological seeds and local varieties of edible fruits: morphology
through time**

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Presentata da:	Diego Sabato
Coordinatore Dottorato	Prof. Gianluigi Bacchetta
Tutor	Prof. Gianluigi Bacchetta Dott.ssa Leonor Peña-Chocarro Dott. Gianfranco Venora
Co-tutor	Dott.ssa Laura Sadori

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GENERAL INTRODUCTION

During the recent past, archaeobotanical studies have reached a significant position in the production of archaeological knowledge. The main aims of the archaeobotanical research are to explore the way in which plant resources were used and managed by human groups (Peña-Chocarro *et al.* 2000).

This discipline has an enormous potential for investigating the relationships between human communities and the natural environment, looking at the various aspects of plant use related to both cultivated and wild plants (Peña-Chocarro and Zapata 2003, Peña-Chocarro 2007). Diets, food procurement, agricultural practice (processing, storage, food preparation), rituals, are some of the main topics in which the study of plant remains can provide information (Mercuri 2008, Peña-Chocarro 2009, van der Veen 2011); furthermore, plant remains are a powerful tool to investigate topics related to ancient societies and their evolution, as long-term transformations of environment into a cultural landscape (Jacomet and Brombacher 2005, Birks 2012, Mercuri and Sadori 2012)

Recently Mercuri *et al.* (2014) summarized the state of art of archaeobotany research in Italy, pointing out that the multiproxy and integrated archaeobotanical approach centred on archaeological sites is one of the most specific and developed across Europe.

Sardinia has remained for a long time a blank gap on the map of European archaeobotanical research; records from early periods are rather scarce and are mostly related to casual findings (Castaldi 1987, Sadori *et al.* 1989, Trump 1990, Costantini and Stancanelli 1997, Celant 1999, Celant 2000, Costantini 2002, Celant 2010). Some further researches on macro-remains have been carried out on samples of Phoenician-Punic period; although still pertinent to punctual discoveries (Montanari 2003, van Dommelen and Finocchi 2008, van Dommelen *et al.* 2008a, 2008b Miola *et al.* 2009, Pérez Jordà *et al.* 2010). Bakels (2002) ushered in the first extensive work on archaeobotany in the island, mainly focalized on Bronze Age period, which built up the bases for last newest researches in this field (Ucchesu 2014, Ucchesu *et al.* 2014a, 2014b Sabato *et al.* 2015 [**Chapter 1**]). Further works have been carried out also for the Medieval Age (Becca *et al.* 2013, Bosi and Bandini Mazzanti 2013). Meanwhile, archaeopalynology made also great progresses in Sardinia in the last two decades, producing important information on environmental changes in the Holocene period (Acquaro *et al.* 2001, López *et al.* 2005, Pittau *et al.* 2012, Di Rita and Melis 2013). Two archaeological sites have been studied within this thesis: a late Bronze Age well at Sa Osa, near Cabras (Sabato *et al.* 2015 [**Chapter 1**]) and Phoenician-Punic amphorae contexts from Santa Giusta lagoon [**Chapter 2**]. The most remarkable discovery was the find of *Cucumis melo* seeds at Sa Osa, since it represents the earliest evidence from Western Europe and one of the most ancient finds of this *taxon*. Zohary *et al.* (2012) suggested that melon cultivation might have begun during the Bronze Age in the Near East and/or in Africa, although Janick *et al.* (2007) highlighted that this crop played a marginal role until at least the Classical period. Sweet melon became widely spread in Europe only in 15th century AD rather later its introduction into the Iberian Peninsula during the Arab domination (Paris *et al.* 2012). The exceptional nature of this record encouraged me to enhance the information about these finds by further analyses described in [**Section 2**]. The identification of seeds is not always easy, especially on broken and distorted archaeological materials, and many different species can produce similar seeds within the same genus or even among different genus. In addition, it is almost impossible to distinguish varieties within the same species despite the fundamental value that accurate identification may have to trace the presence of edible and useful plants. From the very beginning of this discipline researchers tried to find morphological correlations with

the aim of distinguish between wild and cultivated plants (Stummer 1911). Several works have obtained good results on correlation of seed size, since seeds tendency increase their dimension under cultivation processes (Fuller 2007). In any way the results of these types of works are limited, since the human operator can only manage a limited number of samples and parameters, beside of larger error gap. Compared to conventional measurements, computer-aided morpho-colourimetry is exponentially faster, more accurate, precise and efficient, providing a significantly broader spectrum of measurements of morphological and colourimetric features and, at the same time, replacing subjective estimations with objective quantifications (Bacchetta *et al.* 2008). Several works about the application of image analysis to the diaspores of wild vascular flora have been carried out, providing excellent results of classification within taxonomic units close to infra-generic, infra-specific and intra-population levels (Bacchetta *et al.* 2008, Venora *at al.* 2009a, Venora *at al.* 2009b, Venora *at al.* 2009c, Grillo *et al.* 2010, Bacchetta *et al.* 2011a, 2011b; Pinna *et al.* 2014). Many studies have been focused also on crop wild relatives and landraces (Smykalova *et al.* 2011; Smykalova *et al.* 2013), and recently some authors focused on *Vitis vinifera* varieties (Rivera *et al.* 2007; Terral *et al.* 2010; Bouby *et al.* 2013, Orrú *et al.* 2013a) and two recent works correlated shape features of archaeological grape pips and Sardinian grape cultivars (Ucchesu *et al.* in press, Orrú *et al.* 2013b).

DNA sequence analyses can provide corroboration, resolution, support, and accuracy for those parts of phylogeny for which appropriate morphological data is lacking (Scotland *at al.* 2003). For example, one of the latest works performed with Sardinian grape cultivars correlated shape features with molecular discrimination; in this case the multiproxy approach allowed to achieve a clear discrimination among local cultivars and revealed the synonymy groups of local names attributed on same cultivar (Orrú *et al.* 2013a). Positive results have been obtained both on molecular and seed morphological traits on pumpkin (Liu *et al.* 2013). The goal of [**Chapter 3**] is to compare the phenotypic characterization achieved by seed features with the molecular analysis on melon genotypes, while [**Chapter 4**] and [**Chapter 5**] to correlate melon traditional landraces with archaeological seeds.

Archaeogenetic strongly improved in the last two decades (Palmer *et al.* 2012). In spite of waterlogging does not seem to favour DNA preservation, as hydrolysis is one of the major decay reactions (Schlumbaum *et al.* 2008), waterlogged plant remains have already been used as a good source of ancient DNA (Schlumbaum *et al.* 1998, Manen *et al.* 2003, Elbaum *et al.* 2005, Pollmann *et al.* 2005, Gyulai *et al.* 2008, Speirs *et al.* 2009). Small size degraded DNA fragments may be successfully amplified using the PCR (Polymerase Chain Reaction) designed to target small fragments, as an average length of 50-500b (Pääbo *et al.* 2004, Oliveira *et al.* 2012). Ancient DNA extraction has been successfully carried out on Late Bronze Age seeds from Sa Osa [**Chapter 4**] and some Medieval seeds form Sassari [**Chapter 5**].

THESIS STRUCTURE

This research is divided in two main sections and five chapters:

[SECTION 1] ARCHAEOBOTANY

Aims:

- Determining richness, variety and frequency of plant macro-remains from different archaeological sites,
- Providing data on the subsistence system of inhabitants of the investigated sites,
- Evaluating the range of domestic species used and understanding the role of agriculture and its degree of development,
- Exploring the role of wild plants within the economy of the sites,
- Investigating agrarian practices and crop processing,
- Reconstructing the past landscape,
- Comparing the dataset of plant remains with other Mediterranean areas,
- Improving the archaeobotanical knowledge in Sardinia.

This section is composed of two chapters:

[CHAPTER 1] describes the research carried out on waterlogged plant remains from a Late Bronze Age well found near Cabras (1310-1120 cal BC), in an area named Sa Osa (Central-West Sardinia). Despite the limited set of samples, the combination of macro-remain and pollen analyses in this unique context provided important information for exploring not only local subsistence systems but also human impact on the surrounding environment. Grapes and figs were the most abundant remains together with other fruits and edible vascular plants. Remains of melon and mulberry were identified being the earliest remains of these two species for Western Europe. Their presence may confirm early trade between Nuragic people and the eastern Mediterranean and/or African coasts. Melon seeds have been used for further molecular and morphological researches [Chapter 4]. Intentional selection of wood suggests practices associated to the collection of raw material for specific technological demands. The presence of intestinal parasites in the pollen record points to the possible use of the well as a cesspit, at least in its later use, and this is one of the earliest evidence of this type of structures in prehistoric contexts.

[CHAPTER 2] concerns the plant remains contained in some Phoenician-Punic amphorae and depositional contexts found at the bottom of Santa Giusta lagoon (Central-West Sardinia). These amphorae have been related to two deposition phases, one dated to the 6th-5th century BC and a second dated to the 3rd-2nd century BC. Many of them contained also ovine/caprine bones with butchery marks associated with grapes and other fruits, as plums, sloes, and junipers, which may have played a role in meat preservation. Other fruits and nuts found in the same contexts, such as hazelnuts, walnuts, pine nuts, almonds and olives were probably related to food trade. The few remains of cucurbits such as watermelon and bottle gourd suggest contacts with Africa.

[SECTION 2] APPLIED PLANT BIOLOGY

Aims:

- Implementing statistical classifiers able to recognize and discriminate seeds belonging to different varieties,
- Comparing the groups established using molecular analyses with those achieved by seed morphology,
- Increasing the knowledge about the variation of the current extant melon seed collections,
- Recording local varieties partially, or not longer cultivated due to traditional agriculture abandon,
- Analyzing the variability of morpho-colourimetric seed features,
- Extracting and sequencing ancient DNA of archaeological seeds,
- Matching archaeological seeds molecular and morphological characters to their modern relatives,
- Studying origin and diffusion of ancient cultivation.

This section is composed of three chapters:

[CHAPTER 3] describes the phenotypic characterization achieved by seed features compared with molecular analysis on modern *Cucumis melo* genotypes. A set of 124 accessions of *Cucumis melo* has been selected for molecular and morpho-colourimetric analyses plus an additional selection of accessions of *Cucumis sativus*, *Citrullus lanatus* and *Citrullus colocynthis* used to highlight seed morphology distances among genus and species. A strong correlation has been found between the two characters. Both molecular and seed morpho-colourimetric analyses confirm the existence of two melon subspecies while an intermediate group has also been found. A non-random allelic distribution in SNPs located in specific genomic regions suggests that some of these regions may account for a part of the observed variation in seed size. Six major groups of varieties can be discriminated on the basis on seed traits.

[CHAPTER 4] describes the comparison of molecular and morphological seed features of Late Bronze Age melon seeds found in Sa Osa [Chapter 1] with a set of 172 accessions of melon landraces, including 10 traditional Sardinian cultivars. Both molecular and morphological analyses confirmed that these archaeological seeds did not belong to a wild species, but to a cultivated melon, likely to be an intermediate form between the two melon subspecies. Reasonably, this primitive melon could be attributed to an ancestral non-sweet or low sugar form of *chate*, *flexuosus* or *ameri* varieties. The first two show cucumber-like genotypes that played a central role in early melon selection while *ameri* is thought to be the ancestors of the modern sweet varieties, such as *inodorus* and *cantalupensis*. A possible connection with African and Central Asian accessions has been roughed out.

[CHAPTER 5] describes the comparison of molecular and morphological seed features of Medieval melon and watermelon seeds discovered in a well in via Satta, in the centre of Sassari. Molecular characterization has been carried out on the same reference set described in [Chapter 4] while the morphological comparison of watermelon seeds was based on 36 *Citrullus lanatus* and *C. colocynthis* European, African, Asian and Sardinian landraces. Molecular and morphological analyses matched with both sweet and

non-sweet melon accessions. These varieties, nowadays mainly diffuse in South Mediterranean, North Africa and Central Asia, played an important role on first melon selection. Data suggests that several types of melon were already cultivated in Medieval Age while the present sugary melons became widely diffuse only in later phases, as pointed out in previous research. Morphological characterization of watermelon seeds evidenced a close relation with Sardinian, Spanish and Asian landraces, suggesting that watermelons were already close to modern varieties. Since at the time of findings Aragonian kingdom was commercial and political related with Sardinia and that the same territories were earlier dominated by Arabs, these results agree with the idea that these crops were introduced in Spain from Central Asia through Arab domination

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CHAPTER 1

**ARCHAEOBOTANICAL ANALYSIS OF A BRONZE
AGE WELL FROM SA OSA (CABRAS), SARDINIA: A
WEALTH OF KNOWLEDGE**

**ARCHAEOBOTANICAL ANALYSIS OF A BRONZE AGE WELL FROM SA OSA (CABRAS),
SARDINIA: A WEALTH OF KNOWLEDGE**

**Diego Sabato¹, Alessia Masi², Caterina Pepe², Mariano Uccesu¹, Leonor Peña-Chocarro^{3,4},
Alessandro Usai⁵, Gianna Giachi⁶, Chiara Capretti⁷, Gianluigi Bacchetta¹**

¹ *Centro Conservazione Biodiversità (CCB), Dip. di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Italy*

² *Laboratorio di palinologia e paleobotanica, Dip. di Biologia Ambientale, Università di Roma La Sapienza, Italy*

³ *Escuela Española de Historia y Arqueología en Roma-CSIC, Rome, Italy*

⁴ *GI Arqueobiología, Instituto de Historia, CCHS-CSIC, Madrid, Spain*

⁵ *Soprintendenza per i Beni Archeologici per le province di Cagliari e Oristano, Cagliari, Italy*

⁶ *Soprintendenza per i Beni Archeologici della Toscana, Laboratorio di analisi, Firenze, Italy*

⁷ *Istituto per la Valorizzazione del Legno e delle Specie Arboree , IVALSA-CNR, Sesto Fiorentino, Italy*

Abstract

In 2008, during a rescue excavation in the Sa Osa area, near the town of Cabras (Sardinia, Italy), a Nuragic settlement was discovered. The excavation revealed numerous pits, wells and structures dug by the local communities between the Early Copper Age and the Iron Age. These structures were interpreted as elements of a settlement mainly involved in primary production. The most remarkable structure is Well-N, radiocarbon and archaeologically dated to the Late Bronze Age, which has yielded large amounts of waterlogged plant remains, animal and fish bones and pottery. Despite the limited set of samples, the combination of macro-remain and pollen analyses in this unique context provides important information useful for exploring not only local subsistence systems but also human impact on the surrounding environment.

Grapes and figs are the most abundant remains together with other fruits and edible vascular plants. Remains of melon and mulberry were identified being the earliest remains of these two species for Western Europe. Their presence may confirm early trade between Nuragic people and the eastern Mediterranean and/or African coasts. Intentional selection of wood suggests practices associated to the collection of raw material for specific technological demands.

The presence of intestinal parasites in the pollen record points to the possible use of the well as a cesspit, at least in its later use, and this is one of the earliest evidence of this type of structures in prehistoric contexts.

Key words: archaeobotany, waterlogged macro-remains, Bronze Age, pollen, intestinal parasites, Sardinia

1.1 Introduction

1.1.1 General background

Sardinia is located in the middle of the Mediterranean Basin and because of its strategic position was subjected to the influence of different cultures since prehistoric times. The earliest signs of human presence come from the Mesolithic (9th-8th millennium cal. BC), whereas a stable occupation is documented only during the Early Neolithic (6th millennium cal. BC) (Lugliè 2009). The Nuragic civilization started during the Middle Bronze Age when human ranked communities began to mark their territories by means of monumental tombs, called *Tombe dei giganti*, and cyclopean towers, *Nuraghi*. These buildings were elements of networks for territorial control and had probably multiple functions, e.g. fortified dwellings, animal shelters, storerooms for food, raw materials and artefacts (Ugas 2006). Recent research into settlement patterns, funerary contexts, cult places, pottery and metal production, contacts and exchanges, has allowed increasing our knowledge on the Bronze Age (ca. 2000-930 BC) in Sardinia (Depalmas 2009). At the end of this period, the island played a significant role as part of the various interconnected trade routes between the Western and Eastern Mediterranean (Lo Schiavo 2003, Bernardini and Perra 2012). Nuragic societies developed commercial relationships, mainly with Cyprus which are documented by several copper ox-hide ingots and tools for metal working found in various contexts (Begemann *et al.* 2001, Lo Schiavo *et al.* 2009). During the Iron Age, Sardinia became part of the Phoenician commercial network, and later on, Punic colonies were established along its south-western coast (van Dommelen and Finocchi 2008).

Archaeobotanical data in Sardinia are still scarce. Fruits, cereals and legumes were cultivated at least since the Middle and Late Neolithic, 5th-4th millennium BC (Sadori *et al.* 1989, Trump 1990, Mercuri *et al.* 2014). Research based on several Bronze Age sites throughout the island points to a quite developed agricultural system in the Nuragic communities (Bakels 2002, Uccesu *et al.* 2014a, Sabato *et al.* 2015).

This paper focuses on the archaeobotanical remains (seeds, fruits, pollen, wood and charcoal) recovered from Well-N, a waterlogged context of the Nuragic settlement of Sa Osa.

1.1.2 The site

Sa Osa (39°54'51"N 8°32'32"E, 6 m a.s.l.) is located along the Central West coast of Sardinia (Fig.1a), on the alluvial plain of the *Tirso* river and it is limited by two ponds, Cabras to the North and Santa Giusta to the South.

The climate of this area is typically Mediterranean pluviseasonal oceanic with thermotypes ranging between the upper thermo- and the lower-mesomediterranean and ombrotypes between the upper dry and the lower subhumid (Bacchetta *et al.* 2009).

According to the existing ecosystems, the vegetation of the *Sinis* peninsula and surrounding areas can be categorized into three main types: rocky and dunal coast, wetlands and cultivated areas. The rocky coasts are dominated by halorupicolous and scrubland vegetation of the *Crithmo-*

Limonietea, *Cisto-Lavanduletea* and *Quercetea ilicis* classes, while the coastal sand dunes by psammophilous plant assemblages of the *Cakiletea maritimae*, *Ammophiletea* and *Helichryso-Crucianelletea* classes. The amphibious vegetation of the nearby lagoons is characterised by marshes dominated by helophytic, hydrophytic and salt-water plant communities of the classes *Phragmito-Magnocaricetea*, *Ruppietea*, *Juncetea maritimi*, *Saginetea maritimae*, *Sarcocornietea fruticosae*, *Thero-Salicornietea*. Most of the lowland areas are currently managed for agriculture, with rural environments dominated by cereals, vineyards and olive groves (Fenu and Bacchetta 2008).

Rescue excavations carried out during 2008 and 2009 (Fig.1b), revealed a Nuragic settlement composed of numerous wells and pits related to living spaces. These structures were dug by local communities between the Early Copper and the Iron Age, with emphasis during the Middle and Late Bronze Age (Usai 2011). During its occupation, the settlement was affected by water erosion and sedimentation due to river flooding events (Melis and Sechi 2011). The southern sector of Sa Osa revealed many small and medium-sized oblong pits and cylindrical wells filled in with various types of sediments. These cavities probably had different functions (e.g. dwelling, quarry, water supply) and, at some point, some were used either as refuse pits or for food storage (Usai 2011). Archaeobotanical research on two of these wells was carried out and plant remains, charred cereals (hulled barley and free-threshing wheats) and legumes, and few waterlogged macro-remains, mostly fig and grape remains, were recovered (Ucchesu *et al.* 2014a). The most remarkable structure was the so-called Well-N (Fig.1c) dated to the Late Bronze Age. Well-N fill has been considered as a single Stratigraphical Unit (SU171) probably formed during a short period of time (Usai 2011). The wide range of pottery retrieved shows the typical features of Central West Sardinian productions from the end of the Late Bronze Age (Serreli 2011). A crucible probably used for small scale metallurgy was also found. The presence of a symbolic miniature of a jar and several rare cups, interpreted as lamps, suggests a possible ritual significance for this structure (Usai 2011).

A huge amount of organic material has been preserved in waterlogged conditions (seeds, fruits, wood, charcoal, animal and fish bones and insects) (Sanna 2011). Radiocarbon dating of two grape pips collected at depth 1.40 and 2.15 meters has provided two dates, 1286-1115 2σ cal. BC and 1276-1088 2σ cal. BC (OxA-25106, OxA-25107) (Ucchesu *et al.* 2014a). One new dating on *Cucumis melo* seeds is reported in the Results section (Sabato *et al.* 2015).

1.2 Materials and methods

1.2.1 Macro-remains

Well-N was dug in sandstone and alluvial deposits. It has a cylindrical shape, slightly tapered towards the bottom measuring 1 m in diameter. The structure emerged 2 m above sea level. Sandy brownish sediments characterized the first metre while at 1.40 metres (the approximated level of the water table), the sediment became darker. Below this point, soil became increasingly fine and muddy due to the presence of water. The excavation stopped at a depth of ca. 4.20 meters for security reasons (Serreli 2011).

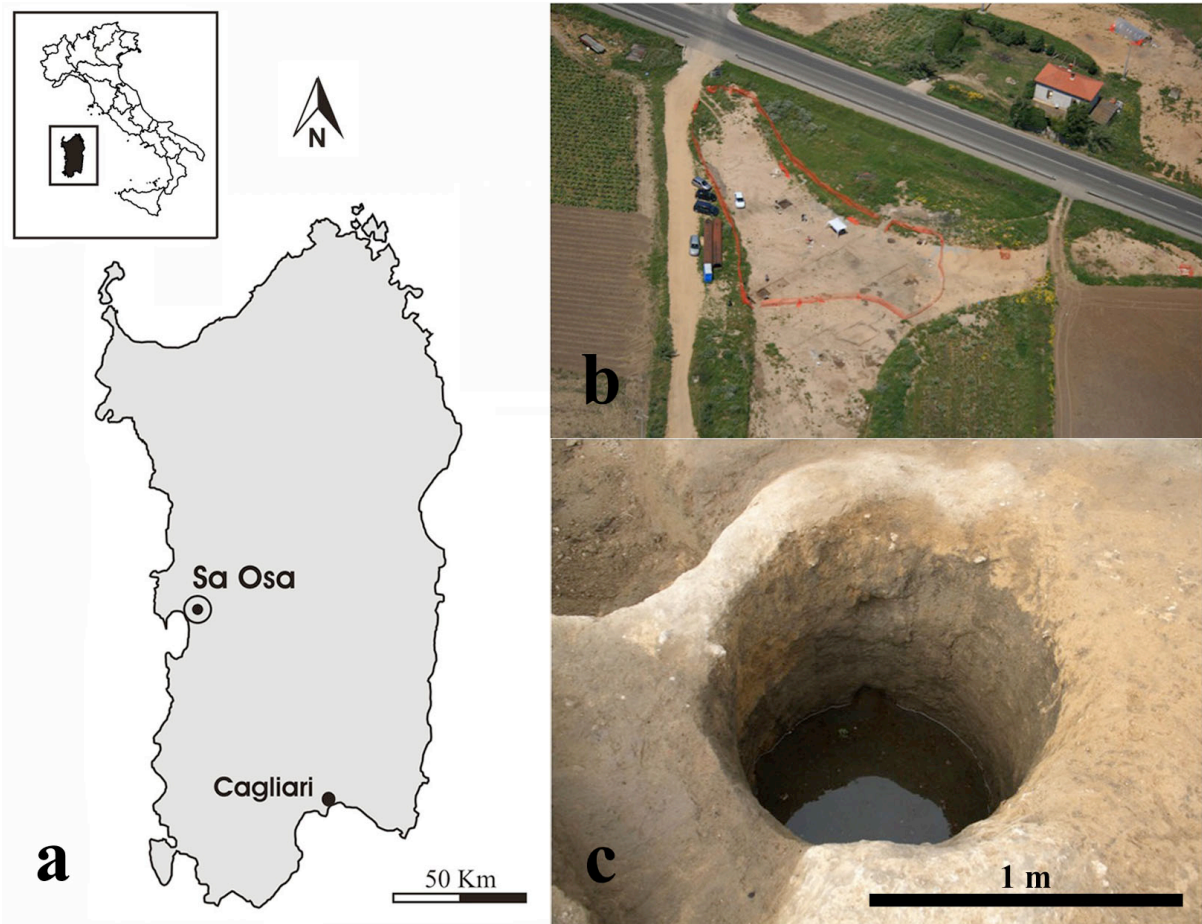


Figure 1. a) Location of the Nuragic site of Sa Osa (Cabras, Sardinia); b) view of the site during the excavation; c) Well-N.

Soil samples from Well-N were taken at different depths between 2.30 and 4.20 metres, for a total of approximately 1.5m³. Three samples were sieved *in-situ*, during the excavation, using 4.0, 2.0 and 0.5 mm meshes, but the total volume of soil processed was not recorded. For the remaining samples different volumes of soil were taken and sub-sampled afterwards: 15 of 30 litres from 2.30/2.60 meters; 3 of 60 litres from 3.80/4.00 meters; 3 of 145 litres from 4.00/4.10 m and 5 of 20 litres from 4.10/4.20. Each subsample, randomly extracted from the total, was then sieved by wash-over using a column of sieves between 4.0 and 0.5 mm. A litre of soil was further sieved with a 0.25 mm mesh. This procedure follows protocols already developed for waterlogged contexts (Jacomet 2013). For each layer, a total of five litres was kept for future analyses. Identifications of seeds/fruits have been carried out using a reference collection and various specialized atlases (Jacomet 2006, Bojňanský and Fargašová 2007, Cappers *et al.* 2012). After identification, waterlogged remains were kept in distilled water and stored at 5° C in the Sardinian Germplasm Bank (BG-SAR).

The larger wood fragments have been identified by the Soprintendenza per i Beni Archeologici della Toscana and the Istituto per la Valorizzazione del Legno e delle Specie Arboree (IVALSA, CNR). The identification was performed following the Italian standard guidelines (UNI.11118:2004) using a transmitted light microscope and comparing the diagnostic characteristics of wood with the reference collection of IVALSA and atlas (Schweingruber 1990). Some of these

pieces revealed woodworking. Small fragments of charcoal and wood were dried and studied at the Departamento de Historia y Geografía, University of Valencia (Spain) using a stereomicroscope and DIC (Differential Interference Contrast) microscopy.

1.2.2 Pollen

Due to the reduced set of pollen samples, palynology was performed to support the study of plant macroremains. The four samples collected were chemically processed following Faegri and Iversen (1989). In order to estimate pollen and non-pollen palynomorphs (NPPs) content, a known amount of *Lycopodium* spores (Stockmarr 1971) was added to each weighted sample. Pollen grain identification was based on atlases (Reille 1992–1998) and on the reference collection from La Sapienza University. The cereal-type pollen has been divided into *Hordeum* group and *Avena/Triticum* group according to Moore *et al.* (1991) while oak pollen *taxa* have been distinguished on the basis of the features reported by Smit (1973). NPPs were counted and identified according to van Geel *et al.* (1986). Their percentages have been calculated using a sum including NPPs and pollen of terrestrial plants. Routine pollen analysis was carried out using a transmitted light microscope.

1.3 Results

1.3.1 Seeds and fruits

Most of the seeds and fruits from Well-N were found in excellent state of preservation due to waterlogging. In addition, some charred material was also retrieved. A total of 35 *taxa* were identified (Tab.1, Fig.2) including fruits and berries (grape, fig, myrtle, sloe, olive, melon, mulberry, blackberry), wild plants and charred cereals and legumes.

The number of *Vitis vinifera* pips and *Ficus carica* achenes is very large representing more than 90% of the total number of remains. For this reason, the number of these two *taxa* has been estimated according to the corresponding volume of thousand items of each *taxon*.

The most remarkable and unexpected find was the identification of ca. 50 seeds of *Cucumis melo* which has been never recorded in this area of the Mediterranean basin during this period. Some fragments recovered at a depth of 3.20 meters have been radiocarbonated by AMS (Beta345402) providing dates between 3260 to 3070 cal BP (1310-1120 cal BC) for 2 σ (95% probability) and 3210-3140 and 3130-3110 and 3090-3080 cal BP for 1 σ (68% probability). One of the three *Morus* sp. endocarps retrieved from the well has been found within the same layer.

Other *taxa* that may have been used for human consumption are *Prunus spinosa* (17 and 10 fragments), *Pistacia lentiscus* (141), *Juniperus oxycedrus* s.l. (5), *Myrtus communis* (247), *Linum cf. usitatissimum* (17), *Rubus* sp. (6) and *Olea europaea* (2).

The few sporadic charred grains found belong to cereals and legumes: two *Triticum aestivum/durum*, a single *Hordeum vulgare* and two *Vicia faba*.

Among the wild plants, *Fumaria* sp. (49), several *Ranunculus* sp. (30) and *Medicago* sp. (24) were the most abundant.

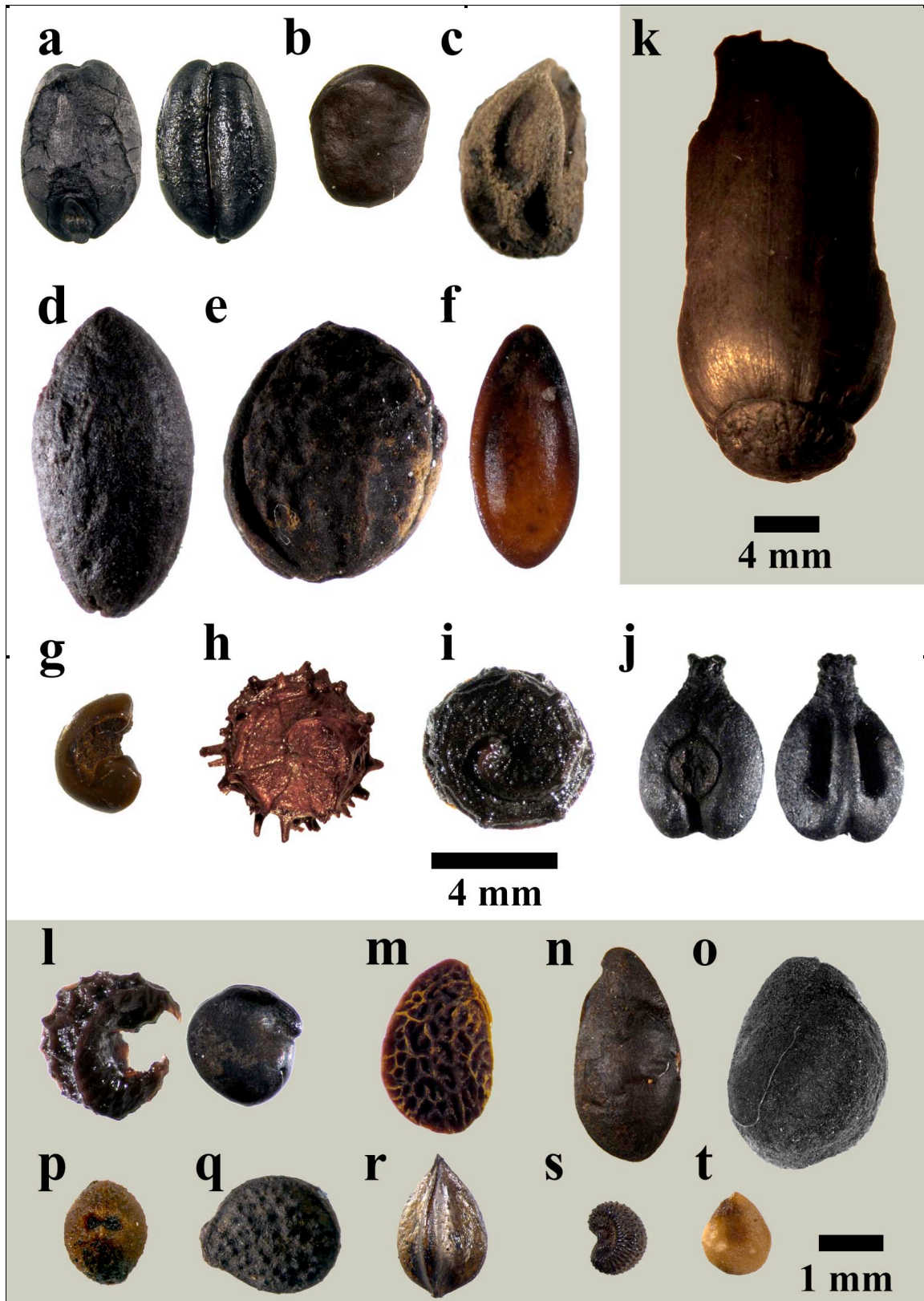


Figure 2. Some of the seeds and fruits from Well-N of Sa Osa: a) *Triticum aestivum/durum* (charred, two sides), b) *Pistacia lentiscus*, c) *Juniperus oxycedrus* s.l., d) *Olea europaea*, e) *Prunus spinosa*, f) *Cucumis melo*, g) *Myrtus communis*, h) *Medicago* cf. *arabica*, i) *Medicago* cf. *littoralis*, j) *Vitis vinifera*, k) *Quercus* sp., l) *Malva* sp. (capsule and seed), m) *Rubus* sp., n) *Linum* cf. *usitatissimum*, o) *Morus* sp., p) *Fumaria* sp., q) *Ranunculus* cf. *trilobus*, r) *Rumex* sp., s) *Silene* sp., t) *Ficus carica*

Depth (m)		2.30/2.60	2.60/2.90	3.10/3.70	3.70/3.80	3.80/4.00	4.00/4.10	4.10/4.20	Total
Studied soil volume (l)		15	?	?	?	3	3	5	
Cereals and legumes									
<i>Hordeum vulgare</i>	C				1				1
<i>Triticum aestivum/durum</i>	C	1				1			2
Cereal fragments	C					1			1
Cereal rachis	W			1					1
<i>Vicia faba</i>	C				2				2
Economic plants									
* <i>Ficus carica</i> estim. n°	W	3750000	187500	337500	37500	337500	300000	37500	4987500
* <i>Vitis vinifera</i> estim. n°	W	5000	600	5000	2000	1900	700	200	15400
* <i>V. vinifera</i> frag. est. n°	W	1080		840	240	840			3000
<i>V. vinifera</i> frag. real n°	W		27	25			120	53	225
<i>Vitis vinifera</i> (petioles)	W	4		20	12	18	7	5	66
<i>Cucumis melo</i>	W	10	8	28	1				47
<i>Juniperus oxycedrus</i> s.l.	W			2	2	1			5
<i>J. oxycedrus</i> (cone frag.)	W					1			1
<i>Linum cf. usitatissimum</i>	W	12	1	2		1	1		17
<i>Morus</i> sp.	W	2		1					3
<i>Myrtus communis</i>	W	132		57	28	20	5	5	247
<i>Olea europaea</i>	W			1		1			2
<i>Pistacia lentiscus</i>	W	11	11	44	20	45	10		141
<i>Prunus spinosa</i>	W	10	3	2	1		1		17
<i>Prunus spinosa</i> (frag.)	W	10							10
<i>Quercus</i> sp.	W				1	1			2
<i>Rubus</i> sp.	W	1	4				1		6
Wild plants									
<i>Ajuga</i> sp.	W			1					1
<i>Carex</i> sp.	W			1				1	2
<i>Chenopodium</i> sp.	W			2					2
<i>Daucus</i> sp.	W	1	1				1	2	5
<i>Fumaria</i> sp.	W	13	4	13		13	3	3	49
<i>Heliotropium</i> sp.	W			2				1	3
Lamiaceae	W						1		1
<i>Malva</i> sp.	W			5					5
<i>Medicago cf. arabica</i>	W	4	4	2	1		1		12
<i>Medicago cf. littoralis</i>	W	1		3	3				7
<i>Medicago</i> sp.	W			5					5
<i>Papaver</i> sp. (capsule fr.)	W	1		1					2
Polygonaceae	W						2		2
<i>Ranunculus cf. arvensis</i>	W			2		2	1		5
<i>Ranunculus cf. trilobus</i>	W		2	5		3	4		14
<i>Ranunculus cf. sardous</i>	W		2	5		4			11
<i>Ranunculus</i> sp.	W			1					1
<i>Rumex</i> sp.	W			4		1	3		8
<i>Silene</i> sp.	W		1			1	1		3
Dicotyledon leaf frag.	W					1			1
Indeterminate	W	2		2			1		5
Total		3756294	188168	343576	39809	340353	300863	37770	5006833

* according to: *F. carica* 1000 seeds = 0.8 ml; *V. vinifera* 1000 seeds = 50.0 ml; *V. vinifera* fragments 120 = 1.0 ml

C = charred; W = waterlogged

Table 1. List of seeds and fruits identified in Well-N. Volume (in ml) of *Ficus carica* for each layer following the table order: 3000, 150, 270, 30, 270, 240, 30. Volume (in ml) of *Vitis vinifera* pips and fragments for each layer following the table order: 250-9frag, 30, 250-7frag., 100-2frag., 95-7frag, 35-1frag, 10

1.3.2 Charcoal and wood

A total of 877 fragments of waterlogged wood and charcoal were analyzed (Tab.2). Results show the presence of twenty different *taxa*, equally distributed between charred/semi-charred (305 fragments) and non-charred wood (351, 209 bark and 22 cork fragments). *Erica* sp. is the most abundant, amounting for almost half of the total remains of both wood and charcoal. Despite their distorted anatomy, several fragments of charred knots are probably related to this *taxon*.

Juniperus sp. is the second most common *taxon* (30% of the total). Eighty-five twigs show common traits: relatively straight with an approximate length of 15 cm, and ca. 8-10 mm wide, corresponding to 5-10 rings. In addition, 52 out of the 64 twigs show latewood at the last ring suggesting that most of them were cut at the end of summer or the beginning of autumn, although the last ring resulted not clearly visible in the remaining 21 pieces.

The twelve *Olea* sp. fragments, all found on depth 3.80/3.40 m, probably belong to a single wood piece. They all have a cylindrical shape, show nearly the same diameter, and some pieces fit together. Other identified *taxa* are: *Pinus* cf. *halepensis* (belonging to the typology of *Pinus pinea/pinaster*), Rosaceae Maloideae (typology of *Crataegus/Sorbus*) and Prunoideae (probably *Prunus spinosa*). Identification to species level cannot be often achieved on the basis of wood anatomy, however *Pistacia* could be probably related to *P. lentiscus* since nowadays it is almost the only *Pistacia* distributed in the island while *P. terebinthus* is limited to the Supramonte area (central-eastern Sardinia). The same applies to *Alnus* cf. *glutinosa* and *Ficus* cf. *carica*; common alder is the only *Alnus* growing in Sardinia while other species of *Ficus* have been introduced to the island only in recent times. Despite the difficulties to distinguish between small fragments of *Ostrya* and *Carpinus*, remains identified as *Carpinus/Ostrya* can be reasonably attributed to the genus *Ostrya*.

1.3.3 Pollen

The sample 4.10/4.20 m has been excluded due to the extremely low pollen content. The total terrestrial pollen count in the other three samples analyzed ranges from 127 to 277 grains. Pollen preservation is quite good and concentration ranges from 1,786 to 32,884 pollen grains/g. A total of thirty-seven *taxa* has been identified (Fig.3). Pollen from trees and shrubs is not abundant and it mainly belongs to the Mediterranean sclerophyllous vegetation (*Juniperus*, Ericaceae, *Olea*, *Cistus*, *Pistacia*, *Phillyrea*, *Quercus* cf. *suber* and *Q. ilex* type) including few deciduous elements (*Vitis*, *Alnus*, deciduous oaks of the *Quercus* gr. *pubescens*, *Ostrya/Carpinus orientalis*).

The majority of the identified *taxa* belong to herbaceous plants. Brassicaceae, present in all samples with percentages ranging from 5.4% (3.80/4.00 m) to 40.9% (4.00/4.10 m), is the most abundant *taxon*. All Brassicaceae pollen grains can be included in the same morphotype with a diameter of ca. 20 µm showing high similarities to *Nasturtium officinale* and *Camelina sativa* pollen grains, both present in Sardinia, although *C. sativa* is considered as invasive exotic plant in the island (Podda *et al.* 2012). Since pollen grains have been found in clumps (and so counted as single units), it is likely that they derive from pieces of anther either preserved in faecal remains or transported by insects (Dimbleby 1985, Mercuri 2008, Bosi *et al.* 2011).

Depth (m)		2.30/2.60	2.60/2.90	3.10/3.70	3.70/3.80	3.80/4.00	4.00/4.10	4.10/4.20	Total
Studied soil volume (l)		15	?	?	?	3	3	5	
<i>Alnus cf. glutinosa</i>	W		1	4	3				8
<i>Alnus/Corylus</i>	W			4					4
Asteraceae	C							1	1
<i>Carpinus/Ostrya sp.</i>	C			2					2
<i>Erica sp.</i>	W		2	22	23	1	4	13	65
<i>Erica sp.</i>	C	44	5	55	20	13	21	5	163
<i>Erica sp.</i>	S				2	1			3
cf. <i>Erica sp.</i> (knurls)	C	22	2	11	13	6		12	66
<i>Ficus cf. carica</i>	W			13	3	1		1	18
<i>Ficus cf. carica</i>	C		1	1	3			1	6
<i>Juniperus sp.</i>	W	11	18	21	9	13	3	14	89
<i>Juniperus sp.</i> (twings)	W		1	65		1		18	85
<i>Juniperus sp.</i>	C			6	1	1		4	12
<i>Olea sp.</i>	W					12			12
<i>Pinus cf. halepensis</i>	W	14	4	4	2			17	41
<i>Pinus sp.</i>	W	2		1					3
<i>Pistacia sp.</i>	C	1		1					2
<i>Prunus sp.</i>	C			2	2	13		4	21
<i>Quercus gr. pubescens</i>	C			3		3			6
<i>Quercus suber</i> (cork)	W	18	3	1					22
<i>Rhamnus/Phillyrea</i>	C			2			1	1	4
Rosaceae maloideae	C			3		1			4
<i>Tamarix sp.</i>	C			2					2
Thymelaeaceae	C				1				1
Dicotyledon Agiosperm	W				1			4	5
Dicotyledon Agiosperm	C	4		5					9
Monocotyledon Agiosp.	C	1		1		1			3
Bark	W			112	22	47		28	209
Indeterminate	W			18	3	2		4	27
Total		117	37	359	108	116	29	127	877

C = charred; W= waterlogged; S = semi-charred;

Table 2. List of wood and charcoal identified in Well-N.

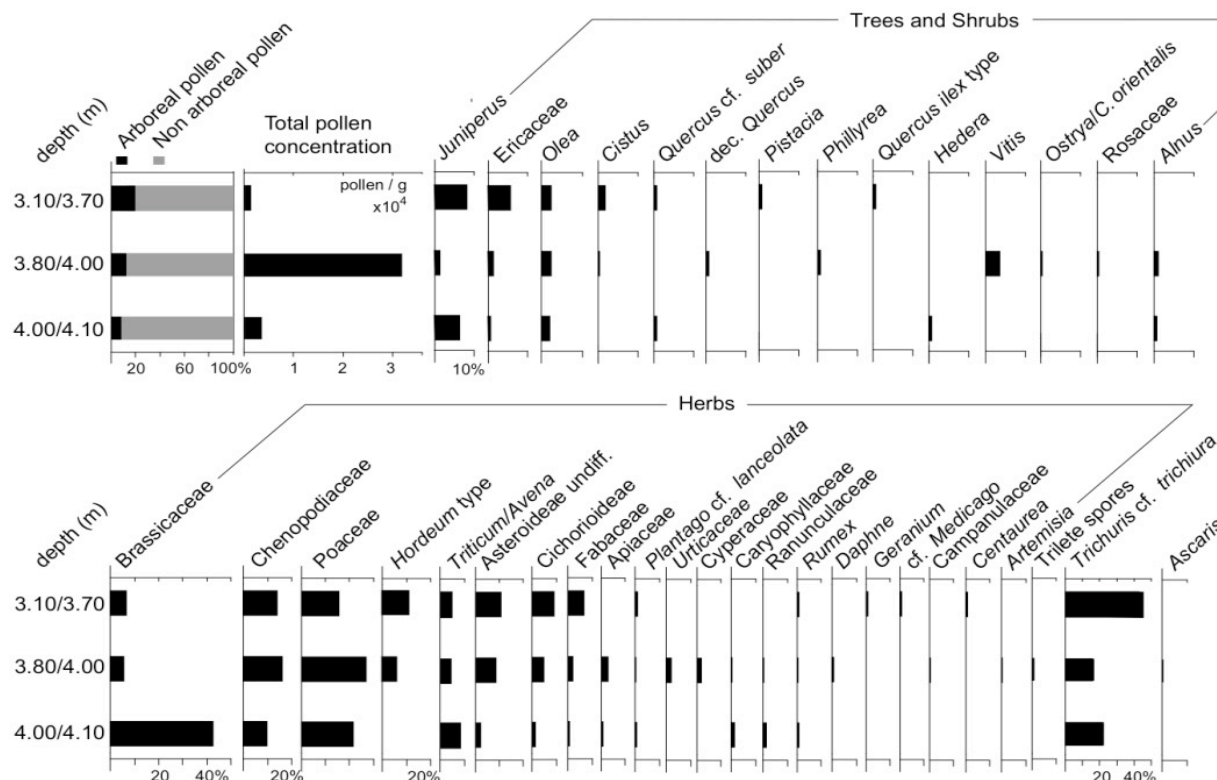


Figure 3. Results of pollen analysis from Well-N, percentage values and total pollen concentration are shown.

Poaceae are also abundant, peaking at 27% at 3.80/4.00 m while Chenopodiaceae range from 9.4% at 4.00/4.10 m to 16.2% at 3.80/4.00 m. Many herbaceous *taxa* probably belong to cultivated and ruderal plants: *Hordeum* group, *Avena/Triticum* group, Fabaceae, *Plantago*, *Rumex*, Cichorioideae. Both the *Hordeum* and the *Avena/Triticum* groups include cultivated and wild forms, but the high percentages of these two pollen types suggest that they come from cereal crops.

Pollen of *Vitis* sp. has been retrieved in only one sample at the depth of 3.80/4.00 m (3.6%). The identified NPPs (Non Pollen Palynomorphs) are eggs of intestinal parasites belonging to *Ascaris* (only one) and *Trichuris* cf. *trichiura* which show high percentages (44.3% at 3.10/3.70 m) in all samples.

1.4 Discussion

Although common in large parts of Europe, waterlogged plant remains are unusual in the Mediterranean area where plant preservation is generally by charring. Examples of early contexts with waterlogged plant remains include the Neolithic sites of *La Draga*, in northeastern Spain (Antolín and Buxó 2011) and *La Marmotta* (Rome) (Fugazzola Delpino *et al.* 1993). The well and cistern fills of the Iron Age villages of *Tossal de les Bannes* and *Illeta dels Banyets* (Valencia, Spain) (Pérez-Jordá 2013) show some affinity with the contexts of Sa Osa. There are, however, other examples from the Mediterranean such as the Middle Bronze Age pit from *San Lorenzo a Greve* (Florence) (Mariotti Lippi *et al.* 2010), the more recent Classical contexts from North Italy (Bosi *et al.* 2011, Rinaldi *et al.* 2013), the harbour of *Irun* (Basque Country) (Peña-Chocarro and Zapata 2005), *Iesso* (Catalonia) (Buxó and Piqué 2008), *Ostia* (Rome) (Pepe *et al.* 2013, Sadori *et al.* 2014) or *Caesarea Maritima* (Israel) (Ramsay 2010) and the medieval contexts of the Basque country (Peña-Chocarro *et al.* 2014) and North Italy (Bandini Mazzanti *et al.* 2005, Bosi *et al.* 2009).

Archaeological evidence suggests that fill from Well-N is related to a single Stratigraphic Unit (Serreli 2011, Usai 2011). The three radiocarbon dates carried out on grape and melon seeds, collected at different depths and analyzed in two independent laboratories, together with the evidence from the pottery, point to a Late Bronze Age deposit (Serreli 2011). The morphology of the grape pips from all layers is homogeneous (Orrù *et al.* 2013), and other grape pips (likely wild) were found in the two other Middle Bronze Age wells from the same site (Ucchesu *et al.* 2014b)

The plant assemblage identified at Sa Osa shows a variety of cultivated and wild plants which represent both the rich diversity of species used by the Sa Osa population and the vegetation that characterized the environment of the area. Amongst them, fruits represent an important part of the record of useful plants.

The record of *Cucumis melo* is of great importance as it represents the earliest evidence from Western Europe and one of the most ancient examples of this *taxon*. Zohary *et al.* (2012) suggested that melon cultivation may have begun during the Bronze Age in the Near East and/or in Africa, although this crop played a marginal role until at least the Classical period (Janick *et al.* 2007). Melon became widely spread in Europe in Medieval times when introduced to the Iberian Peninsula during

the Arab domination (Paris *et al.* 2012). The earliest known representations of melon come from Egypt during the second half of the 2nd millennium BC (Darby *et al.* 1977, Manniche 1989) and the oldest archaeobotanical finds are reported from the same area during the Neolithic (Fahmy 2001). Data from the Near East and Greece comes from the Bronze and the Iron Age (Kroll 1982, 1984, van Zeist *et al.* 2003). A few melon seeds from the Roman period have been retrieved in Pompeii (Murphy *et al.* 2013) and in Northern Italy (Castelletti *et al.* 2001, Rinaldi *et al.* 2013), the harbour of Rome (Pepe *et al.* 2013) and in Northern Europe (Livarda 2011). The early presence of melon in Sardinia suggests that its introduction during the Late Bronze Age could have followed the trade routes that linked the island to the Mycenaean and Minoan world through Cyprus for the exchange of metals (Begemann *et al.* 2001, Lo Schiavo *et al.* 2009). Archaeological research has evidenced that commercial relations between Cyprus and Sardinia were not only based on the trade of oxhide ingots, but exchanges included a variety of products such as tools for metal and wood working and luxury objects (Lo Schiavo 2012). Plants such as mulberry, could have had as well a role in these commercial relationships with Eastern Mediterranean and/or Africa. Although mulberry was imported from Asia only in historical times, *Morus* sp. has been recorded in a Punic channel of the ancient port of Carthage (van Zeist *et al.* 2001) directly connected with Sardinia up to the foundation of permanent colonies in the South-West coasts of the island (van Dommelen and Finocchi 2008). The absence of melon and mulberry records in Europe during the same period may indicate a limited and local spread of these species during the Bronze Age.

The most recurrent *taxa* were *Vitis vinifera* and *Ficus carica*. Despite the great number of fig achenes, this species is not represented in the pollen diagram probably due to its reduced pollen dispersion. According to Kislev *et al.* (2006), archaeobotanical evidence suggests that fig cultivation was practiced in the Near East already by the 12th millennium BP but this data has been criticized (Lev-Yadun *et al.* 2006).

In the case of the grape, previous research on the seed morphology of the Sa Osa specimens has suggested a close relationship between the Sa Osa grape pips and modern Sardinian grapevine varieties (Orrù *et al.* 2013). Recently, further research confirmed this data (Ucchesu *et al.* 2014b). Bearing in mind that *Vitis* pollen travels short distances, the high pollen percentage found in one of the Sa Osa samples points to the proximity of grape vines to the site, which is also evidenced in the curve of the Mistras lagoon during the same period (Di Rita and Melis 2013). In the Mediterranean basin and particularly, in southern Greece and Cyprus cultivated pips are documented during the first half of the 3rd millennium BC while in the southern Balkans the earliest records are from the 2nd millennium BC (Kroll 1991). In Italy, the earliest record comes from Tuscany, from the Middle Bronze Age (Mariotti Lippi *et al.* 2000, Bellini *et al.* 2008), but it is only during the Iron Age that cultivation developed (Marvelli *et al.* 2013). In Sardinia, *Vitis* pips are found from the Middle Bronze Age (Bakels 2002, Celant 2010, Tanda *et al.* 2012, Ucchesu *et al.* 2014b). The beginning of viticulture in the western Mediterranean basin during the Bronze Age has been debated (Stika and Heiss 2013). In the Iberian Peninsula, the archaeological record suggests a direct relationship between the introduction of

viticulture and the first contacts with Phoenicians and Greeks (Gómez-Bellard *et al.* 1993, Buxó and Piqué 2008) and a similar trend is found in Southern France (Bouby *et al.* 2013, Brun 2011).

The scarce presence of cereals and legumes is probably related to the type of preservation. In fact, charred cereals and legumes are abundant in the other wells of the same site (Ucchesu *et al.* 2014a).

The presence of *Prunus spinosa* endocarps could indicate an intentional harvest although due to the wide spread of this species in the area an accidental incorporation into the well cannot be totally excluded. Similarly myrtle, juniper, mastic tree, and blackberry produce all edible fruits that may have arrived into the well as part of food refuse. All of these *taxa* could be easily collected in the surrounding area being an important complement to the diet of the local populations.

Pollen data indicates that the surroundings of Sa Osa were covered by Mediterranean scrub communities dominated by *Juniperus* and Ericaceae. Juniper is also recorded in the wood and seed assemblages. The big size of the cones of *Juniperus oxycedrus* s.l. suggests that the specimens from Sa Osa belong to this species which is also the commonest in the Western coast of Sardinia (Bacchetta *et al.* 2009). The juniper twigs identified are quite homogeneous in size, age and most of them were collected in the same period, at the end of the summer/autumn, when the wood is harder and less flexible suggesting that collection for specific uses, e.g. building material, was practiced. Twig selection for building purposes has been already put forward for the Middle Bronze Age site of Terramara di Montale although not specifically for *Juniperus* (Mercuri *et al.* 2006).

The phytocoenoses of *Erica multiflora* is very common in the Sinis Peninsula (Fenu and Bacchetta 2008) as well as the association of *Erica arborea* and *Arbutus unedo* in the mountains around Sa Osa (Bacchetta *et al.* 2009). The large amount of charcoal, semi-charred and non-charred wood fragments of *Erica* sp. could be related to its use as fuel as its wood has a high heating power in which probably explains its use in metallurgical activities such as iron reduction (Mariotti Lippi *et al.* 2000, Sadori *et al.* 2010). *Erica* wood fragments may be associated to the crucible found in the same context (Usai 2011).

Pistacia lentiscus is recorded in all samples. One of the most popular products of the mastic tree is its resin, a well-known product in the eastern Mediterranean (Greece and Turkey), Sardinia (Thi Mai *et al.* 2014, Loi 2013) and in some northern African communities (Morales *et al.* 2013). The oil extracted from *Pistacia* seeds is also used as oil lamp and for cooking.

Pollen analysis shows a predominance of herbaceous plants with a clear overrepresentation of synanthropic taxa, from which many are linked to human activities as it is the case of cereals and Brassicaceae. The morphology of Brassicaceae pollen grains (one morphotype with a grain diameter of ca. 20 mm) indicates two possible species: *Nasturtium officinale* and *Camelina sativa*. While the second seems to be recently introduced in Sardinia, the first one is widespread in the area (Podda *et al.* 2012). It is probable that *Nasturtium officinale* (watercress) was gathered from the wild and used as a food plant as recorded in the ethnographic record (Atzei 2009, Ranfa *et al.* 2014). Besides watercress, other plants of water environment are equally represented in the pollen assemblage. Mainly

Chenopodiaceae but also *Artemisia*, other Asteroideae, and Poaceae pollen can be related to marshy areas. Vegetation from coastal marshes is also common in the pollen diagram of the nearby lagoon of Mistras (Di Rita & Melis 2013). Arboreal pollen is rather low and most *taxa* come from the *maquis* (typical evergreen sclerophyllous vegetation).

All pollen samples show high percentages of eggs of *Trichuris* cf. *trichiuria* (whipworm) while at depth 3.80/4.00 m these appear together with an *Ascaris* egg (mawworm). *Trichuris* and *Ascaris* are parasites of wild and domestic animals, mostly mammals, including humans. Finding these parasites in ancient contexts is unusual as to the chemical composition of the egg shell (chitin) requires anoxic conditions, such as those of pits and latrines, to get preserved (Florenzano *et al.* 2012). The association of these parasites with humans is well known from prehistoric times already. In particular, in Europe, eggs of *Ascaris* have been recorded in Upper Palaeolithic contexts associated to the Neanderthal man of *Arcy sur Cure* (France) (Carvalho Gonçalves *et al.* 2003) and also in the Similaun man (*Ötzi*), who lived c. 4150 BC (Oeggl 2009). However, most of the records come from medieval contexts (Bouchet *et al.* 2003, Bosi *et al.* 2011, Brinkkemper and van Haaster 2012, Florenzano *et al.* 2012). The absence of other species of *Trichuris* suggests that Well-N was possibly used as a cesspit being this the most ancient example of this type of structure in Sardinia and one of the oldest in Italy.

1.5 Conclusions

The analysis of plant remains from Well-N of Sa Osa has confirmed the high potential of the discipline for investigating plant use during the Bronze Age and exploring past landscapes in Sardinia.

Apart from the limited presence of cereal remains represented by charred remains of barley and naked wheats, most of the plant material correspond to fruits and berries preserved by waterlogging. The presence of intestinal parasites amongst the material recovered from Well N together with the presence of species such as figs, grapes, melon amongst others which may have gone through the intestinal tract, points to a change of the use of this well as a latrine, at least in the last phase, where probably other food remains may have been thrown in or arrived accidentally to it.

The plant assemblage shows the importance of fruits and berries in the plant diet of Sa Osa inhabitants and it reveals a significant diversity. And a quite developed agricultural system. Apart from cereals, the Sa Osa community made use of both wild and cultivated fruits (figs, grapes, olives, melon, elderberry, myrtle, sloe, lentisk, etc). One of the most interesting finds is represented by the seeds of melon which are the most ancient examples of this species in the Mediterranean Basin. Equally interesting is the presence of *Morus* sp. which also represents an early find.

Pollen analysis has been carried out on a limited number of samples but results highlight the presence of the typical Mediterranean vegetation.

Furthermore, the study of wood and charcoal has provided with information on the particular selection of wood species for specific uses such as building.

Although future work in other structures of the site may offer further interesting data, the analysis of plant material from Well-N has provided with a fascinating insight into plant use during the Bronze Age.

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CHAPTER 2

PHOENICIAN-PUNIC TRADE: AMPHORAE

CONTENTS FROM SANTA GIUSTA LAGOON,

SARDINIA (ITALY)

PHOENICIAN-PUNIC TRADE: AMPHORAE CONTENTS FROM SANTA GIUSTA LAGOON, SARDINIA (ITALY)

Diego Sabato¹, Leonor Peña-Chocarro^{2, 3}, Carla Del Vais⁴, Ignazio Sanna⁵, Gianluigi Bacchetta¹
Mariano Ucchesu¹

¹*Centro Conservazione Biodiversità (CCB), University of Cagliari, Italy.*

²*Escuela Española de Historia y Arqueología en Roma (EEHAR)-CSIC, Roma, Italy*

³*GI Arqueobiología, Centro de Ciencias Humanas y Sociales (CCHS)-CSIC, Madrid, Spain.*

⁴*Dipartimento di Storia, Beni Culturali e Territorio, University of Cagliari, Italy.*

⁵*Soprintendenza per i Beni Archeologici per le province di Cagliari e Oristano, Italy.*

Abstract

Research carried out in Santa Giusta lagoon since 2006 has revealed the presence of Phoenician-Punic archaeological contexts of exceptional importance. Several transport amphorae, together with domestic pottery and ceramics associated with funerary and ritual uses, have been recovered. Two deposition phases have been distinguished, one dated to the 6th-5th century BC and a second dated to the 3rd-2nd century BC.

Many amphorae contained ovine/caprine bones with butchery marks associated with grapes and other fruits. The waterlogged conditions favoured the preservation of organic materials, including pinecones, seeds and fruits of several *taxa* and wood remains.

Fruits and nuts such as *Corylus avellana*, *Juglans regia*, *Prunus dulcis*, *Pinus pinea* and *Olea europaea* are probably related to food trade while other edible plants such as *Vitis vinifera*, *Prunus domestica*, *Prunus spinosa*, and *Juniperus oxycedrus* may have played a role in meat preservation. The few remains of cucurbits, such as *Citrullus lanatus* and *Lagenaria siceraria*, suggest commercial contacts with other colonies in Northern Africa.

Keywords: Sardinia, Phoenician-Punic, amphorae, archaeobotany

2.1 Introduction

2.1.1 General background

Punic archaeology has long been the subject of intense interest and debate in which agricultural issues have been always central. Punic agricultural prosperity has been often highlighted in modern historiography and a significant place has been given to Mago and his 28-volume agricultural treatise translated into Latin by the Roman Senate as a proof of his renowned agrarian excellence. Leaving aside whether the fame of Punic agriculture is really mirrored in Mago's work or as it has been suggested (Kring 2008) its purpose enters into more philosophical and political realms, it is all the more remarkable that over the years so little attention has been paid to the analysis of plant remains as direct evidence of plant cultivation and the great deal of information that can be drawn from them.

Punic archaeobotany is still in its infancy due to the lack of properly sampled contexts. Apart from the seminal work of van Zeist *et al.* (2001) in Carthage, which shows the enormous potential of the discipline to explore food production and agricultural topics, research into the role of plants in Punic subsistence has been very limited. In Sardinia, plant remains are also scarce due to the little spread of systematic flotation. With the exception of few cases (Bakels 2002, Wetterstrom 1987), proper sampling and the application of recovery techniques have started to be applied only recently (Buosi *et al.* 2015, Pérez Jordà 2010, Uccesu *et al.* 2014a, Sabato *et al.* 2015 [**Chapter 1**]) producing results that consent to throw light on the use of plants in the past. Although the datasets are very reduced, plant remains from the Punic period in Sardinia are documented in sites such as *Pinn'e Maiolu* (Bakels 2002), *Ortu Comidu* (Wetterstrom 1986), *Nora* (Marinval and Cassien 2001, Miola *et al.* 2009) and *Truncu'e Molas* (Pérez Jordà 2010) demonstrating the presence of cereals (free-threshing wheats and barley), legumes (broad beans and lentils) and fruits (grapes).

Despite the specific context of the material studied (transport amphorae), this research offers the possibility of exploring the range of plants used during the Punic period and contributing to the development of archaeobotany in Sardinia.

2.1.2 The site

The pond of Santa Giusta is located in the north-central part of the Gulf of Oristano, close to the mouth of river Tirso (Fig. 1a). It has a round shape with an area of about 790 hectares that can reach a maximum of 900 hectares during the autumn-winter season. The brackish water of the lagoon is depth between 40 and 150 cm. Local people and archaeologists already knew the archaeological presences in the lagoon since at least the 70s. The underwater excavation of the Santa Giusta started in 2006 by Soprintendenza per i Beni Archeologici per le province di Cagliari e Oristano and the University of Cagliari. Phoenician-Punic amphorae were found belonging to two different phases, 6th-5th and 3rd-2nd century BC (Del Vais and Sanna 2009).

The various surveys carried out in the Northeast of the lagoon have identified two main areas of dispersion, which have been called Area A and B (Fig. 1b, 1c) (Del Vais and Sanna 2012). Both groups of amphorae contained numerous plants and animal remains which have been preserved in excellent condition thanks to the silt of the lagoon. Animal bones were identified as remains of ovines/caprines with evident butchery traces and prove the use of the amphorae as containers for transporting meat, but specific archaeozoological studies have to be carried out.

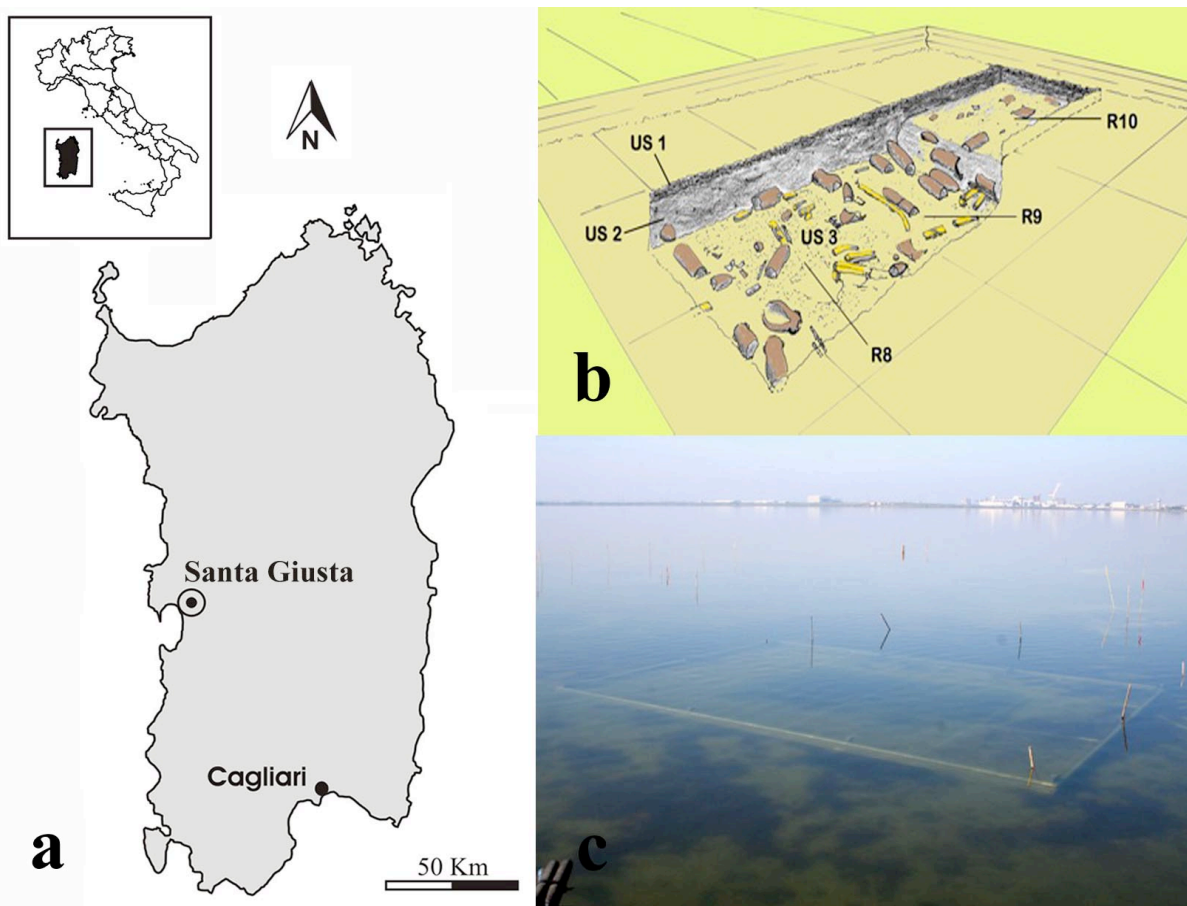


Figure 1. a) Location of Santa Giusta lagoon; b, c) The excavation area (in: Del Vais and Sanna 2009).

2.2 Materials and Methods

During the excavation, a total of 41 soil samples were collected from inside the amphorae and 51 samples were sucked from the deposition layers using a sub aquatic pump. The total number of contexts analysed in this study is 82, which have been divided into two groups according to the chronological period they belong to, either 6th/5th century BC or 3rd/2nd centuries BC. Thirteen samples come from amphorae dated to the 6th/5th centuries BC while eighteen have been dated to the 3rd/2nd centuries. The content of two further cups dated to the 3rd/2nd century was also considered but it was sterile. Several samples (51) were also collected from deposition layers. In the latter case, it is likely we are dealing with part of the amphorae content spread around due to breakage in antiquity. Only 11 of the samples from deposition layers have been attributed with security to the 3rd/2nd centuries BC

while the remaining 40 are dated to a period between the transition from the 6th to the 5th century and the transition from the 3rd to the 2nd century BC due to the difficulty of attributing the soft silt layers to specific chronologies.

The samples were water sieved by the Soprintendenza dei Beni Archeologici della Sardegna with a coarse mesh of 2 mm., and all waterlogged material was kept in distilled water and stored in a fridge at +5°C temperature at the Sardinian Germplasm Bank (BG-SAR). Due to the mesh size used, plant remains below 2 mm were not collected.

Identifications were carried out using the reference collection of the Sardinian Germplasm Bank and various specialized atlases for seed and fruit identification (Beijerinck 1947, Bojňanský and Fargašová 2007, Cappers 2012). The botanical nomenclature follows Zohary *et al.* (2012) and Pignatti and Anzalone (1982).

2.3 Results

Samples provided more than 4,000 macro-remains that were found in an excellent state of preservation (Tab.1). A wide range of species has been identified (Fig. 3, 4) including fruits such as grapes (*Vitis vinifera*), plums (*Prunus domestica*), sloes (*Prunus spinosa*), almonds (*Prunus dulcis*), olives (*Olea europaea*), junipers (*Juniperus oxycedrus* cf. *macrocarpa*) and nuts such as hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*) and pine nuts (*Pinus pinea* and *P. halepensis*) together with several pine cones (Fig.4). Furthermore, a few seeds of watermelon (*Citrullus lanatus*) and bottle gourd (*Lagenaria siceraria*), rarely found archaeologically, have been as well retrieved. Two seeds in both groups of samples represent the watermelon, while the bottle gourd has been only recorded in one sample from a deposition layer dated to the 3rd/2nd centuries. A high number of the aquatic pondweeds (*Potamogeton* sp.) was also recorded.

No significant differences emerge between the two groups samples (amphorae and depositional layers). The occurrence of almonds, watermelon and bottle gourd in the most recent samples is the only difference, although their scarce presence cannot rule out that they were also present in the oldest contexts.

Amongst the juicy fruits, grapes are the most abundant. They appear in both the interior of amphorae and in the various deposition layers in all periods. They are more abundant in the samples from the amphorae and particularly in the group of the oldest ones. It is likely that the specimens recovered from the deposition layers come from broken vessels. Seeds from plums have been found in low numbers in both amphorae and deposition layers. Other fruits are the olive that appears in all samples and chronologies and the sloe and the juniper, which are also represented in all samples.

	Layers (40) 6 th /3 rd cent. BC	Layers (11) 3 rd /2 nd cent. BC	Amphorae (13) 6 th /5 th cent. BC	Amphorae (18) 3 rd /2 nd cent. BC	Total
<i>Citrullus lanatus</i>	1	-	-	1	2
<i>Corylus avellana</i>	-	-	-	5	5
<i>C. avellana</i> fragments	-	-	2	-	2
<i>Juniperus oxycedrus</i> cf. <i>macrocarpa</i>	3	2	6	3	14
<i>Juglans regia</i> fragments	2	-	-	1	3
<i>Lagenaria siceraria</i>	-	1	-	-	1
<i>Olea europaea</i>	29	9	7	11	56
<i>Olea europaea</i> fragments	16	1	2	-	19
<i>Pinus halepensis</i> cones	3	-	-	1	4
<i>Pinus pinea</i> cones	-	1	-	1	2
<i>P. pinea</i> pine cone fragments	21	14	-	-	35
<i>P. pinea</i>	6	6	-	6	18
<i>P. pinea</i> fragments	46	60	2	6	114
<i>Potamogeton</i> sp.	75	-	1,362	664	1,437
<i>Prunus domestica</i>	4	-	1	5	10
<i>P. domestica</i> fragments	1	-	-	-	1
<i>Prunus dulcis</i>	2	-	-	8	10
<i>P. dulcis</i> fragments	8	3	-	5	16
<i>Prunus spinosa</i>	34	6	5	1	46
<i>P. spinosa</i> fragments	8	-	-	-	8
<i>Prunus</i> sp.	8	-	4	4	16
<i>Quercus</i> sp.	1	-	-	-	1
<i>Quercus</i> sp. fragments	1	-	-	-	1
<i>Vitis vinifera</i>	140	27	1,240	273	1,680
Indeterminate (wild plants)	11	-	-	-	11
Total	420	130	2,631	995	3,512

Table 1. List of seeds and fruits identified.

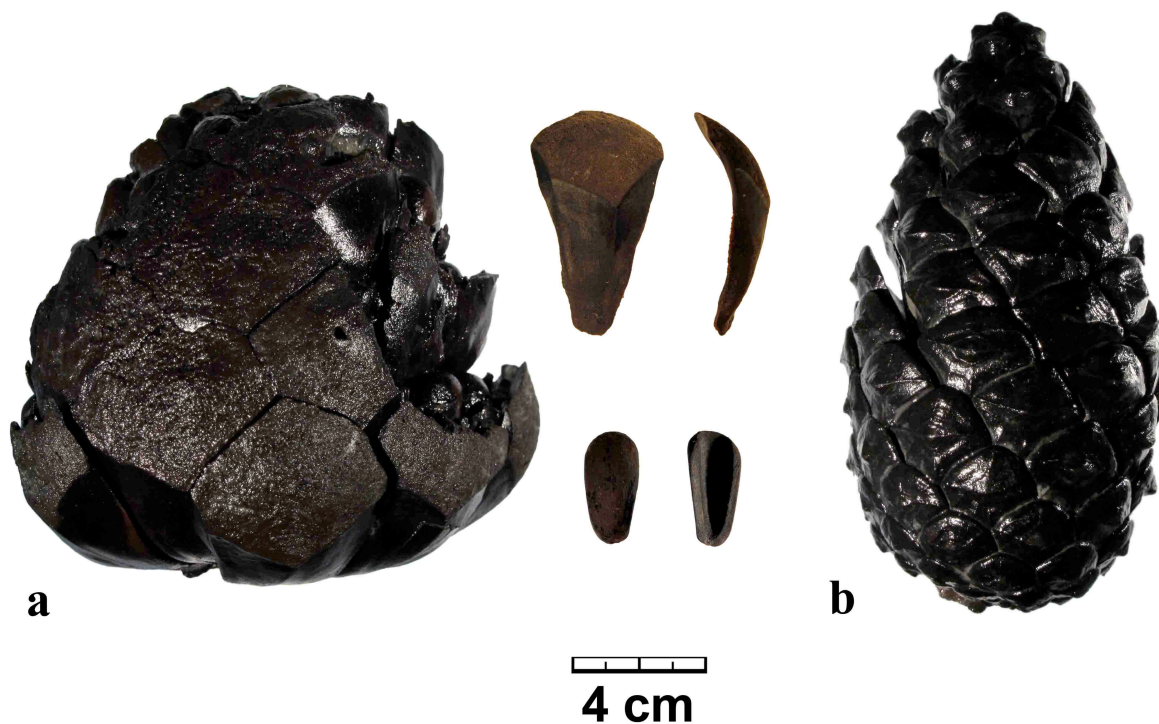


Figure 3. Waterlogged remains of: a) *Pinus Pinea* cone, bract scale and pine nut b) *Pinus halepensis* cone.

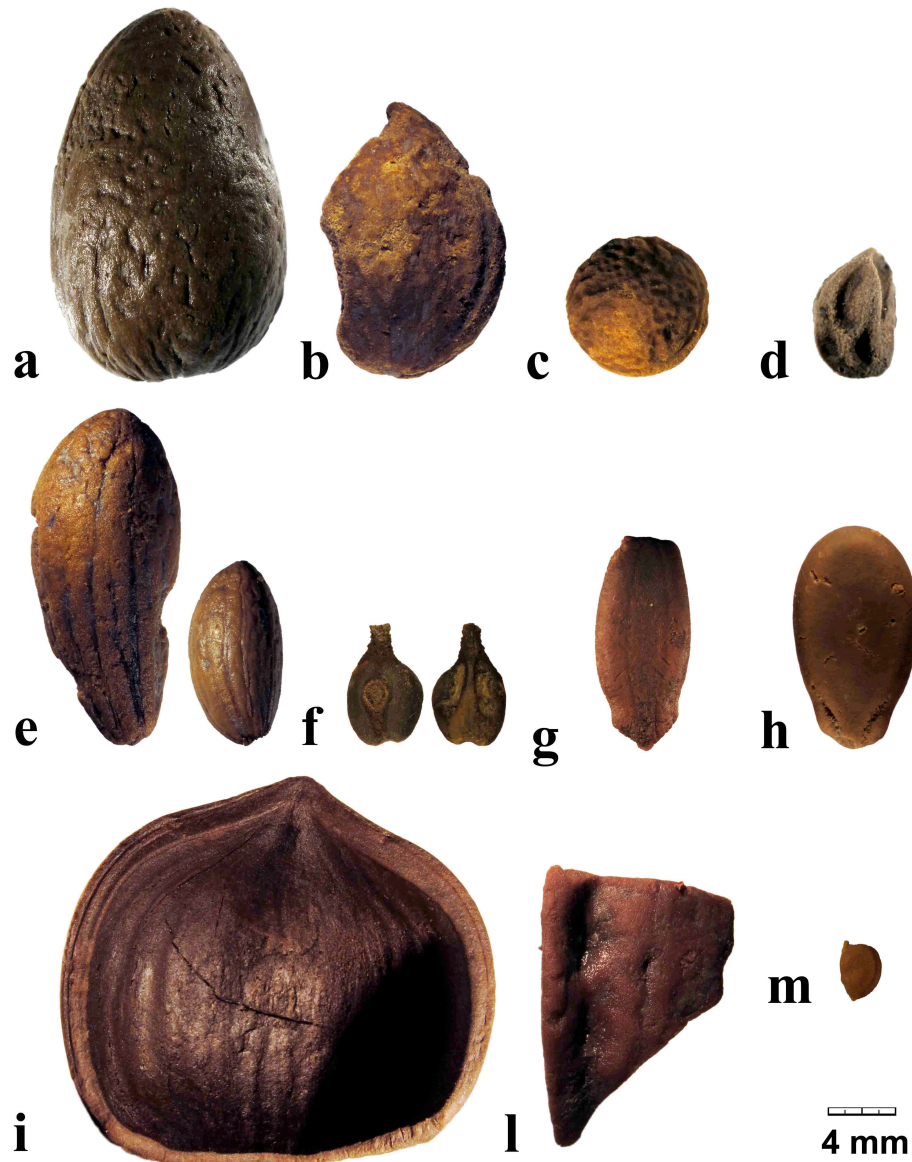


Figure 4. Waterlogged remains of a) *Prunus dulcis* (almond), b) *Prunus domestica* (plum), c) *Prunus spinosa* (sloe), d) *Juniperus oxycedrus* cf. *macrocarpa* (juniper), e) *Olea europaea* (olive), f) *Vitis vinifera* (grape), g) *Lagenaria siceraria* (bottle gourd), h) *Citrullus lanatus* (watermelon), i) *Corylus avellana* (hazelnut), l) *Juglans regia* (walnut), m) *Potamogeton* sp. (pondweed).

2.4 Discussion

The material analysed for this paper is mainly composed of plants of economic importance being the fruits the dominant category. Cereals are absent probably due to the preservation conditions as waterlogging does not favour their conservation; cereal grains are preserved mainly in a charred condition. The same applies to legumes tend to decompose easily under waterlogged conditions. However, despite their absence both plant categories played an important role in the economy of Sardinian communities. In part because the Carthaginian occupation of the island has long been understood in terms of a colonial exploitation of the agrarian and mineral resources. The most abundant species is the grape that has been recovered from all contexts and periods. Due to the

concentration of remains in some of the vessels, particularly in the oldest material, it is likely that some of the amphorae contained grapes or dried raisins.

Many of the amphorae recovered contained remains of ovine/caprine bones suggesting the trading of meat pieces. Meat could be preserved salted, marinated, smoked, dried and also dipped in wine into which different additives were added (fruits, berries, etc.) which could explain the presence of grape pips. Textual evidence points to the role of wine as ingredient in many foods such as meat marinades, fish sauces, cheeses and desserts (McGovern 1999). Grape as well as other species such as plums and perhaps sloes (identified amongst the archaeobotanical remains) may have played a role in meat preservation. Some authors (Sampels 2013) emphasize the antioxidant capacity of many fruits, spices and berries (as plums and grapes) due to their content in phenols, anthocyanins and ascorbic acid. Furthermore, modern food industry uses plum products and grape seed extracts in food processing for improving the colour of finished meat rising the degree of acceptability of foods (Karre *et al.* 2013). Other species such as the strong scented berries of juniper, which have been found at the site and grow widely in Sardinia, could be considered as additives to improve wine flavour (Atzei 2003). Similar examples of grape pips associated to ovine/caprine bones are also documented in some amphorae from Nora (Poplin 1980, Marival and Cassien 2001). Furthermore, a 10th century AD receipt reported from Didimo in *Geoponica* (Book 19th 9.5, translated by Meana *et al.* 1998) suggests the use of grape pomace without separating the skin from the berry, to preserve sheep, goat and deer salted meat. The remains found at Nora were interpreted in this line (André 1981). For Santa Giusta, this could be also the case, at least for some of the grape seeds found. Such a practice would allow recycling the wine waste produced in the numerous wineries active in the area (Pérez Jordà *et al.* 2010). *Vitis* pollen have been recorded since the 5th century BC levels at *Tharros* (Acquaro *et al.* 2001) and is recorded at the transition from the middle Bronze Age to the Punic Period (approx. from 3500 to 2500 cal BP) in the lake of Mistras (Di Rita and Melis 2013). Grape pollen and pips have been found huge amount in the same area since Bronze Age (Lovicu *et al.* 2011, Orrù *et al.* 2013, Uccesu *et al.* 2014b).

Grape pips may have also come from amphorae containing wine as, in antiquity, wine could include grape residues (pedicels, skins, pips). It remains unclear whether their variable presence in the archaeological record represented different grades of wine quality. Apart from grape pips, none of these remains have been reported from Santa Giusta. In some of the amphorae, there is evidence of treatment with a pitch coating (Del Vais and Sanna 2009) to reduce the permeability of the amphorae walls during transport of liquids pointing most probably wine. One of the commonest materials used for vessel coating was pine resin, which beyond waterproofing the pottery added also a pleasant flavour to the wine. It also helped to preserve wine by preventing oxygen from entering through the pores of the clay and therefore avoiding the multiplication of the bacteria responsible for the conversion of wine into vinegar. It has been suggested that it also contributed to lessen unpleasant taste or odour (McGovern 1996). Pliny, in *Natural History* (16.38), described the use of pine pitch for

lining storage jars. Pine resin from Aleppo pine (*Pinus halepensis*) has been traditionally used in Greece to produce the retsina, a popular resinated wine. Several pinecones have been retrieved belonging to two different species: *Pinus halepensis* and *P. pinea* from which also seeds have been identified. Their presence in the samples is difficult to interpret. In antiquity, pinecones sealed with clay were used as stoppers in amphorae (Twede 2002) while steeped in the wine helped to avoid the wine becoming vinegar. But, in order to enhance wine taste and scent, additives other than resin were also added (fruits, berries, spices, herbs, honey, etc.).

The presence of walnuts, hazelnuts, almonds and pine nuts amongst the remains suggests that also dry fruits were part of the traded items. Although *Corylus avellana* and *Juglans regia* are considered introduced plants in Sardinia (Podda *et al.* 2012), pollen of the genus *Corylus* and *Juglans* is present in the same area since at least 5300 BP (Di Rita and Melis 2013) and evident in the 5th century BC (Acquaro *et al.* 2001). *Juglans* is considered an indicator of human activities (Di Rita and Melis 2013). In northern Italy *Corylus* is recorded since the Neolithic (Rottoli and Castiglioni 2009). As far as pine (*P. halepensis* and *P. pinea*) is concerned, it could be of local provenience although nowadays pines only thrive naturally in two restricted areas in the southwest, Porto Pino and Portixeddu-Buggerru (Mossa 1990). However, walnuts, hazelnuts and pine nuts identified in Punic Carthage have been interpreted as exotic food, since these plants do not grow naturally in the area (van Zeist *et al.* 2001). It is yet unclear whether these *taxa* were present in the island before the Phoenician colonization or if the colonists introduced them.

Seeds of *Olea europea* show various shapes and sizes representing perhaps different varieties belonging to cultivated and/or wild specimens. The record of olives in Sardinia goes back to at least the Middle Bronze Age, 1800-1300 BC. Olive stones were identified at the site of *Duos Nuraghes* (Bakels 2002) and *Olea* pollen appears in *Tharros* (Acquaro *et al.* 2001) and in the *Mistras* diagram (Di Rita and Melis 2013) where moderate frequencies (up to 5%) are detected between 5300-1600 BP indicating that olive trees were extensively exploited. Bearing in mind the enormous importance of olive in the Mediterranean it is surprising that olive cultivation was not a common practice in Sardinia according to the archaeobotanical data (Di Rita and Melis 2013). The association of olive stones with amphorae remains open to various possibilities. On the one hand, olives or olive oil could have been traded and on the other, it also is likely that olives were ingredients of the meat dressing transported.

Citrullus lanatus (watermelon) and *Lagenaria siceraria* (bottle gourd) only appeared in the latest phase, in samples from the 3rd century BC, indicating, perhaps, a later introduction. In both cases the number of seeds is limited (only 3 seeds in total). It is believed that the area of domestication of *Citrullus lanatus* is located in Africa where it was growing at least since the beginnings of the 4th millennium BP (Zohary *et al.* 2012). The oldest representation comes from an Egyptian wall painting from a tomb at *Meir* dated to the Old Kingdom (3100-2180) as reported by Janick *et al.* (2007). However, remains of cultivated watermelon are only found in Egypt during the 2nd millennium BC. Hepper (1990) reported them from Tutankhamen tomb and this species has been also found in Sudan

(van Zeist 1983). Wasylikowa and van der Veen (2004) suggest the possibility that some seeds from sites in Egypt may have been erroneously identified as other *Citrullus* species being in fact *C. lanatus*. Cox and Van der Veen (2008) reported further remains of watermelon seeds dated to the 1st millennium BC such as those from the site of *Raybun*, in South Arabia (Levkovskaya and Filatenko 1992), in the Hera temple of Samos, Greece (Kučan 1995) and Israel (Schultze-Motel 1974). For Europe, but also from northern Africa, remains have been retrieved in a number of sites dated to the Roman period (Castelletti *at al.* 2001, Rinaldi *et al.* 2013), for further details see Cox and van der Veen (2008). Watermelon was probably known by Phoenicians and the relative colonies. The presence of *Lagenaria siceria* is probably related to its traditional use as water container, a practice still common today in Sardinia (Atzei 2003) and Africa and widely diffused in Roman times (Schlumbaum and Vandorpe 2012).

Seeds of *Potamogeton* sp. (pondweed) were probably within the sediment that filled the amphorae during the deposition. This water plant thrives in shallow freshwater, but some species such as *Potamogeton pectinatus* (common in Santa Giusta pond) have shown some salinity tolerance (Van Wijk *et al.* 1988). The lagoon in Phoenician and Punic age was probably smaller than it is today (Del Vais and Sanna 2012). Lugliè (2001) reports a possible submerged ancient river bed in the south of the Area A, in phase with the findings of 6th-5th century BC. The abundant record of pondweeds both within the layers and inside the amphorae may perhaps suggest a low freshwater level

2.5 Conclusions

Waterlogged plant remains from various amphorae dedicated to the transport of goods dated to two different periods, the 6th-5th and the 3rd-2nd centuries BC were retrieved in Santa Giusta lagoon. Plant composition was similar in both assemblages suggesting some continuity in the use of the species identified. The absence of staples such as cereals is probably related to the preservation of the material under waterlogged conditions, which does not favour cereal grain preservation. Cereals get usually preserved by charring. Other species such as walnuts, hazelnuts and pine nuts were probably part of trade routes, which may have involved specific categories of products with high commercial value. In addition, basic products were locally produced. Palynological research in Sardinia has shown the presence of pine and other fruit trees although it is yet unclear whether they were cultivated in limited areas. The relatively large size of the fruits suggests a strong selection of the best specimens.

The occurrence of fruits, mainly grapes, and spices together with animal bones with clear butchery marks can be understood as a particular way of meat conservation as suggested by similar finds in other sites.

The record of two cucurbits, watermelon and bottle gourd, points to contacts with other colonies of the Northern coast of Africa, although these may have occurred only in the most recent phase. It is unlikely that these crops were locally cultivated. The spread of watermelon in Europe occurred during Roman colonization, when it played only a marginal role, while proper cultivation of this crop only

occurred later, in medieval times.

The analysis of plant remains from Santa Giusta lagoon has allowed exploring aspects of Phoenician/Punic trade in Mediterranean basin. This is especially important because archaeobotanical data from these periods is rather scarce not only in Sardinia but also in the remaining Mediterranean region. The dataset studied for this paper is a small part of the total available so further research will certainly contribute to clarify many of other issues raised in this paper and improve our knowledge about Punic agriculture.

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CHAPTER 3

SEEDS MORPHO-COLOURIMETRIC ANALYSIS AS COMPLEMENTARY METHOD TO MOLECULAR CHARACTERIZATION OF MELON DIVERSITY

SEEDS MORPHO-COLOURIMETRIC ANALYSIS AS COMPLEMENTARY METHOD TO MOLECULAR CHARACTERIZATION OF MELON DIVERSITY

Diego Sabato¹, Cristina Esteras², Oscar Grillo¹⁻³, Belén Picó², Gianluigi Bacchetta¹

¹ *Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Italy.*

² *Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Universitat Politècnica de València, Spain.*

³ *Stazione Consorziale Sperimentale di Granicoltura per la Sicilia (SSGS), Caltagirone, Italy.*

Abstract

Melon has undergone an intense process of selection and crossbreeding, resulting in many landraces distributed all over Europe, Africa and Asia. Due to this huge variability, the systematic position of this *taxon* has been reviewed many times in the last two decades.

The goal of this article is to compare the phenotypic characterization achieved by seed features with the molecular analysis on melon genotypes. A set of 124 accessions of *Cucumis melo* has been selected for molecular and morpho-colourimetric analyses plus an additional selection of accessions of *Cucumis sativus*, *Citrullus lanatus* and *Citrullus colocynthis* used to highlight seed morphology distances among genus and species.

Genotyping was performed on the basis of 211 polymorphic SNPs and was executed using the iPLEX[®] Gold MassARRAY Sequenom technology. A total of 137 parameters were specifically designed to evaluate seeds colour, size, shape and texture.

Both molecular and seed morpho-colourimetric analyses confirm the existence of two melon subspecies while an intermediate group has also been found. A non random allelic distribution in SNPs located in specific genomic regions suggests that some of these regions may account for a part of the observed variation in seed size. Six major groups of varieties can be discriminated on the basis on seed traits.

Keywords: Cucurbitaceae; *Cucumis melo*; genetic characterization; old landraces; seed image analysis; wild crop relatives.

3.1 Introduction

3.1.1 General background

The Cucurbitaceae family consists of two well defined subfamilies (Zanonioideae and Cucurbitoideae) with about 130 genera and 800 *taxa* according to most recent classifications, outlining varying degrees of circumscriptive cohesiveness (Jeffrey 2005, Jeffrey and De Wilde 2006). Among them, the most economically important species are *Cucumis melo* L. (melon), *Cucumis sativus* L. (cucumber), *Citrullus lanatus* (Thunb.) Matsum & Nakai (watermelon) and *Cucurbita* L. spp. (gourds and squashes).

Melon is worldwide diffused and comprises wild, feral and cultivated varieties, including sweet melons used for dessert and non-sweet ones consumed raw, pickled or cooked (Kirkbride, 1993, Bates and Robinson, 1995). The origin of domesticated melon is not fully understood yet, Africa has been traditionally thought to be the centre of origin of this species, as the presence of melon is attested in Egypt since at least the third millennium BC (Zohary *et al.* 2012) and since the second millennium BC in Western Mediterranean (Sabato *et al.* 2015 [Chapter 1]). However, recent studies postulate that the origin-distribution centre include the Australia-Malaysia area (Renner *et al.* 2007). In addition, due to the high level of variation found in Asia, especially in India, melon could have originated there and then reached Africa (Renner *et al.* 2007, Sebastian *et al.* 2010). Other theories suggest that two independent domestications took place (Jeffrey 1980, Esquinas-Alcazar and Guilick 1983, Mallick and Mausi 1986, Bates and Robinson 1995). First representations show fruits likely belonging to the non-sweet melon varieties *chate* and *flexuosus* (with long cucumber-like fruits) (Janick *et al.* 2007). The presence of round sweet melons in the Mediterranean basin till the Classical Age is uncertain, but it is well proven since the 11th century AD by Arabian trade with Central Asia (Paris *et al.* 2012).

Although *C. melo* has been traditionally separated into two subspecies according to ovary hairiness, subspecies *melo* and *agrestis* (Naudin 1859), nowadays this classification mainly responds to molecular clustering. Different varieties have been reported within each subspecies (Naudin 1859, Munger and Robinson 1991). Pitrat *et al.* (2000) recognized 16 varieties: *cantalupensis* Naudin and *reticulatus* Ser. (cantaloupes, muskmelons), *inodorus* H.Jac. (winter melons, casaba melons), *flexuosus* L. (snake melons), *chate* Hasselq. (cucumber melons), *adana* Pangalo, *chandalak* Gabaev, *ameri* Pangalo (Asian melons), *chito* C.Morren (American melons), *dudaim* L. (pocket melons), and *tibish* Mohamed within the subsp. *melo* L. and *acidulus* Naudin, *conomon* Thunb., *makuwa* Makino and *chinensis* Pangalo (pickling melons), and *momordica* Roxb. (snap melons) within subsp. *agrestis* Naudin. In later revisions, Pitrat (2008) merged some varieties and Esteras *et al.* (2009, 2013), after further molecular studies, moved *tibish* and *chito* into the subspecies *agrestis*. Additional simplified systems have also been proposed (Nesom 2011). Some of these varieties are quite heterogeneous, and accessions displaying intermediate features are difficult to classify. On the other hand, the wild forms of *agrestis* are usually referred to as *C. melo* subsp. *agrestis* var. *agrestis*, following early

classifications. They are mainly distributed in North and Eastern Africa and the Indian sub-continent (Roy *et al.* 2012), but free-living forms of small size fruited melons have been found in Northern Australia, Southern USA and Central America. Among all the varieties, the sweet *cantalupensis*, *reticulatus* and *inodorus* melons are the ones with the most commercial interest worldwide (Pitrat, 2008).

3.1.2 Analyses

In order to establish genetic relationships among the aforementioned subspecies and varieties, several molecular studies have been carried out in melon, employing different marker systems like RFLPs, RAPDs, AFLPs, ISSRs, SSRs and lately SNPs (reviewed in: Esteras *et al.* 2012). Most of them support the division at sub-specific level and some have contributed to better reclassify some of the varieties (Stepansky *et al.* 1999, Deleu *et al.* 2009, Esteras *et al.* 2009, 2013). In addition, a high diversity has been reported within the subspecies *agrestis*, which has been used in breeding mainly to introduce disease resistances to commercial types. Geographically, more variation has been described near the centres of domestication (Africa and India) compared to other distribution areas (Blanca *et al.* 2012). SNPs are considered to be high-quality markers and are mostly used for genome-wide surveys of genetic diversity in high to medium-throughput genotyping platforms (Fan *et al.* 2006, Steermers and Gunderson 2007, Gabriel *et al.* 2009). The number of this kind of markers available in melon has largely increased in the last few years (Blanca *et al.* 2011, 2012). The availability of these large SNPs collections (<http://melogene.net/>) has encouraged diversity studies in melon on a genome-scale. Esteras *et al.* (2013) reported the first application of a GoldenGate platform to genotype a melon core collection with 768 markers distributed throughout the genome, demonstrating the usefulness of this SNP set for genetic diversity and population structure studies. Furthermore, the recent publication of the sequence of the melon genome (Garcia-Mas *et al.* 2012) will also promote this kind of surveys, providing an additional resource to map these newly identified SNPs.

Regarding phenotypic variability in this crop, several studies have been carried out with core collections representative of the species (Stepansky *et al.* 1999, Esteras *et al.* 2009, Leida *et al.* in press), and with germplasm from specific centres of origin and diversity (reviewed in: Esteras *et al.* 2012, Raghmi *et al.* 2014). These assays have basically focused on fruit traits and response to biotic and abiotic stress and many QTLs controlling these traits have been already mapped in the melon genome (Diaz *et al.* 2011). In contrast to other species for which extensive efforts have been made in mapping QTLs for seed properties (Cai *et al.* 2012), and even in cloning the underlying genes (Orsi and Tanksley 2009), only some studies have included seed traits in melons. Some of them report the correlation of seed traits to botanical classification and origin (Stepansky *et al.* 1999, Yashiro *et al.* 2005, Tanaka *et al.* 2007). Fujishita and Nakagawa (1973) pointed out that seed size is one important trait for variety identification in melon. Fujishita (1980) described *makuwa* and *conomon* varieties with seeds smaller than 9 mm, *reticulatus* with seeds larger than 9 mm and *momordica* with

intermediate seeds. Moreover in a recent study Tanaka *et al.* (2013) correlated seed length and weight to chloroplast genome variation, using accessions belonging to six varieties and some unclassified accessions. However, seed traits have not been extensively analysed in large collections, representing the whole diversity of the species, mainly due to the difficulty to measure these tiny characteristics. In other cucurbits like *Cucurbita pepo*, seed traits have been used as discriminating factors since early studies (Decker and Newsom 1988), and correlation between seed and fruit traits has been reported (Paris and Nerson 2003). The existence of a similar correlation in melon needs to be demonstrated, but it would facilitate genetics studies of seed characteristics by using the available genetic information on fruit traits.

Since the inception of the taxonomy, hierarchical classifications have been constructed on the basis of morphology and it seems that these classifications are congruent with most of phylogenetic levels. DNA sequence analyses can provide corroboration, resolution, support, and accuracy for those parts of phylogeny for which appropriate morphological data is lacking (Scotland *et al.* 2003). For example, one of the latest works performed with Sardinian grape cultivars correlated shape features with molecular discrimination, in this case the multiproxy approach allowed to achieve a clear discrimination among local cultivars and revealed the synonymy groups of local names attributed on same cultivar (Orrú *et al.* 2013a). Positive results have been obtained as well on molecular and seed morphological traits on pumpkin (Liu *et al.* 2013). Morpho-colourimetric evaluations are commonly employed as tools to assess shape, size and colour of objects, in order to relate these quantitative physical characteristics with qualitative aspects (Venora *et al.* 2009, Grillo *et al.* 2010). Compared to conventional measurements, computer-aided morpho-colourimetry is exponentially faster, more accurate, precise and efficient, providing a significantly broader spectrum of measurements of morphological and colourimetric features and, at the same time, replacing subjective estimations with objective quantifications (Venora *et al.* 2007a, Bacchetta *et al.* 2008). Several works about the application of image analysis to the diaspores of wild vascular flora have been carried out, providing excellent results of classification within taxonomic units close to infra-generic, infra-specific and intra-population levels (Bacchetta *et al.* 2008, Bacchetta *et al.* 2011a, 2011b, Grillo *et al.* 2012, Pinna *et al.* 2014). Many studies have been focused also on crop wild relatives and landraces (Venora *et al.* 2007b, Smykalova *et al.* 2011, Smykalova *et al.* 2013), and recently many authors focused on the *Vitis vinifera* complex (Rivera *et al.* 2007, Terral *et al.* 2010, Orrú *et al.* 2013a, 2013b).

The knowledge of the existing diversity in melon is essential, not only for the conservation of this genetic diversity, but also for its exploitation in commercial breeding, as this species displays crossability problems with other species of the genus *Cucumis*.

The goals of this research are to:

- compare the groups established using molecular analyses with those achieved by seed characters;
- analyse the variability of morpho-colourimetric seed features;
- implement statistical classifiers able to discriminate among the studied varieties;
- increase the knowledge about the variation of the current extant melon seed collections;

3.2 Material and methods

3.2.1 Seed lots detail

The starting material was a core collection of 200 melon accessions, including wild relatives, feral types, landraces, breeding lines and commercial cultivars from 54 countries representing the putative origin areas and diversification centres of the species. This collection was established on the framework of a previous project (MELRIP 2007-2010, Esteras *et al.* 2009, 2013). It has been multiplied and conserved at the COMAV Genebank (Institute for the Conservation and Breeding of the Agrobiodiversity, www.comav.upv.es). The full collection was genotyped with AFLP and SNPs markers, and extensively phenotyped for plant and fruit traits at COMAV.

According to the previously generated phenotypic and genotypic data, we selected a subset of 124 accessions, originated from 48 countries and representing all varieties. Fruits were collected at the optimum maturity stage corresponding to the complete morphologic and chromatic seed development. To avoid over-representation of single plant and/or fruit features, seeds from the highest number of plants and fruits available for each accession have been taken. Undeveloped (stenospermocarpic), hard deformed and sterile seeds were excluded. Fruit weight phenotypes of this subset of accessions produced in these previous phenotyping assays were used to study the correlation between fruit and the seed traits analysed in the present work. Details of accessions are provided in **Annex 1**. Representative pictures of fruits and seeds of each variety can be found in Fig. 1 and 2. This sub-collection included accessions from both subspecies *melo* and *agrestis*. Within the subspecies *melo*, 41 accessions belonging to the *cantalupensis*, *reticulatus* and *inodorus* varieties were assayed, including representatives of most commercial market classes, but also old landraces selected in different countries. Moreover, 26 landraces of the *ameri* variety (including the close *ameri*, *adana* and *chandalack* varieties), mainly coming from Eastern Europe, Central and Western Asia and Northern Africa, were also considered. This variety is rarely found in the commercial chain and is thought to be the origin of modern *cantalupensis* and *inodorus* cultivars (Pitrat *et al.* 2000). Additionally, 10 African and Asian *flexuosus* melons, one representative of the cucumber-like melon *chate* from Southern Italy, and three ornamental aromatic *dudaim* from central Asia, were analysed.

The best-represented varieties of the subspecies *agrestis* were *conomon* (with the closely related *chinensis* and *makuwa*) and *momordica* as they are widely used as breeding materials, and included accessions from Far-Eastern countries and India. Several non-sweet and non-climacteric

agrestis accessions were assayed: six *acidulus* accessions from Central Africa, two *tibish* from Sudan (considered to be the most primitive form of melon) (Pitrat *et al.* 2000), one *chito* and nine wild *agrestis* melons with small fruits from Africa, India and America. Also seven accessions that do not fit any of these varieties, showing intermediate characteristics, were included. Accessions of *C. melo* subsp. *agrestis* var. *agrestis* were considered as a separate group, because they represent the wild form of melon whereas all the remaining varieties are cultivated forms.

With the purpose to evidence morphology distances in genus and species levels, a small set of close relatives of melon were used (Fig. 3). An overall amount of 21 accessions of *Cucumis sativus*, 18 of *Citrullus lanatus* and nine of *Citrullus colocynthis*, were selected [Annex 2]. All accessions were supplied by COMAV Genebank and represent mainly Iberian Peninsula landraces and Far East, African and Mediterranean ones.

3.2.2 Molecular analysis

The DNA was extracted from young leaves using the CTAB method with minor changes (Esteras *et al.* 2012). DNA concentrations in TE buffer were adjusted to 50 ng/μl, with the PicoGreen fluorescence being measured on an ABI7900 apparatus (Applied Biosystems). Genotyping was done with a total of 211 polymorphic SNPs (Single Nucleotide Polymorphism), evenly distributed throughout the genome, that were selected from the SNP melon collection available in the Melogene database (<http://www.melogene.net/>) and *in silico* identified in two previous re-sequencing analysis (Blanca *et al.* 2011, 2012). Genotyping was performed using the iPLEX[®] Gold MassARRAY Sequenom technology at the epigenetic and genotyping unit of the University of Valencia (Unitat Central d'Investigació en Medicina UCIM). This genotyping technology relies on Single Base Extension (SBE) using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest to generate different allelic products. Using the MALDI-TOF mass spectrometry, the distinct mass of the extended primer identifies the SNP allele (Gabriel *et al.* 2009).

The genotyping results were employed to perform a cluster analysis using the PowerMarker software (Liu and Muse 2005). Nei's genetic distance (Nei *et al.* 1983) was used, and the support values for the degree of confidence at the nodes of the dendrogram were analysed by bootstrap re-sampling 1,000 times. Phylip 3.69 software (Felsenstein 1997) was employed to construct the consensus tree and TreeView32 (Page 1996) to visualize it. In addition, the number of alleles, the frequency of the most common allele (MAF) and the polymorphism information content (PIC) were calculated for each *locus* with PowerMarker.

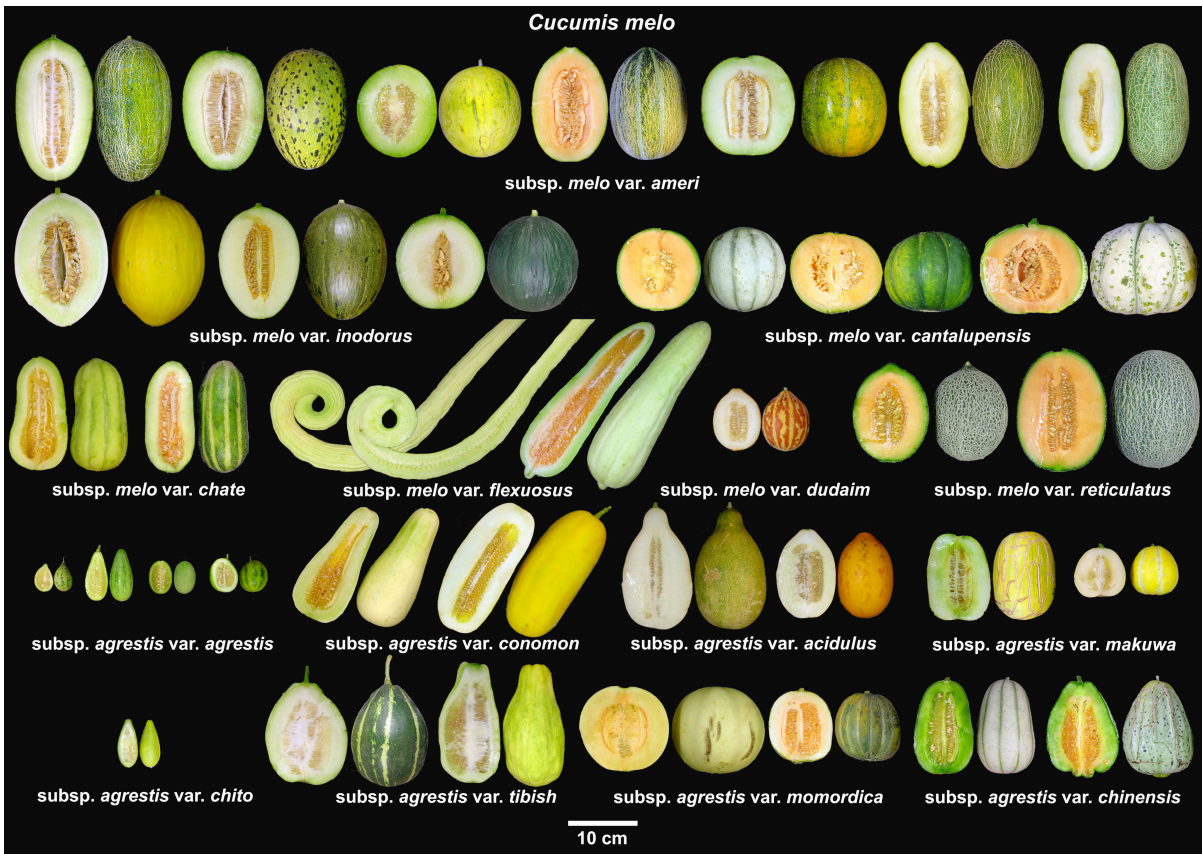


Figure 1. fruits and seeds of each melon variety included in the study

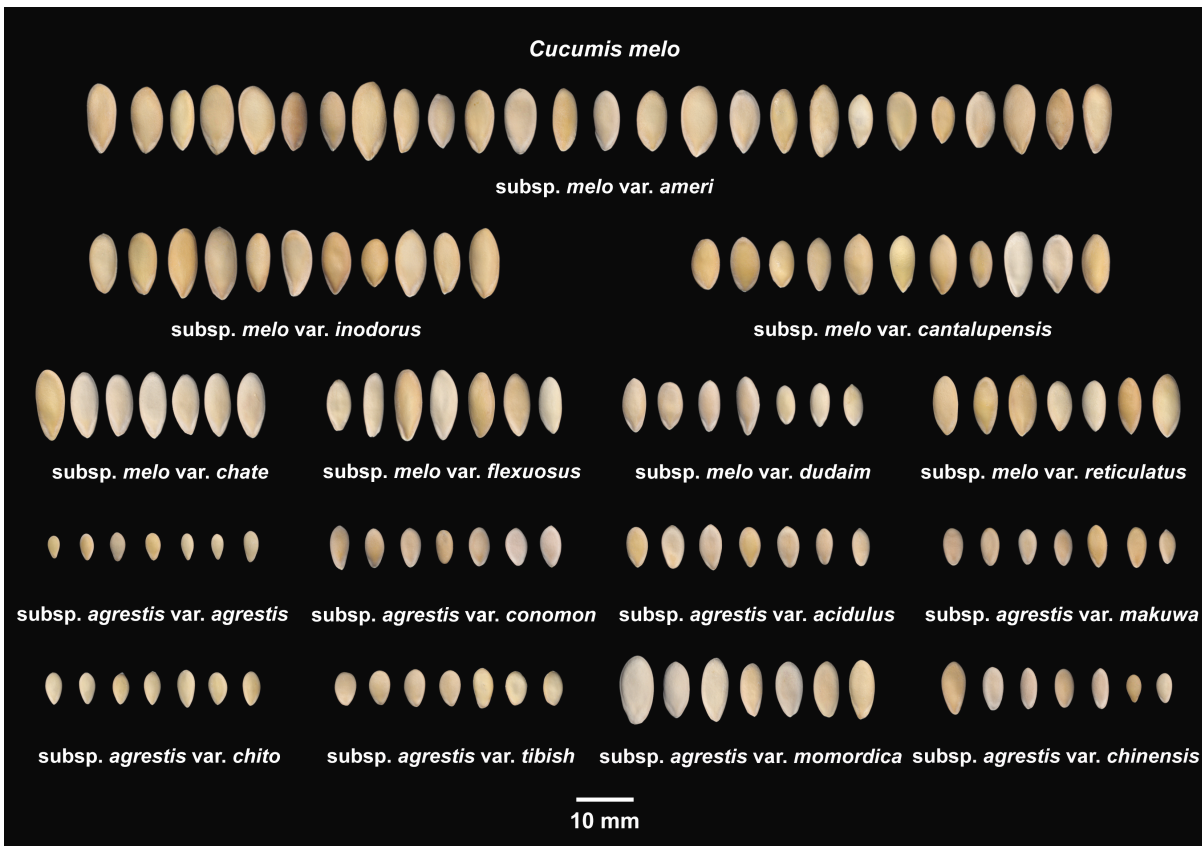


Figure 2. fruits and seeds of each melon variety included in the study

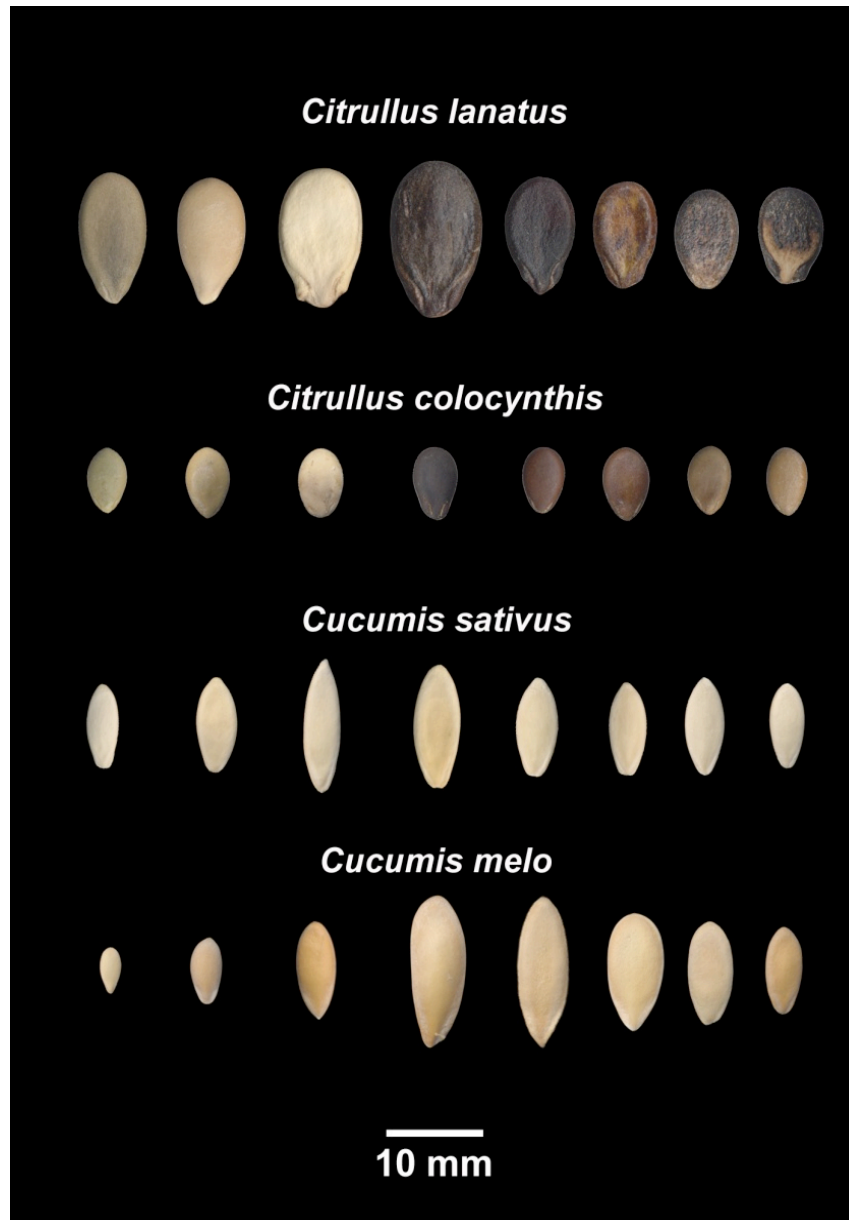


Figure 3. Representative seeds of watermelon, colocynth, melon and cucumber.

The population structure underlying the genotyped collection, the number of populations and the probability of each accession belonging to each inferred population, was analysed using STRUCTURE v2.2 (Pritchard *et al.* 2000) in Esteras *et al.* (2013). Information about markers employed in the genotyping assay and summary statistics generated in the genotyping analysis with PowerMarker software is provided in **Annex 3**. Detailed information for each SNP marker, such as sequence, allele variation and location is available in the Melogene database. Both the database of the melon genome, melonomics (<http://melonomics.net>), and the SNP melon collection available in melogene were used to select and analyse the variation of the melon orthologue of a gene underlying a major QTL associated to seed size in tomato, Seedweight 4.1 (Sw4.1) (Orsi and Tanksley 2009). This is an ABC transporter orthologous to the *Arabidopsis* ABC transporter gene At4g39850, also associated with variation in both seed length and width in this model species. This family of proteins is used in transmembrane transport of diverse substances.

Most SNPs used in this study were employed in previous mapping experiments and their position in the genetic map is known (Esteras *et al.* 2013). This genetic position was used to check the allelic distribution in the germplasm collection of SNPs located in regions of the genetic map in which QTLs for fruit size were previously located (Diaz *et al.* 2011), and to confirm if differential allelic distributions were also related with differences in the seed traits measured in the present study.

3.2.3 *Seed morpho-colourimetric analysis*

Before image acquisition, the scanner was calibrated for colour matching, using the Kodak Q60 R2 Colour Input Target reference image, following the protocol of Shahin and Symons (2003) as suggested by Venora *et al.* (2009). Two images were acquired for each sample, one with black and the other one with white background, using a flatbed scanner (Epson EU22), with a resolution of 400 dpi and 24 bit-depth, in RGB colour model and stored in TIFF format. Sub-samples consisting of 100 seeds were randomly chosen from the original seed lots and arranged on the scanner tray for scanning in such a way that they did not touch each other. When the original accession was numerically lower than 100 units, the analysis was executed on the whole seed lot. All images were analysed with KS-400 release 3.0 image analysis software by Carl Zeiss Vision GmbH (Oberkochen, Germany). A macro, expressly developed for the characterization of cultivated leguminous seeds (Venora *et al.* 2009), was partially modified to perform automatically all the analysis procedures, reducing the execution time and contextual mistakes in the analysis process. In order to increase the discrimination power, this macro was further enhanced adding algorithms able to compute many other size, shape and colour features of each seed in the images. A total of 20 parameters, specifically designed to evaluate seed colour, were measured together with 17 features descriptive of seed dimensions, 78 shape Elliptic Fourier Descriptors (EFDs) able to define seeds contour shape, and further 22 Haralik's features to assess seed surface texture, for an overall amount of 137 morpho-colourimetric parameters (Table 1).

Colour parameters	
Rmean	Red channel mean value of seed pixels (grey levels)
R_SD	Standard Deviation of Red channel value
Gmean	Green channel mean value of seed pixels (grey levels)
G_SD	Standard Deviation of Green channel value
Bmean	Blue channel mean value of seed pixels (grey levels)
B_SD	Standard Deviation of Blue channel value
Hmean	Hue channel mean value of seed pixels (grey levels)
H_SD	Standard Deviation of Hue channel value
Lmean	Lightness channel mean value of seed pixels (grey levels)
L_SD	Standard Deviation of Lightness channel value
Smean	Saturation channel mean value of seed pixels (grey levels)
S_SD	Standard Deviation of Saturation channel value
Dmean	Density channel mean value of seed pixels (grey levels)
D_SD	Standard Deviation of Density channel value
S	Skewness, asymmetry degree of intensity values distribution (grey levels)
K	Kurtosis, peakness degree of intensity values distribution (densitometric units)
H	Energy measure of the increasing intensity power (densitometric units)
E	Entropy Dispersion power (bit)
Dsum	Sum of Density values of the seed pixels (grey levels)
SqDsum	Sum of the Squares of density values (grey levels)
Shape parameters	
A	Area (mm ²)
P	Perimeter (mm)
Pconv	Convex Perimeter (mm)
PCrof	Crofton's Perimeter (calculated using the Crofton's formula) (mm)
Pconv/PCrof	Ratio between convex and Crofton's perimeters
Dmax	Maximum diameter of the seed (mm)
Dmin	Minimum diameter of the seed (mm)
Dmin/Dmax	Ratio between minimum and maximum diameters
Sf	Shape Factor = $(4 \times \pi \times \text{area}) / \text{Perimeter}^2$ (normalized value)
Rf	Roundness Factor = $(4 \times \pi \times \text{area}) / \text{max diameter}^2$ (normalized value)
Ecd	Diameter of a circle with an area equivalent to that of the seed (mm)
EAmx	Maximum axis of an ellipse with equivalent area (mm)
EAmn	Minimum axis of an ellipse with equivalent area (mm)
Cpt	Compact grade = $(\sqrt{2} (4/\pi) \times \text{area}) / \text{Dmax}$
C	Curl = ratio between maximum diameters and Fiber lengths
Fl	Fiber length (mm)
Cvx	Convexity = ratio between Crofton's Perimeters and real Perimeters
EFDs 1 to 78	Elliptic Fourier Descriptors
Texture parameters	
Haralik 1	Angular second moment
HaralikSD1	Standard Deviation of Angular second moment
Haralik 2	Contrast
HaralikSD2	Standard Deviation of Contrast
Haralik 3	Correlation
HaralikSD3	Standard Deviation of Correlation
Haralik 4	Sum of square: variance
HaralikSD4	Standard Deviation of Sum of square: variance
Haralik 5	Inverse difference moment
HaralikSD5	Standard Deviation of moment
Haralik 6	Sum average
HaralikSD6	Standard Deviation of Sum average
Haralik 7	Sum variance
HaralikSD7	Standard Deviation of Sum variance
Haralik 8	Sum Entropy
HaralikSD8	Standard Deviation of Sum Entropy
Haralik 9	Entropy
HaralikDS9	Standard Deviation of Entropy
Haralik 10	Difference variance
HaralikSD10	Standard Deviation of Difference variance
Haralik 11	Difference Entropy
Haralik SD11	Standard Deviation of Difference Entropy

Table 1. List of characters analysed in morpho-colourimetric analysis

3.3 Results

3.3.1 Molecular analysis

The 211 polymorphic SNPs used in this study were quite informative with PIC (Polymorphism Information Content) average values ranging from 0.01 to 0.48 (see Supplementary data). Table 2 shows the polymorphism detected in each of the analysed group of accessions. The highest degree of polymorphism was found in the *ameri* accessions (91.00%), followed by *flexuosus* (85.78%) which also displays the highest genetic diversity (0.32), being *cantalupensis* and *inodorus* less variable (63.51% and 63.98% respectively). Within subsp. *agrestis* the highest polymorphism level was found in *momordica* (73.93%), with a genetic diversity of 0.30, being the wild *agrestis* less variable (46.92%).

<i>C. melo</i> subsp. <i>melo</i>		<i>C. melo</i> subsp. <i>agrestis</i>	
<i>ameri</i>	91.00% / 0.2559 ^a	<i>agrestis</i>	46.92% / 0.1535
<i>cantalupensis</i>	63.51% / 0.2022	<i>conomon</i>	42.65% / 0.1243
<i>dudaim</i> ^b	-	<i>chito</i> ^b	-
<i>flexuosus</i>	85.78% / 0.3193	<i>acidulus</i>	42.65% / 0.1504
<i>inodorus</i>	63.98% / 0.1609	<i>makuwa</i>	5.21% / 0.0197
<i>reticulatus</i>	53.55% / 0.1873	<i>momordica</i>	73.93% / 0.3044
<i>chate</i> ^b	-	<i>chinensis</i>	53.35% / 0.1715
		<i>tibish</i>	

^a Nei's gene diversity (1973).

^b not calculated, none or less than three genotypes analysed in this group

Table 2. Polymorphism level and gene diversity in the different groups according to SNP analysis.

The relationships among the varieties assayed in this study are shown in the NJ tree, constructed with the polymorphic SNPs (Fig. 4). This tree supports the sub specific division and the intermediate position of *momordica*, *flexuosus* and *dudaim* varieties. Within *C. melo* subsp. *melo*, *inodorus* and *cantalupensis* varieties, are clustered apart, with two differentiated clusters within *inodorus* (one containing mainly the Spanish types and a more disperse one with African and Eastern Europe types), and two clusters of *cantalupensis* (commercial Charentais types, and other *cantalupensis* and *reticulatus* types). Accessions of *ameri* variety appear mixed with *inodorus* and *cantalupensis*. In the *C. melo* subsp. *agrestis* the African *agrestis* and *tibish* varieties, and the Asian *conomon*, *makuwa* and *chinensis* ones, could be clearly separated in two groups. Most of the other varieties of this subspecies were intermediate between these two groups.

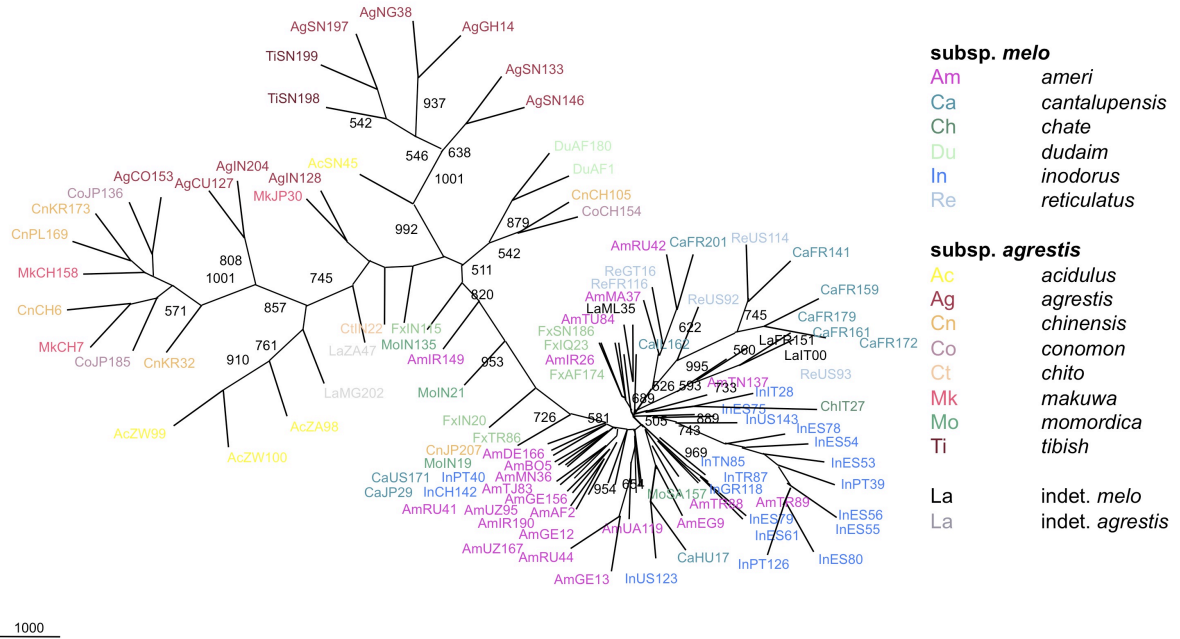


Figure 4. NJ tree constructed with SNPs results. Each group of accessions according to botanical variety is represented in a different colour. Only Bootstrap values higher than 500 are showed.

Annex 1 includes the population or populations (accessions with admixture) to which each accession is assigned according to the Structure analysis (Esteras et al. 2013). The *cantalupensis* accessions corresponded mostly to two structured populations (Charentais French types, population 1, and American *reticulatus*, population 2). A similar **situation** was found in the *inodorus* accessions, with a differentiated population of the Spanish melons (population 3), and a second from Northern Africa and Eastern Europe (population 4). In both varieties some traditional or improved materials showed an admixture of one or two *cantalupensis* and/or *inodorus* populations. Some Central Asian *ameri*, *ananas* and *chandalack* accessions belonged to a differentiated population (population 5), but most of them showed admixture of two or more populations. *Agrestis* accessions were mostly separated into the exotic Eastern *conomon* (population 6) and the African wild *agrestis* plus *acidulus* and *tibish* (population 7), despite some degree of admixture of population 6 and 7 was found in the latter groups.

3.3.2 Seed morpho-colourimetric analysis

In order to assess the phenotypic differences among genus and species hierarchy, the LDA was applied, considering all accessions of *Cucumis melo*, *C. sativus*, *Citrullus lanatus* and *C. colocynthis*. The discrimination of these species, based on 16,096 seeds, is quite clear. All species were discriminated with a high percentage, overall correct identification was 97.6% (Table 3). Misclassification between the two genera, *Cucumis* and *Citrullus*, was fairly close to zero. Also classification errors between the two respective species within the same genus were not significant (1.1%-5.4% between *C. melo* and *C. sativus*, 0.7%-5.2% between *C. lanatus* and *C. colocynthis*).

	<i>Cucumis melo</i>	<i>Cucumis sativus</i>	<i>Citrullus lanatus</i>	<i>Citrullus colocynthis</i>	Total n°
Seeds number					
<i>Cucumis melo</i>	11,472	123	-	-	11,595
<i>Cucumis sativus</i>	112	1,950	-	-	2,062
<i>Citrullus lanatus</i>	76	1	1,412	10	1,499
<i>Citrullus colocynthis</i>	16	-	49	875	940
Percentage					%
<i>Cucumis melo</i>	98.9	1.1	-	-	100.0
<i>Cucumis sativus</i>	5.4	94.6	-	-	100.0
<i>Citrullus lanatus</i>	5.1	0.1	94.2	0.7	100.0
<i>Citrullus colocynthis</i>	1.7	-	5.2	93.1	100.0

- 97.6% overall classification

Table 3. Results of cross validated LDA analysis on *Cucumis melo*, *Cucumis sativus*, *Citrullus lanatus* and *Citrullus colocynthis*. First part of the table reports the amount of analysed seeds, the second part the respective percentage. The value of the number of an item crossed with itself and the other items indicates the number/percentage of seeds correctly classified as the same group, e.g. among the 11,595 of *C. melo* seeds, 11,472 (98.9%) have been correctly classified as melon, 123 (1.1%) as *C. sativus* and none as *C. colocynthis* or *C. lanatus*.

Figure 5 reports the 2D scatter-plot graph of the discrimination among *Cucumis* and *Citrullus* species. The four *taxa* are clearly grouped and the distance between the two genera, *Cucumis* and *Citrullus*, is higher than that between the two species within the same genus. *C. colocynthis* and *C. lanatus* seeds are more distant than *C. melo* and *C. sativus* seeds.

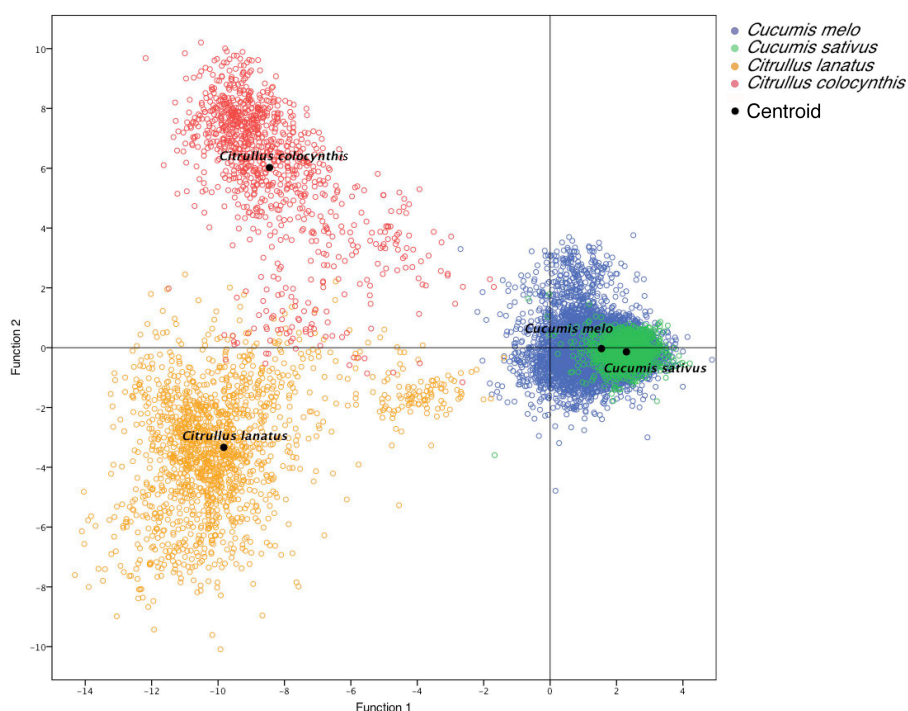


Figure 5. Scatter plot graph based on LDA analysis discrimination of *Cucumis melo*, *Cucumis sativus*, *Citrullus lanatus* and *Citrullus colocynthis*. Small points represent single seed data, black points represent their average (centroid). Spatial arrangement of points suggests similarity and dissimilarity of groups, but just first two functions of 3 available can be used for the graphical representation. The variance on X axis is 74.4% and on Y axis is 15.1%, the remaining 10.5% is distributed on the non represented third function.

Table 4 shows the first 10 discriminant parameters according to the *F-to-remove* value. As expected, the most important seed character was related to seed colour, mainly the mean red channel value (Rmean). It means that colour, and in particular the wavelengths related to the red light, is the most reliable feature to distinguish among these *taxa*. Seed dimension also plays an important role in discrimination. Minimum diameter (Dmin), Area (A) and some derived measures such as the maximum axis of the ellipse with equivalent area (Eamax), were of great importance. Also two texture parameters (Haralik11 and 5), resulted in being useful for the *taxa* identification, mainly related to differences between the spotted and wrinkled watermelon seeds and the smooth and monochromatic melon and cucumber seeds.

Parameter	<i>F-to-remove</i>	<i>Tolerance</i>	<i>Wilks' lambda</i>
1 Rmean	3526.6	0.045	0.008
2 Dmin	914.2	0.020	0.005
3 Area	755.6	0.019	0.005
4 Haralik11	651.4	0.197	0.005
5 Haralik5	595.1	0.001	0.005
6 FD18	593.8	0.290	0.005
7 SDsum	570.9	0.008	0.005
8 Eamax	532.5	0.126	0.005
9 Sf	526.4	0.009	0.005
10 Cpt	404.2	0.005	0.005

Table 4. First 10 factors used for discrimination among species in order of decreasing *F-to-remove*, that describes the power of each variable in the model. The *Tolerance* indicates the proportion of a variable variance not accounted by other independent variables in the equation. *Wilks' lambda* is a direct measure of the proportion of variance in the combination of dependent variables that is unaccounted for by the independent variable.

Discrimination of melon seeds between the two *C. melo* subspecies was also clear with an overall correct identification of 93.2% (Table 5). Seeds of the subspecies *melo* were correctly classified in 98.2% of cases, with only 146 seeds out of the 8,125 analysed seeds misattributed to the subspecies *agrestis*. Wild melons were also correctly classified in 96.4% of cases, with the remaining 3.6% of the cases misclassified as seeds belonging to the same subspecies and not to subspecies *melo*. Subspecies *agrestis* was correctly discriminated in 76.7% of cases.

	<i>C. melo</i> subsp. <i>melo</i>	<i>C. melo</i> subsp. <i>agrestis</i>	<i>C. melo</i> subsp. <i>agrestis</i> (wild)	Total n°
Seed number				
<i>C. melo</i> subsp. <i>melo</i>	7,979	146	-	8,125
<i>C. melo</i> subsp. <i>agrestis</i>	486	2,008	124	2,618
<i>C. melo</i> subsp. <i>agrestis</i> (wild)	-	31	821	852
Percentage				%
<i>C. melo</i> subsp. <i>melo</i>	98.2	1.8	-	100.0
<i>C. melo</i> subsp. <i>agrestis</i>	18.6	76.7	4.7	100.0
<i>C. melo</i> subsp. <i>agrestis</i> (wild)	-	3.6	96.4	100.0

- 93.2% overall classification

Table 5. Results of cross validated LDA analysis on melon subspecies.

Unlike the inter-genera classification, infraspecific classification is mainly due to seed size parameters (Table 6). Area and Eecd are the first two parameters used to distinguish between subspecies, the colour traits being less important. Nevertheless, R_SD, S_SD, B_SD, L_SD colour values were useful to differentiate *melo* seeds, with a darker cream colour, from lighter *agrestis* seeds.

Parameter	<i>F-to-remove</i>	<i>Tolerance</i>	<i>Wilks' lambda</i>
1 Eecd	532.1	0.006	0.112
2 Area	333.0	0.003	0.108
3 Gmean	224.8	0.018	0.106
4 R_SD	188.1	0.006	0.106
5 S_SD	170.4	0.039	0.106
6 B_SD	144.7	0.023	0.105
7 L_SD	118.6	0.003	0.105
8 Energy	107.0	0.032	0.104
9 Entropy	99.4	0.017	0.104
10 HaralikSD7	99.0	0.022	0.104

Table 6. First 10 factors used for discrimination among subspecies in order of decreasing *F-to-remove*.

Table 7 shows the cross-validated results of melon varieties classification. Correct classification percentages of varieties belonging to subspecies *agrestis* (ranging from 55.8% to 93.3%) was greater than that of *melo* varieties (ranging from 37.1% to 86.1%). Overall correct identification was of 64.9%. Wild melons formed a homogeneous group, with only a 6.7% of misclassification with other related types of the *agrestis* subspecies.

The other two varieties of subspecies *agrestis*, more genetically related to the wild types, are *tibish* and *acidulus*. Accessions of both varieties included in this study were all from Africa except one from Sri Lanka, and their seeds were quite well discriminated, 79.5% and 80.6% respectively. A lower degree of discrimination (ranging from 54.2% to 70.6%) was found in accessions of the *conomon* and related varieties, *chinensis* and *makuwa*, misclassifications mostly occurred among them. Within the subspecies melon, seed analysis gave a 20% of *ameri* classified as *inodorus* and vice versa. Also a 24.4% and 16.4% of misclassification with the *ameri* type was found in *cantalupensis* and *reticulatus*, respectively. *Momordica* was misidentified in several other varieties, mainly belonging to subspecies *melo* (*ameri*, *flexuosus* and *reticulatus*). It was not possible to define this variety as a determined group, since seeds can be correctly classified as *momordica* only in 55.8% of cases. Figure 6 shows the spatial position occupied by each variety where affinity distances can be deduced. Varieties belonging to subspecies *agrestis*, except from *momordica* variety, are distributed mainly in the left quadrant, rather than subspecies *melo* that occupies the right side. An intermediate group across the two subspecies, formed with *momordica*, *dudaim*, *flexuosus* and *chate* varieties, can be easily determined. Both dimension and colour traits proved to be key parameters (Table 8). Area and Eecd were again the most discriminant factors followed by colour parameters.

	<i>ameri</i>	<i>inodorus</i>	<i>cantalupensis</i>	<i>reticulatus</i>	<i>chate</i>	<i>flexuosus</i>	<i>dudaim</i>	<i>momordica</i>	<i>acidulus</i>	<i>tibish</i>	<i>chinensis</i>	<i>conomon</i>	<i>makuwa</i>	<i>chito</i>	<i>agrestis</i>	<i>indet. melo</i>	<i>indet. agrestis</i>	Total n°
Seed number																		
<i>ameri</i>	1569	479	164	114	27	54	6	45	12	-	-	-	-	1	-	41	3	2515
<i>inodorus</i>	456	1516	64	93	12	11	1	23	1	1	-	-	-	-	-	20	1	2199
<i>cantalupensis</i>	233	57	354	182	51	37	-	7	4	1	10	-	-	-	-	9	10	955
<i>reticulatus</i>	112	24	68	444	-	1	18	-	-	-	-	-	-	-	-	14	-	681
<i>chate</i>	6	-	-	-	85	5	-	2	-	-	-	-	-	-	-	-	-	98
<i>flexuosus</i>	147	2	16	12	21	637	22	47	1	1	-	-	-	-	-	1	-	907
<i>dudaim</i>	42	-	-	13	1	-	229	1	7	1	-	-	-	1	-	-	-	295
<i>momordica</i>	68	31	2	18	2	32	1	198	-	-	-	-	-	-	-	3	-	355
<i>acidulus</i>	14	2	15	-	-	7	1	1	458	10	19	1	19	3	-	1	17	568
<i>tibish</i>	-	-	-	-	-	-	4	-	18	124	-	1	1	-	-	-	8	156
<i>chinensis</i>	23	1	24	-	-	-	4	-	16	1	317	80	46	30	42	-	1	585
<i>conomon</i>	-	-	-	-	-	-	-	-	10	14	52	195	24	-	-	-	1	296
<i>makuwa</i>	-	-	-	-	-	-	-	-	8	17	55	30	274	-	4	-	-	388
<i>chito</i>	-	-	-	-	-	-	1	-	-	2	-	-	-	83	4	-	-	90
<i>agrestis</i>	-	-	-	-	-	-	-	-	-	15	13	-	22	7	795	-	-	852
<i>indet. melo</i>	60	126	44	60	-	-	-	-	27	5	5	-	-	-	-	114	34	475
<i>indet. agrestis</i>	-	-	6	2	-	6	-	1	29	1	1	-	-	-	-	4	130	180
Percentage																		%
<i>ameri</i>	62.4	19.0	6.5	4.5	1.1	2.1	0.2	1.8	0.5	-	-	-	-	-	-	1.6	0.1	100.0
<i>inodorus</i>	20.7	68.9	2.9	4.2	0.5	0.5	-	1.0	-	-	-	-	-	-	-	0.9	-	100.0
<i>cantalupensis</i>	24.4	6.0	37.1	19.1	5.3	3.9	-	0.7	0.4	0.1	1.0	-	-	-	-	0.9	1.0	100.0
<i>reticulatus</i>	16.4	3.5	1-	65.2	-	0.1	2.6	-	-	-	-	-	-	-	-	2.1	-	100.0
<i>chate</i>	6.1	-	-	-	86.7	5.1	-	2.0	-	-	-	-	-	-	-	-	-	100.0
<i>flexuosus</i>	16.2	0.2	1.8	1.3	2.3	70.2	2.4	5.2	0.1	0.1	-	-	-	-	-	0.1	-	100.0
<i>dudaim</i>	14.2	-	-	4.4	0.3	-	77.6	0.3	2.4	0.3	-	-	-	0.3	-	-	-	100.0
<i>momordica</i>	19.2	8.7	0.6	5.1	0.6	9.0	0.3	55.8	-	-	-	-	-	-	-	0.8	-	100.0
<i>acidulus</i>	2.5	0.4	2.6	-	-	1.2	0.2	0.2	80.6	1.8	3.3	0.2	3.3	0.5	-	0.2	3.0	100.0
<i>tibish</i>	-	-	-	-	-	-	2.6	-	11.5	79.5	-	0.6	0.6	-	-	-	5.1	100.0
<i>chinensis</i>	3.9	0.2	4.1	-	-	-	0.7	-	2.7	0.2	54.2	13.7	7.9	5.1	7.2	-	0.2	100.0
<i>conomon</i>	-	-	-	-	-	-	-	-	3.4	4.7	17.6	65.9	8.1	-	-	-	0.3	100.0
<i>makuwa</i>	-	-	-	-	-	-	-	-	2.1	4.4	14.2	7.7	70.6	-	1.0	-	-	100.0
<i>chito</i>	-	-	-	-	-	-	1.1	-	-	2.2	-	-	-	92.2	4.4	-	-	100.0
<i>agrestis</i>	-	-	-	-	-	-	-	-	-	1.8	1.5	-	2.6	0.8	93.3	-	-	100.0
<i>indet. melo</i>	12.6	26.5	9.3	12.6	-	-	-	-	5.7	1.1	1.1	-	-	-	-	24.0	7.2	100.0
<i>indet. agrestis</i>	-	-	3.3	1.1	-	3.3	-	0.6	16.1	0.6	0.6	-	-	-	-	2.2	72.2	100.0

- 64.9% overall classification

Table 7. Results of cross validated LDA analysis on melon varieties

In order to set up statistically solid groups, according to morpho-colourimetric data, varieties with similar phenotypic characters were clustered. Six different macro-groups were isolated: the ameri/inodorus group, the cantaloupe group (*cantalupensis* and *reticulatus*), the intermediate group (*dudaim*, *chate*, *flexuosus* and *momordica*), the African agrestis group (*tibish* and African *acidulus*), the conomon group (*conomon*, *chinensis*, *makuwa* and Asian *acidulus*) and the wild type group (*agrestis* and *chito*). In Table 9, cross validated results of LDA is shown, while Table 10 reports the main features that contribute to *taxa* discrimination. Again Area was one of the most important discriminatory parameters, together with Eecd and some colour descriptors. Overall correct identification was of 78.3%. Most macro-groups resulted in being correctly classified, with percentages up to 73.5%, except for cantaloupe group that reached 61.9% of correct identification, confirming a high overlapping of these ecotypes with the ameri/inodorus group, which anyway can be

correctly isolated in 85.1% of cases. Indeterminate *melo* and *agrestis* groups, formed with non-classifiable accessions, were totally scattered in their respective subspecies and intermediate forms.

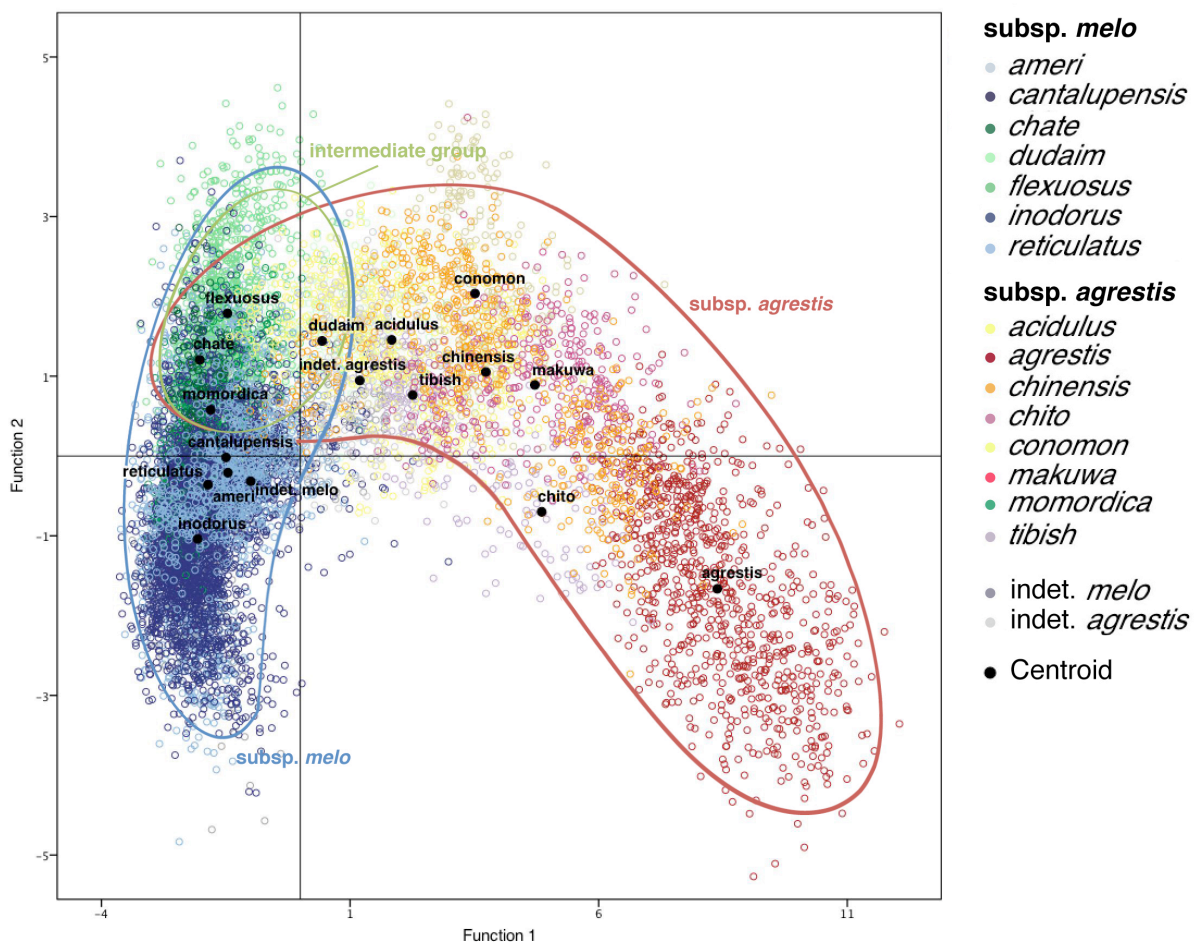


Figure 6. Scatter plot graph based on melon varieties. Small points represent single seed data, black points represent their average (centroid). Spatial arrangement of points suggests similarity and dissimilarity of groups, but just first two functions of 16 available can be used for the graphical representation. The variance on X axis is 70.1% and on Y axis is 8.2%, the remaining 21,7% is distributed over the 16 non represented functions. Major areas occupied of subspecies *agrestis* and *melo* varieties and their intermediate forms are marked.

Parameter	<i>F-to-remove</i>	<i>Tolerance</i>	<i>Wilks' lambda</i>
1 Area	221.7	0.008	0.005
2 Eecd	156.4	0.004	0.004
3 SqDsum	100.5	0.018	0.004
4 Gmean	97.1	0.023	0.004
5 H_SD	86.7	0.254	0.004
6 Rf	74.7	0.047	0.004
7 B_SD	69.8	0.024	0.004
8 R_SD	57.4	0.006	0.004
9 Pconv	54.6	0.001	0.004
10 Dmax	49.0	0.002	0.004

Table 8. First 10 factors used for discrimination among varieties in order of decreasing *F-to-remove*.

	cantaloupe grp.	ameri/inodorus grp.	intermediate grp.	African <i>agrestis</i> grp.	conomon grp.	wild types	indet. <i>melo</i>	indet. <i>agrestis</i>	Total
Seed number									n°
cantaloupe grp.	1027	443	135	3	10	-	13	5	1,636
ameri/inodorus grp.	462	4,006	193	2	1	-	42	8	4,714
intermediate grp.	76	306	1,230	32	5	-	2	4	1,655
African <i>agrestis</i> grp.	16	13	17	610	51	-	2	15	724
conomon grp.	27	28	1	39	1,096	73	3	2	1,269
wild types	-	-	-	24	46	870	-	2	942
indet. <i>melo</i>	84	232	3	31	10	-	95	20	475
indet. <i>agrestis</i>	15	1	10	27	1	-	2	124	180
Percentage									%
cantaloupe grp.	61.9	27.9	8.3	0.2	0.6	-	0.9	0.4	100.0
ameri/inodorus grp.	9.9	85.1	4.1	-	-	-	0.8	0.1	100.0
intermediate grp.	5.6	18.5	73.5	1.1	0.7	-	0.2	0.4	100.0
African <i>agrestis</i> grp.	2.1	1.3	3.2	87.9	1.8	-	0.3	3.5	100.0
conomon grp.	1.8	2.3	0.1	2.2	87.6	5.4	0.4	0.1	100.0
wild types	-	-	-	0.7	5.6	93.3	-	0.3	100.0
indet. <i>melo</i>	16.2	51.4	0.8	7.8	1.9	-	18.5	3.4	100.0
indet. <i>agrestis</i>	6.1	0.6	5.6	13.9	1.7	-	1.1	71.1	100.0

- 78.3% overall classification

Table 9. Results of cross validated LDA analysis on groups of variety with higher similarity: cantaloupe grp. (*cantalupensis* and *reticulatus*), ameri/inodorus grp., intermediate grp. (*chate*, *dudaim*, *flexuosus* and *momordica*), African *agrestis* grp. (African *acidulus* and *thibis*), conomon grp. (*conomon*, *chinensis*, *makuwa* and Asian *acidulus*), wild type grp. (*agrestis* and *chito*) and *agrestis* and *melo* indeterminate landraces.

Parameter	<i>F-to-remove</i>	<i>Tolerance</i>	<i>Wilks' lambda</i>
1 Gmean	159.86	0.022	0.015
2 Area	125.42	0.001	0.015
3 Eecd	122.42	0.001	0.015
4 H_SD	106.21	0.234	0.015
5 Dmin/Dmax	78.28	0.024	0.014
6 B_SD	71.56	0.023	0.014
7 SqDsum	68.77	0.011	0.014
8 Cpt	67.38	0.004	0.014
9 HaralikSD5	59.32	0.041	0.014
10 EAmx	57.08	0.003	0.014

Table 10. First 10 factors used for discrimination among macro-groups in order of decreasing *F-to-remove*.

3.3.3 Integration of molecular data and seed/fruit phenotypes

High positive correlation ($r=0.921$) was found between the seed parameter Area and fruit weight, measured in a previous phenotyping assay. The coefficient of determination (R^2) amounted to 0.849.

The analysis of the allelic frequencies of some SNPs located in genomic regions in which QTLs involved in fruit weight have been reported (Diaz *et al.* 2011), reveals some regions in which allelic distribution seems to be non random in different seed size groups of accessions, with specific alleles

being more frequent in accessions with small/large seeds. For example, accessions with low values of the Seed parameter Area have high frequencies of one of the two alleles of 3 SNPs located in Linkage group I (LGI), in regions in which QTLs for fruit weight have been reported, CMPSNP711 and AI_17-E07 (located at 45,2 and 46,8 cM, respectively) and CMPSNP731 (located at 80,4 cM), whereas similar frequencies for the two alleles are observed in the SNPs located in other regions of this LGI. In fact, the ANOVA shows significant differences in the average Seed Area for these three markers between accessions belonging to the two homozygous genotypic classes (mean \pm standard deviation of homozygous a, allele more frequent in large seed accessions = 39.9 ± 10.9 , 39.1 ± 10.8 and 39.2 ± 11.4 ; homozygous b, allele more frequent in small seed accessions = 25.0 ± 14.3 , 19.4 ± 10.4 , and 18.9 ± 10.5).

The best hit of the *Arabidopsis thaliana* gene At4g39850 with the melon unigene collection of melonomics was found with MELO3C018991, located in CM3.5_scaffold00035 from 2610482 to 2613142. This scaffold is anchored to the melon genetic map in LGVII at 32,1cM. A differential allelic distribution in small *versus* large seed size accessions, similar to that found markers of LGI, was found in SNP CMPSNP262 (located in LGVII at 30,5cM). Information about the natural genetic variation of the gene MELO3C018991 was obtained from the melogene database of SNPs. A single SNP in this gene was found. It was a non-synonym mutation (C/T aa 249 S/N). According to the sequence information provided by the melogene data base, one allele had been sequenced in three pools composed of *inodorus*, *momordica* and *agrestis acidulus* accessions (including most of the accessions of these groups analysed in this study that have large or intermediate seed size), whereas the alternative allele had been sequenced in the *conomon* pool of accessions (also including many of the *conomon* accessions analysed in the present study, all with small seed size).

3.4 Discussion

The division of melon in two subspecies, *C. melo* subsp. *melo* and *C. melo* subsp. *agrestis*, already described elsewhere (Silberstein *et al.* 1999, Monforte *et al.* 2003, Deleu *et al.* 2009, Esteras *et al.* 2009, Blanca *et al.* 2011), is well supported by both molecular and seed morpho-colourimetric analyses, with some accessions in intermediate positions. The high degree of admixture of populations of both subspecies, found with SNPs in the *flexuosus*, *chate*, *dudaim* and *momordica* varieties, is in agreement with the high molecular variability previously reported in them (Esteras *et al.* 2013) and with the reported idea that from these varieties evolved most of the current melon populations. In fact, in the largest melon re-sequencing assay performed by Blanca *et al.* (2012), *momordica* was the most heterogeneous variety, and shared the highest percentage of SNPs with other varieties of both subspecies. All of these non-sweet varieties have limited diffusion through Africa and Near East. The same pattern can be evidenced by morpho-colourimetric analysis which shows the intermediate position of these varieties between the two subspecies. Similar achievements were found for *C. melo* subsp. *agrestis* var. *agrestis*, the wild forms of melons, which resulted in being isolated from the

cultivated accessions. Among cultivars, *chito* was one of the subspecies *agrestis* varieties that showed more misclassification with the wild *agrestis* group, consistently with the history of this group. This is a small type of melon naturalized in America that shares many characters with wild *agrestis* as it likely derives from wild African types introduced into the Americas (Pitrat *et al.* 2000), so it cannot be totally considered as cultivated.

Within the subspecies *melo*, *ameri* accessions show high degrees of admixture with all other *melo* varieties. This group of accessions is the most heterogeneous within the cultivated melon and include quite different landraces. The high crossbreeding of with *inodorus* and *cantalupensis* produced a wide range of intermediate forms that does not always permit to isolate *ameri* from the others. These results also agree with the hypothesis that modern *inodorus* and *cantalupensis* derived from these variable Asian melons (Pitrat *et al.* 2000). Few reports describe the variability of Asian types of the subsp. *melo*. In a recent study on Iranian melons, Raghani *et al.* (2014) reported the high diversity in melons from these area and remarked their differences with European/American *inodorus* and *cantalupensis*. Seed image analysis results agree with molecular ones, although the latter are, as expected, more discriminant. Molecular analysis reveals three differentiated groups within *inodorus* and *cantalupensis* (Spanish *inodorus*, Charentais melons and *reticulatus*) that were hard to distinguish on the basis of seed traits.

Within the subspecies *agrestis*, the *conomon* group (*conomon*, *chinensis* and *makuwa*) was quite similar molecularly, also according to previous studies (Blanca *et al.* 2012), and presented closely related seed traits. Despite *acidulus* and *tibish* being molecularly similar to wild *agrestis*, the bigger size of their seeds allows to separate these two varieties from the wild form. However, *acidulus* and *tibish* are quite hardly differentiated on the basis of seed traits having a significant degree of misclassification. Old classification models placed *tibish* in subspecies *melo* (Pitrat *et al.* 2000), but molecular analyses demonstrated its greater similarity to *agrestis* (Esteras *et al.* 2009). Seed morphology agrees with the classification of this primitive melon belonging to subspecies *agrestis*. In line with molecular data, the unclassified landraces seem to be mostly mixed types of different varieties of the subspecies *melo*, mostly *inodorus*, but with some traces of subspecies *agrestis*.

According only to seed morphology it was possible to isolate six different groups of varieties: a group of accessions of *ameri* and *inodorus* closely related to the other group of *cantalupensis* and *reticulatus*, an intermediate group between the two subspecies (*dudaim*, *chate*, *flexuosus* and *momordica*), a group of African *agrestis* varieties (*tibish* and *acidulus*), the *conomon* group formed with *conomon*, *chinensis*, *makuwa* and Asian *acidulus*, and a group formed with wild melon types (*agrestis* and *chito*).

Seed image analysis has proved to be also successful to discriminate between genus and among species and intraspecific groups in cucurbits, resulting in an interesting tool to assist the classification of cucurbit germplasm at different taxonomic levels. At the genus and species levels, data completely fit with the current taxonomy, where species of the same genus are more related than species of

different genus. *Citrullus lanatus* and *C. colocynthis* show higher heterogeneity in seeds morpho-colourimetric features than *Cucumis melo* and *C. sativus*. Despite of *C. colocynthis* being traditionally considered the wild ancestor of *C. lanatus*, genetic analysis showed that the cultivated and Egusi watermelon (var. *lanatus*) and the citron type (var. *citroides*) diverged into separate lineages appearing independently evolved from a common ancestor, possibly *C. ecirrhosus* (Dane and Liu 2007). Furthermore, whereas cucumber and melon are two cultivated crops phylogenetically close related and surely deriving from the same ancestor (Sebastian *et al.* 2010), *C. colocynthis* is a perennial (rarely annual) wild species growing on sandy habitats in desert and semi-desert areas of North Africa, the Near East and South-West Asia as far as India (Jeffrey 2001). All of these differences are clearly reflected on seed morphology.

Despite the importance of seed size in plant evolution and crop domestication, relatively little is known about the genetic and molecular processes underlying natural variation in seed size and morphology. Integration of SNPs and phenotypic data sets provide the opportunity to obtain information about the genetics of seed traits. A strong correlation was found between the seed Area, the most discriminant seed trait among melon accessions, and fruit weight, which agree with the results reported in other Cucurbits (Paris and Nerson 2003). Moreover, the evidence of a non random allelic distribution in large/small seed groups of accessions of SNPs located in some genomic regions in which QTLs involved in fruit weight had been previously located (Diaz *et al.* 2011) suggests that some of these regions may also account for part of the observed variation in seed size. This non random distribution of alleles could be also due to an effect of the structure of the population, then associations of alleles to seed traits must be proved in larger unstructured populations or in populations specifically designed, such as introgression lines with which we are currently working. For example, populations derived from the cross of *acidulus/tibish* and wild *agrestis* could be suited to study the genetics of seed traits as these varieties are molecularly similar, but significantly differ in seed traits. The use of the available genomic tools can also facilitate the identification of candidate mutations involved in seed traits, such as that found in the melon orthologue of the tomato *SW4* (Orsi and Tanksley 2009). Our results raised the possibility that the melon orthologue of *SW4* might also underlie natural variations in seed size in melon, but the association needs to be demonstrated in appropriate populations.

3.5 Conclusions

Molecular analysis recognized some differentiated populations, but also a wide range of mixed types. Despite this molecular admixture, seed image analysis revealed six major groups that can be discriminated on the basis of specific phenotypic traits, mainly associated to seed size and morphology and less to seed colour. The obtained seed groupings are in agreement with the molecular relationships and with the history of the melon varieties. In fact wild *agrestis*, Far eastern *conomon* and African *tibish* and *acidulus* could be clearly distinguished. Discrimination of the cultivated types of the *melo* subspecies (*inodorus*, *cantalupensis*, *reticulatus* and *ameri*) was also possible although less clear, due

to a more intense crossing and breeding process undergone by these commercial groups. The intermediate position of *momordica*, *flexuosus* and *dudaim* groups across the two subspecies is also detected by seed morpho-colourimetric analysis. The identification of the more discriminant specific traits allows the development of a method to classify new seeds in any of the reported groups. A great deal of the extant melon variation is maintained in different seed collections, so this tool would be of great utility to manage their variation and optimize their conservation and use. Also the integration of molecular and seed data would be a useful tool to study the genetics of seed traits.

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3.6 Annex 1: *Cucumis melo* accessions details utilized for morpho-colorimetric and molecular analyses and the corresponding results of Population Structure.

Population Structure	Code	Code2**	subsp.	variety	type	country	local or commercial name	seeds
-	InAL3	-	<i>melo</i>	<i>inodorus</i>	landrace	Albania	Ames 23237	100
5/4	InCN142	In-HamiChi	<i>melo</i>	<i>inodorus</i>	landrace	China	Hami melon	96
3	InES53	In-TeNinvSp2	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Tendral negro de inv.	95
3	InES54	In-BTempSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Blanco tempranillo	81
4	InES55	In-LaHCCSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Hilo carrete	95
4	InES56	In-BLisSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Blanco liso	81
3	InES61	In-PsPiñSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Piel de sapo Piñonet	97
3/5	InES75	La-ErizoSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Eriçó mallorquin	98
3	InES78	In-LaBolasSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Bolas	85
3	InES79	In-AmAoroSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Amarillo oro	95
3	InES80	In-RoSp	<i>melo</i>	<i>inodorus</i>	landrace	Spain	Rochet	99
4	InGR118*	In-BaskGreece	<i>melo</i>	<i>inodorus</i>	landrace	Greece	Baskavas	98
4	InHU17	In-MusHung	<i>melo</i>	<i>inodorus</i>	landrace	Hungary	cukordinnye	99
4/1	InIT28	In-CucumIta	<i>melo</i>	<i>inodorus</i>	landrace	Italy	Cucummarazzo	99
-	InPT120*	-	<i>melo</i>	<i>inodorus</i>	landrace	Portugal	Branco de ribateja	69
3	InPT126*	In-CraPor	<i>melo</i>	<i>inodorus</i>	landrace	Portugal	Crabranco	82
3/4	InPT39	La-MelaoPor	<i>melo</i>	<i>inodorus</i>	landrace	Portugal	Melão	48
5/1	InPT40	La-CascaPor	<i>melo</i>	<i>inodorus</i>	landrace	Portugal	Casca de Carvalho	98
4/1	InTN65	In-AsliTun	<i>melo</i>	<i>inodorus</i>	landrace	Tunisia	Aslí	98
4	InTN85	In-MaazTun	<i>melo</i>	<i>inodorus</i>	landrace	Tunisia	Maazoon	98
-	InTR104	-	<i>melo</i>	<i>inodorus</i>	landrace	Turkey	Kırkağaç - PI 169305	98
4	InTR87	In-WTTur	<i>melo</i>	<i>inodorus</i>	breeding-line	Turkey	Winter type-PI 169329	99
-	InTR91	-	<i>melo</i>	<i>inodorus</i>	landrace	Turkey	Kırkağaç	100
4	InUS123	In-CGBUSA	<i>melo</i>	<i>inodorus</i>	breeding-line	USA	Casaba golden beauty	92
3/4	InUS143	In-HoneyDewUSA	<i>melo</i>	<i>inodorus</i>	breeding-line	USA	Honeydew green flesh	97
1	CaFR141*	Can-GyFran	<i>melo</i>	<i>cantalupensis</i>	breeding-line	France	Gynadou	98
1	CaFR159*	Can-NOFran	<i>melo</i>	<i>cantalupensis</i>	breeding-line	France	Nantais Oblong	96
1/5	CaFR161	Can-NCFran	<i>melo</i>	<i>cantalupensis</i>	landrace	France	Noir des Carmes	98
1	CaFR172	Can-PGFran	<i>melo</i>	<i>cantalupensis</i>	landrace	France	Petit Gris de Rennes	100
1/5	CaFR179	Can-PresFran	<i>melo</i>	<i>cantalupensis</i>	landrace	France	Prescott Fond Blanc	100
1	CaFR201	Can-VedFran	<i>melo</i>	<i>cantalupensis</i>	breeding-line	France	Vedrantais	94
-	CaHU18	-	<i>melo</i>	<i>cantalupensis</i>	landrace	Hungary	Ezüst ananasz	96
1/4/5	CaIL162	Can-NYIs	<i>melo</i>	<i>cantalupensis</i>	breeding-line	Israel	Noy Israel	99
5/1	CaJP29	Can-PearlJa	<i>melo</i>	<i>cantalupensis</i>	breeding-line	Japan	Pearl - PI 266947	93
5/1	CaUS171*	Can-PSUSA	<i>melo</i>	<i>cantalupensis</i>	breeding-line	USA	Persian Small Type	81
2/1	ReFR116*	Can-ASLFran	<i>melo</i>	<i>reticulatus</i>	breeding-line	France	ASL	95
2	ReGT16	Can-SUD2Guat	<i>melo</i>	<i>reticulatus</i>	landrace	Guatemala	SUD-CU-2	90
-	ReLY33	-	<i>melo</i>	<i>reticulatus</i>	landrace	Libia	Khiar	100
2	ReUS114*	Can-HBJUSA	<i>melo</i>	<i>reticulatus</i>	breeding-line	USA	Ar Hale's Best Jumbo	98
1/2	ReUS92	Can-GCHUSA	<i>melo</i>	<i>reticulatus</i>	breeding-line	USA	Golden Champlain	100
1/2	ReUS93	Can-GHUSA	<i>melo</i>	<i>reticulatus</i>	breeding-line	USA	Golden Honey	98
-	ReYU97	-	<i>melo</i>	<i>reticulatus</i>	landrace	ExYugoslavia	YUG	100
-	AmAF109	-	<i>melo</i>	<i>ameri</i>	landrace	Afganistan	Safed Sarda	98

5/4	AmAF2	Am-3584Afg	<i>melo</i>	<i>ameri</i>	landrace	Afganistan	PI 125951	98
5/1/2/4/3	AmBO5	La-Bol	<i>melo</i>	<i>ameri</i>	landrace	Bolivia	Bol-84	91
5/4/1	AmDE166*	Am-OpalGer	<i>melo</i>	<i>ameri</i>	landrace	Germany	Opalkugel	95
4	AmEG9	Am-KafEgy	<i>melo</i>	<i>ameri</i>	landrace	Egypt	Kafr hakim - PI 288233	100
5	AmGE12	Am-NanaGeorg	<i>melo</i>	<i>ameri</i>	landrace	Georgia	Nanatri	100
4/5	AmGE13	Am-KolGeor	<i>melo</i>	<i>ameri</i>	landrace	Georgia	Koljonitza-PI 314427	85
5/4	AmGE156*	Am-NesviGeor	<i>melo</i>	<i>ameri</i>	landrace	Georgia	Mucha nesvi	91
6/7/1	AmIR149*	Am-KhaIran	<i>melo</i>	<i>ameri</i>	landrace	Iran	Khatoni	74
5	AmIR190*	La-SousIran	<i>melo</i>	<i>ameri</i>	landrace	Iran	Souski	98
5/4/2	AmIR26	Am-6053Iran	<i>melo</i>	<i>ameri</i>	landrace	Iran	PI 140632	99
-	AmKZ106	-	<i>melo</i>	<i>ameri</i>	landrace	Kazakhstan	Imljskaja	92
1/5/4/6	AmMA37	Am-Afr1Mor	<i>melo</i>	<i>ameri</i>	landrace	Morocco	AFR-C-1	92
5/4	AmMN36	Am-ChandMon	<i>melo</i>	<i>ameri</i>	landrace	Mongolia	Chandalack	79
5	AmRU41	Am-KorcaRus	<i>melo</i>	<i>ameri</i>	landrace	Russia	Korça	99
2	AmRU42	Am-ApelRus	<i>melo</i>	<i>ameri</i>	landrace	Russia	Apelsinaja	92
4	AmRU44	Am-KuvRus	<i>melo</i>	<i>ameri</i>	landrace	Russia	Kuvinska - PI 506460	99
5/4	AmTJ83	Am-TokTaj	<i>melo</i>	<i>ameri</i>	landrace	Tajikistan	Tokash	98
5/6/1/4	AmTN84	Am-BattiTun	<i>melo</i>	<i>ameri</i>	landrace	Tunisia	Battikh	90
4/5	AmTR88	Am-AltimTur	<i>melo</i>	<i>ameri</i>	landrace	Turkey	Altimbis - PI 169331	88
4	AmTR89	Am-HassanTur	<i>melo</i>	<i>ameri</i>	landrace	Turkey	PI 169368	97
5/3/4	AmTU137	Am-GalaTun	<i>melo</i>	<i>ameri</i>	landrace	Tunisia	Galaoui	98
4/3/2/5	AmUA119*	Am-BirUkr	<i>melo</i>	<i>ameri</i>	landrace	Ukraine	Birjucekutskaja	99
-	AmUA90	-	<i>melo</i>	<i>ameri</i>	landrace	Ukraine	Salgirska - PI 506459	100
5	AmUZ167	Am-OuzUzb	<i>melo</i>	<i>ameri</i>	landrace	Uzbekistan	Ouzbeque	84
5	AmUZ95	Am-KokUzb	<i>melo</i>	<i>ameri</i>	landrace	Uzbekistan	Kokcha	98
-	AmUZ96	-	<i>melo</i>	<i>ameri</i>	landrace	Uzbekistan	Kizil-uruk	81
1/5	ChIT27	Chate-CarIta	<i>melo</i>	<i>chate</i>	landrace	Italy	Carosello	98
-	DuAF1	-	<i>melo</i>	<i>dudaim</i>	landrace	Afganistan	Dudaim	100
6/7/2	DuAF180	Dud-QPMAfg	<i>melo</i>	<i>dudaim</i>	landrace	Afganistan	Queen's pocket melon	97
-	DuGE296	-	<i>melo</i>	<i>dudaim</i>	landrace	Georgia	Dudaim	98
5	FxAF174	Flex-TarehAfg	<i>melo</i>	<i>flexuosus</i>	landrace	Afganistan	PI 222187	40
7/6/5	FxIN115	Flex-AryaInd	<i>melo</i>	<i>flexuosus</i>	landrace	India	Arya	99
5/6/7	FxIN20	Flex-Co20Ind	<i>melo</i>	<i>flexuosus</i>	landrace	India	Snakemelon	96
5/3/7	FxIQ23	Flex-KhiIraq	<i>melo</i>	<i>flexuosus</i>	landrace	Irak	khia taaruzi	98
5/3/7/1	FxSD186*	Flex-SilkaSud	<i>melo</i>	<i>flexuosus</i>	landrace	Sudan	Silka	100
-	FxTR16	-	<i>melo</i>	<i>flexuosus</i>	landrace	Turkey	Acur	96
-	FxTR21	-	<i>melo</i>	<i>flexuosus</i>	landrace	Turkey	Acur	95
-	FxTR4	-	<i>melo</i>	<i>flexuosus</i>	landrace	Turkey	Siyak	96
-	FxTR54	-	<i>melo</i>	<i>flexuosus</i>	landrace	Turkey	Adsiz	98
5/4/2/6	FxTR86	Flex-AcukTur	<i>melo</i>	<i>flexuosus</i>	landrace	Turkey	Acuk - PI 167057	89
-	LaET11	-	<i>melo</i>	indet.landrace	landrace	Etiopia	Popone	111
1/5/4	LaFR151*	La-KroFran	<i>melo</i>	indet.landrace	landrace	France	Kroumir	96
1/2/3/6	LaIT00	Can-PopIta	<i>melo</i>	indet.landrace	landrace	Italy	Popone d'oro	98
5/1/7/4	LaML35	La-KankMali	<i>melo</i>	indet.landrace	landrace	Mali	Kankani - PI 490388	72
-	AcLK148*	-	<i>agrestis</i>	<i>acidulus</i>	landrace	Sri Lanka	Kekiri	97
7	AcSN45	Ac-G22843Se	<i>agrestis</i>	<i>acidulus</i>	landrace	Senegal	PI 436534	100
-	AcSN46	-	<i>agrestis</i>	<i>acidulus</i>	landrace	Senegal	PI 436532	96

7/6	AcZA98	Ac-5384Zamb	<i>agrestis</i>	<i>acidulus</i>	landrace	Zambia	PI 505602	86
6/7	AcZW100	Ac-TGR1551Zimb	<i>agrestis</i>	<i>acidulus</i>	landrace	Zimbabwe	TGR1843-PI 482429	93
6/7	AcZW99	Ac-TGR1843Zimb	<i>agrestis</i>	<i>acidulus</i>	landrace	Zimbabwe	TGR 1551-PI 482420	96
6/7	AgCO153	Ag-MeloncCol	<i>agrestis</i>	<i>agrestis</i>	wild	Colombia	Meloncillo	100
6/7	AgCU127*	Ag-Cuba	<i>agrestis</i>	<i>agrestis</i>	wild	Cuba	CUBA	96
7	AgGH14	Ag-15591Gha	<i>agrestis</i>	<i>agrestis</i>	wild	Ghana	PI 185111	98
7/6	AgIN128	Ag-CallInd	<i>agrestis</i>	<i>agrestis</i>	wild	India	Callosus	97
7/6	AgIN204	Ag-WChInd	<i>agrestis</i>	<i>agrestis</i>	wild	India	Wild chibbar	96
7	AgNG38	Ag-Co38Nig	<i>agrestis</i>	<i>agrestis</i>	wild	Nigeria	CO38	91
7	AgSN133*	Ag-FadSud	<i>agrestis</i>	<i>agrestis</i>	wild	Sudan	Fadasi	95
7	AgSN146*	Ag-HumSud	<i>agrestis</i>	<i>agrestis</i>	wild	Sudan	Humaid	91
7	AgSN197*	Ag-TendSud	<i>agrestis</i>	<i>agrestis</i>	wild	Sudan	Tendelti	88
6/5	CnCH105	Con-GouChi	<i>agrestis</i>	<i>chinensis</i>	landrace	China	Gogua	96
6	CnCH6	Con-Co6Chi	<i>agrestis</i>	<i>chinensis</i>	landrace	China	makuwa	98
1/6	CnJP207	Con-YapuJa	<i>agrestis</i>	<i>chinensis</i>	landrace	Japan	Yamato Purinsu	96
6	CnKR173	Con-SCKo	<i>agrestis</i>	<i>chinensis</i>	landrace	Korea	Songwhan Charmi	95
6	CnKR32	Con-Pat81Ko	<i>agrestis</i>	<i>chinensis</i>	landrace	Korea	PAT-81	100
6	CnPL169*	Con-PaulPol	<i>agrestis</i>	<i>chinensis</i>	landrace	Polonia	Paul	100
6/5	CoCH154*	Con-MielChi	<i>agrestis</i>	<i>conomon</i>	landrace	China	Miel blanc	98
-	CoJP136	-	<i>agrestis</i>	<i>conomon</i>	landrace	Japan	Freeman's cucumber	99
6	CoJP185	Con-ShiroJa	<i>agrestis</i>	<i>conomon</i>	landrace	Japan	Shiro uri okayama	99
6	MkCH158*	Con-NanChi	<i>agrestis</i>	<i>makuwa</i>	landrace	China	Nanbukin	96
6	MkCH7	Con-LongtChi	<i>agrestis</i>	<i>makuwa</i>	landrace	China	Longtian - PI 618854	96
-	MkJP188	-	<i>agrestis</i>	<i>makuwa</i>	landrace	Japan	Omaru Gin makuwa	100
6	MkJP30	Con-GMJJa	<i>agrestis</i>	<i>makuwa</i>	landrace	Japan	Ginsen mak.-PI420176	96
7/6/1	CtIN22	Chi-VellInd	<i>agrestis</i>	<i>chito</i>	landrace	India	Velleri - PI 164320	90
6/7/1	MoIN135*	Mom-FPInd	<i>agrestis</i>	<i>momordica</i>	landrace	India	Faizabadi phoont	95
5	MoIN19	Mom-KhaInd	<i>agrestis</i>	<i>momordica</i>	landrace	India	Kharbuja	99
6/5/7	MoIN21	Mom-PI124Ind	<i>agrestis</i>	<i>momordica</i>	landrace	India	PI 124112	93
4/5	MoSA157*	La-NajdAS	<i>agrestis</i>	<i>momordica</i>	breeding-line	Saudi Arabia	NADJ	68
7	TiSN198*	Tibish-DSud	<i>agrestis</i>	<i>tibish</i>	landrace	Sudan	Tibish Djebel	98
7	TiSN199*	Tibish-KSud	<i>agrestis</i>	<i>tibish</i>	landrace	Sudan	Tibish Khurtagat	58
6/7	LaMG202*	La-VoaMad	<i>agrestis</i>	indet.landrace	landrace	Madagascar	Voatango	81
6/7/5	LaZA47	La-TransSAfr	<i>agrestis</i>	indet.landrace	landrace	South Africa	wild type - PI 282448	99

PI accessions were provided by NPGS (USDA)

*accessions from MELRIP project

** code in Leida *et al.* in press

- Only morpho-colourimetric analysis

3.7 Annex 2: *Cucumis sativus*, *Citrullus lanatus* and *Citrullus colocynthis* accessions details utilized for morpho-colorimetric analyses.

Code	species	variety	Country	location	local/commercial name	seeds
CyAF82	<i>C. colocynthis</i>		Afghanistan			89
CyDZ33	<i>C. colocynthis</i>		Algeria	Ighil, Béchar		100
CyDZ34	<i>C. colocynthis</i>		Algeria	Ighil, Béchar		91
CyES49	<i>C. colocynthis</i>		Spain	Murcia	Citruyus	95
CyES72	<i>C. colocynthis</i>		Spain	Almería		91
CyES99	<i>C. colocynthis</i>		Spain	Arona.Tenerife	Sandía	86
CyIT59	<i>C. colocynthis</i>		Italy		Coloquintida	99
CyMA4	<i>C. colocynthis</i>		Morocco	Tinghir		106
CyZA83	<i>C. colocynthis</i>		South Africa			72
LcAO08	<i>C. lanatus</i>	<i>citroides</i>	Angola	Humpata.Mulenga, Huila		79
LcES64	<i>C. lanatus</i>	<i>citroides</i>	Spain	Casas Altas, Valencia	Sandía para mermeladas	81
LcES67	<i>C. lanatus</i>	<i>citroides</i>	Spain	Ademuz, Valencia	Sandía	86
LnAO22	<i>C. lanatus</i>	<i>lanatus</i>	Angola	Namibe.Kanbongue		104
LnAO53	<i>C. lanatus</i>	<i>lanatus</i>	Angola	Luanda		75
LnAO55	<i>C. lanatus</i>	<i>lanatus</i>	Angola	Luanda		68
LnDZ25	<i>C. lanatus</i>	<i>lanatus</i>	Algeria	Mostaganem		85
LnDZ59	<i>C. lanatus</i>	<i>lanatus</i>	Algeria	Ammes, Béchar		94
LnDZ78	<i>C. lanatus</i>	<i>lanatus</i>	Algeria	Mustapha Ben Brahims		78
LnES51	<i>C. lanatus</i>	<i>lanatus</i>	Spain	Rota, Cádiz	Sandía de Rota	89
LnES62	<i>C. lanatus</i>	<i>lanatus</i>	Spain	Moraleda, Granada	Sandía inverniza	78
LnES81	<i>C. lanatus</i>	<i>lanatus</i>	Spain	.Mallorca	Sandía de pinyol blanc	65
LnGR32	<i>C. lanatus</i>	<i>lanatus</i>	Greece	Navplion, Argholidha		97
LnKZ81	<i>C. lanatus</i>	<i>lanatus</i>	Kyrgyzstan	Lenin Dzho		89
LnMA3	<i>C. lanatus</i>	<i>lanatus</i>	Morocco	Khmelat		91
LnSY93	<i>C. lanatus</i>	<i>lanatus</i>	Syria	Damasco		45
LnUZ78	<i>C. lanatus</i>	<i>lanatus</i>	Uzbekistan	San Salar		80
SaCN05	<i>C. sativus</i>		China	Ju, Shandong	Chun Huang Gua	100
SaCN06	<i>C. sativus</i>		China	Xinzheng, Henan	Shou Pi Qing Huang Gua	100
SaCN07	<i>C. sativus</i>		China	Haicheng, Liaoning	Qing Jiang Xian Gua	99
SaCN08	<i>C. sativus</i>		China	Haicheng, Liaoning	Cao Huang Gua	98
SaCN09	<i>C. sativus</i>		China	Jianping, Liaoning	Jian Ping Xian Qiu Huang Gua	98
SaES37	<i>C. sativus</i>		Spain	Badajoz	Pepino enano	100
SaES39	<i>C. sativus</i>		Spain	Tramacastilla, Teruel	Pepino	106
SaES42	<i>C. sativus</i>		Spain	Quicena, Huesca	Pepino gordo	98
SaES44	<i>C. sativus</i>		Spain	Rueda de JalónZaragoza	Pepino antiguo	100
SaES72	<i>C. sativus</i>		Spain	Ugíjar, Granada	Pepino	99
SaES72	<i>C. sativus</i>		Spain	Cebreros, Ávila	Pepino pequeño blanco	97
SaES80	<i>C. sativus</i>		Spain	Benaoján, Málaga	Pepino del terreno	99
SaIN58	<i>C. sativus</i>		India	Calcutta	Pepino	97
SaJP04	<i>C. sativus</i>		Japan		Sunpu-Fushinari	98
SaRU05	<i>C. sativus</i>		Russia		Nezysnrij	97
SaRU06	<i>C. sativus</i>		Russia		Nezysnrij	96
SaRU25	<i>C. sativus</i>		Russia		Bercizozskij	100
SaRU26	<i>C. sativus</i>		Russia		Posrednik 97-J	97
SaRU27	<i>C. sativus</i>		Russia		Plodozitys 147	100
SaRU363	<i>C. sativus</i>		Russia			97
SaRU463	<i>C. sativus</i>		Russia			86

3.8 Annex 3 SNPs details: Information about the 211 SNP markers employed in the genotyping assay and summary statistic results generated in genotyping analysis with PowerMarker software.

Marker name	LG	cM	Ref.*	Major Allele Frequency	Genotype n°	Allele n°	Gene Diversity*	Heterozygosity	PIC
snv87591**	1			0.607	3	2	0.477	0.087	0.363
AI_09-F07	1	0	X	0.888	3	2	0.198	0.029	0.179
CMPSNP1095	1	3.2	X	0.791	3	2	0.330	0.126	0.276
CMPSNP83	1	18.1	X	0.510	3	2	0.500	0.078	0.375
AI_17-E07	1	45.2	X	0.612	3	2	0.475	0.078	0.362
CMPSNP711	1	46.8	X	0.500	3	2	0.500	0.049	0.375
snv219731	1			0.816	3	2	0.301	0.039	0.256
CMPSNP410	1	59.6	X	0.544	3	2	0.496	0.078	0.373
F116	1	69.2	X	0.617	3	2	0.473	0.087	0.361
AI_05-G01	1	72.4	X	0.874	3	2	0.221	0.019	0.196
CMPSNP731	1	80.4	X	0.675	3	2	0.439	0.049	0.343
CMPSNP204	1	86.8	X	0.791	3	2	0.330	0.029	0.276
CMPSNP774	2	0	X	0.524	3	2	0.499	0.039	0.374
CMPSNP431	2	4.8	X	0.558	3	2	0.493	0.049	0.372
CMPSNP502	2	32.6	X	0.716	3	2	0.407	0.039	0.324
CMPSNP1057	2	37.4	X	0.883	3	2	0.206	0.019	0.185
snv173017	2	37.4		0.883	3	2	0.206	0.019	0.185
snv27560	2			0.752	3	2	0.373	0.087	0.303
AI_14-H05	2	40.6	X	0.583	3	2	0.486	0.117	0.368
snv235996	2			0.617	3	2	0.473	0.107	0.361
CMPSNP128	2	50.2	X	0.820	3	2	0.295	0.029	0.251
CMPSNP246	2	53.4	X	0.714	3	2	0.409	0.049	0.325
snv231408	2			0.529	3	2	0.498	0.078	0.374
CMPSNP1003	2	58.2	X	0.723	3	2	0.400	0.068	0.320
CMPSNP886	2	63	X	0.607	3	2	0.477	0.068	0.363
snv14547	2			0.544	3	2	0.496	0.058	0.373
snv14545	2			0.701	3	2	0.419	0.088	0.331
snv14541	2			0.670	3	2	0.442	0.039	0.344
snv14540	2			0.926	3	2	0.136	0.010	0.127
CMPSNP658	2	77.9	X	0.612	3	2	0.475	0.039	0.362
CMPSNP566	2	86	X	0.626	3	2	0.468	0.029	0.359
CMPSNP94	2	90.9	X	0.748	3	2	0.377	0.039	0.306
AI_18-E05	3	3.2	X	0.694	3	2	0.425	0.087	0.334
snv58869	3			0.714	3	2	0.409	0.029	0.325
snv58852	3			0.718	3	2	0.405	0.039	0.323
snv58849	3			0.796	3	2	0.325	0.019	0.272
snv58847	3			0.728	3	2	0.396	0.058	0.318
CMPSNP275	3	4.8	X	0.568	3	2	0.491	0.029	0.370
CMPSNP540	3	8	X	0.623	3	2	0.470	0.049	0.360
CMPSNP165	3	24.3	X	0.515	4	3	0.509	0.078	0.389
snv232359	3			0.539	3	2	0.497	0.049	0.373
snv78115	3			0.480	5	3	0.572	0.039	0.478
CMPSNP769	3	42.8	X	0.578	3	2	0.488	0.029	0.369
CMPSNP164	3	46.2	X	0.675	3	2	0.439	0.049	0.343
CMPSNP998	3	56.3	X	0.602	3	2	0.479	0.039	0.364
snv201818	3			0.912	3	2	0.161	0.020	0.148
snv174716	3			0.743	3	2	0.382	0.107	0.309
CMPSNP595	3	61.1	X	0.752	3	2	0.373	0.068	0.303
CMPSNP712	3	62.7	X	0.529	3	2	0.498	0.068	0.374
snv209888	3			0.990	2	2	0.019	0.000	0.019
CMPSNP480	4	0	X	0.545	3	2	0.496	0.070	0.373
snv_METC120280_217	4			0.529	3	2	0.498	0.184	0.374
CMPSNP787	4	6.4	X	0.757	3	2	0.368	0.078	0.300
CMPSNP1132	4	11.2	X	0.563	3	2	0.492	0.058	0.371
PS_34-C02	4	17.6	X	0.684	3	2	0.432	0.068	0.339
CMPSNP907	4	20.8	X	0.665	3	2	0.446	0.068	0.346
snv143844	4			0.526	3	2	0.499	0.071	0.374
CMPSNP264	4	32.1	X	0.519	3	2	0.499	0.068	0.375
snv2392	4			0.927	3	2	0.135	0.029	0.126
CMPSNP147	4	48.3	X	0.553	3	2	0.494	0.117	0.372
snv161722	4			0.617	3	2	0.473	0.146	0.361
AI_03-F03	4	53.1	X	0.631	3	2	0.466	0.117	0.357
CMPSNP352	4	54.7	X	0.646	3	2	0.458	0.068	0.353
CMPSNP852	4	62.7	X	0.655	3	2	0.452	0.029	0.350
CMPSNP607	4	69.1	X	0.757	3	2	0.368	0.039	0.300
CMPSNP677	4	77.1	X	0.665	3	2	0.446	0.029	0.346
snv59920	4			0.937	3	2	0.118	0.010	0.111
snv133267	4			0.689	3	2	0.428	0.078	0.337
CMPSNP24	4	86.8	X	0.623	3	2	0.470	0.069	0.360
PS_07-E07	4	101.5	X	0.563	3	2	0.492	0.078	0.371

snv30491	4			0.665	3	2	0.446	0.107	0.346
SC51-3375	4	114.6	X	0.859	3	2	0.242	0.010	0.213
CMPSNP898	5	0	X	0.655	3	2	0.452	0.126	0.350
CMPSNP387	5	18.5	X	0.636	3	2	0.463	0.068	0.356
CMPSNP437	5	26.5	X	0.665	3	2	0.446	0.049	0.346
CMPSNP726	5	41.2	X	0.845	3	2	0.262	0.039	0.228
snv152833	5			0.893	3	2	0.191	0.019	0.173
snv178174	5			0.874	3	2	0.221	0.039	0.196
PSI_25-H03	5/8	59.4	X	0.515	3	2	0.500	0.039	0.375
CMPSNP788	5	50.9	X	0.617	3	2	0.473	0.029	0.361
SSH9G15	5	52.5	X	0.673	3	2	0.440	0.059	0.343
snv203948	5			0.709	3	2	0.413	0.019	0.328
snv203941	5			0.922	3	2	0.143	0.019	0.133
60k41.243	5	73.4	X	0.626	3	2	0.468	0.068	0.359
CMPSNP1155	5	79.8	X	0.646	3	2	0.458	0.087	0.353
AI_13-H12	5	89.4	X	0.660	3	2	0.449	0.058	0.348
CMPSNP735	5	94.2	X	0.738	3	2	0.387	0.039	0.312
snv85853	5			0.845	3	2	0.262	0.019	0.228
CMPSNP925	6	1.6	X	0.642	3	2	0.460	0.069	0.354
CMPSNP218	6	8	X	0.607	3	2	0.477	0.087	0.363
CMPSNP571	6	20.8	X	0.529	3	2	0.498	0.087	0.374
snv10873	6			0.758	4	3	0.376	0.040	0.319
snv10871	6			0.646	3	2	0.458	0.146	0.353
snv10863	6			0.660	3	2	0.449	0.155	0.348
snv102438	6			0.840	3	2	0.269	0.049	0.233
CMPSNP1167	6	25.6	X	0.864	3	2	0.235	0.039	0.207
CMPSNP433	6	32	X	0.694	3	2	0.425	0.068	0.334
CMPSNP3	6	43.2	X	0.646	3	2	0.458	0.049	0.353
snv80700	6			0.743	3	2	0.382	0.068	0.309
CMPSNP292	6	49.6	X	0.607	3	2	0.477	0.087	0.363
snv180558	6			0.665	3	2	0.446	0.068	0.346
snv180557	6			0.583	3	2	0.486	0.058	0.368
CMPSNP295	6	49.6	X	0.825	3	2	0.288	0.078	0.247
snv26555	6			0.583	3	2	0.486	0.058	0.368
snv_METC113399_986	6			0.752	3	2	0.373	0.068	0.303
CMPSNP1038	6	57.6	X	0.837	3	2	0.273	0.050	0.236
CMPSNP1021	6	57.6	X	0.568	3	2	0.491	0.068	0.370
A_38-F04	6	70.7	X	0.539	3	2	0.497	0.087	0.373
AI_13-F02	6	85.3	X	0.535	3	2	0.498	0.059	0.374
CMPSNP378	6	86.9	X	0.650	3	2	0.455	0.039	0.351
snv31558	7			0.926	3	2	0.136	0.010	0.127
AI_05-F11	7	4.9	X	0.525	3	2	0.499	0.049	0.374
snv237194	7			0.675	3	2	0.439	0.087	0.343
CMPSNP249	7	11.3	X	0.650	3	2	0.455	0.078	0.351
CMPSNP262	7	30.5	X	0.636	3	2	0.463	0.087	0.356
CMPSNP579	7	30.5	X	0.524	3	2	0.499	0.058	0.374
CMPSNP1009	7	32.1	X	0.853	3	2	0.251	0.059	0.219
CMPSNP287	7	35.3	X	0.694	3	2	0.425	0.029	0.334
snv227297	7			0.767	3	2	0.357	0.019	0.294
snv227298	7			0.770	3	2	0.355	0.010	0.292
snv227300	7			0.519	3	2	0.499	0.068	0.375
snv116229	7			0.995	2	2	0.010	0.010	0.010
CMPSNP56	7	43.3	X	0.650	3	2	0.455	0.097	0.351
CMPSNP465	7	59.4	X	0.680	3	2	0.435	0.019	0.341
CMPSNP415	7	72.2	X	0.626	3	2	0.468	0.029	0.359
CMPSNP12	8	0	X	0.597	3	2	0.481	0.087	0.365
CMPSNP766	8	4.8	X	0.636	3	2	0.463	0.029	0.356
CMPSNP718	8	11.2	X	0.587	3	2	0.485	0.068	0.367
snv72549	8			0.578	3	2	0.488	0.087	0.369
snv72552	8			0.563	3	2	0.492	0.097	0.371
CMPSNP97	8	19.2	X	0.578	3	2	0.488	0.437	0.369
CMPSNP44	8	22.4	X	0.680	3	2	0.435	0.039	0.341
AI_21-D08	8	28.8	X	0.614	2	2	0.474	0.000	0.362
CMPSNP181	8	35.2	X	0.574	3	2	0.489	0.020	0.369
snv145912	8			0.995	2	2	0.011	0.011	0.011
F013	8	48.1	X	0.646	3	2	0.457	0.063	0.353
CMPSNP1066	8	79.2	X	0.607	3	2	0.477	0.010	0.363
CMPSNP553	9	0	X	0.524	3	2	0.499	0.078	0.374
CMPSNP173	9	3.2	X	0.626	3	2	0.468	0.087	0.359
P5.64	9	8	X	0.617	3	2	0.473	0.126	0.361
snv156328	9			0.932	2	2	0.127	0.000	0.119
snv156330	9			0.951	3	2	0.092	0.039	0.088
snv181016	9			0.510	3	2	0.500	0.109	0.375
CMPSNP1077	9	19.2	X	0.592	3	2	0.483	0.039	0.366
CMPSNP320	9	20.8	X	0.728	3	2	0.396	0.078	0.318
CMPSNP144	9	22.4	X	0.607	3	2	0.477	0.029	0.363
snv81780	9			0.558	3	2	0.493	0.068	0.372

snv81788	9			0.612	3	2	0.475	0.058	0.362
snv96603	9			0.789	3	2	0.333	0.029	0.277
snv96602	9			0.932	3	2	0.127	0.019	0.119
snv96589	9			0.529	3	2	0.498	0.107	0.374
CMPSNP1035	9	33.6	X	0.617	3	2	0.473	0.068	0.361
CMPSNP159	9	36.8	X	0.691	3	2	0.427	0.029	0.336
snv235812	9			0.743	3	2	0.382	0.068	0.309
snv16859	9			0.544	3	2	0.496	0.049	0.373
snv16858	9			0.699	3	2	0.421	0.058	0.332
CMPSNP1133	9	59.2	X	0.573	3	2	0.489	0.117	0.370
CMPSNP890	9	64	X	0.525	3	2	0.499	0.070	0.374
psi36-10864	10	0	X	0.530	3	2	0.498	0.100	0.374
psi36-839	10	0	X	0.520	3	2	0.499	0.059	0.375
CMPSNP172	10	1.6	X	0.743	3	2	0.382	0.068	0.309
snv72138	10			0.660	3	2	0.449	0.078	0.348
snv238357	10			0.582	3	2	0.487	0.041	0.368
CMPSNP528	10	8	X	0.529	3	2	0.498	0.068	0.374
snv92882	10			0.714	3	2	0.409	0.068	0.325
CMPSNP65	10	14.4	X	0.748	3	2	0.377	0.058	0.306
snv57827	10			0.757	3	2	0.368	0.058	0.300
CMPSNP762	10	23.9	X	0.748	3	2	0.377	0.050	0.306
CMPSNP671	10	28.8	X	0.529	3	2	0.498	0.118	0.374
snv86654	10			0.535	3	2	0.498	0.040	0.374
snv96678	10			0.752	3	2	0.373	0.049	0.303
snv212837	10			0.752	3	2	0.373	0.107	0.303
snv87950	10			0.621	3	2	0.471	0.039	0.360
snv38521	10			0.772	3	2	0.352	0.165	0.290
snv38520	10			0.510	3	2	0.500	0.071	0.375
snv38519	10			0.801	3	2	0.319	0.029	0.268
CMPSNP550	10	38.5	X	0.660	3	2	0.449	0.350	0.348
CMPSNP426	11	0	X	0.607	3	2	0.477	0.049	0.363
HS_35-E11	11	16.4	X	0.743	3	2	0.382	0.087	0.309
snv223744	11			0.665	3	2	0.446	0.049	0.346
PSI_41-B07	11	27.6	X	0.694	3	2	0.425	0.029	0.334
snv205766	11			0.500	3	2	0.500	0.446	0.375
CMPSNP389	11	47.7	X	0.602	3	2	0.479	0.233	0.364
CMPSNP30	11	66	X	0.608	3	2	0.477	0.098	0.363
CMPSNP315	11	90.9	X	0.711	3	2	0.411	0.049	0.327
CMPSNP475	11	98.9	X	0.535	3	2	0.498	0.059	0.374
CMPSNP122	11	100.5	X	0.607	3	2	0.477	0.049	0.363
snv236017	11			0.922	2	2	0.143	0.000	0.133
snv5835	11			0.913	3	2	0.159	0.117	0.147
snv5837	11			0.942	3	2	0.110	0.019	0.104
snv_METC085477	11			0.597	3	2	0.481	0.068	0.365
CMPSNP385	12	4.8	X	0.762	3	2	0.363	0.049	0.297
CMPSNP310	12	9.8	X	0.505	3	2	0.500	0.058	0.375
snv152951	12			0.874	3	2	0.221	0.019	0.196
snv152951	12			0.874	3	2	0.221	0.039	0.196
AI_35-A08	12	16.4	X	0.617	3	2	0.473	0.068	0.361
AI_09-G07	12	18.1	X	0.740	3	2	0.385	0.140	0.311
CMPSNP285	12	21.4	X	0.908	3	2	0.167	0.049	0.153
snv234449	12			0.675	3	2	0.439	0.107	0.343
CMPSNP361	12	37	X	0.583	3	2	0.486	0.078	0.368
CMPSNP5	12	58.1	X	0.743	3	2	0.382	0.029	0.309
snv106138	12			0.568	3	2	0.491	0.049	0.370
FR14F22	12	67.7	X	0.524	3	2	0.499	0.058	0.374
P02.03	12	69.3	X	0.631	3	2	0.466	0.097	0.357
snv59053				0.879	3	2	0.213	0.029	0.191
snv22707				0.871	3	2	0.224	0.040	0.199
snv230197				0.985	2	2	0.029	0.029	0.029
snv230201				0.539	3	2	0.497	0.204	0.373
snv230204				0.791	3	2	0.330	0.146	0.276
snv230212				0.971	2	2	0.057	0.059	0.055
snv234334				0.617	3	2	0.473	0.107	0.361

* References in which these markers were experimentally validated and where additional information is available. X= Esteras *et al.* 2013

** Markers beginning by snv are new SNPs selected from Melogene database

CHAPTER 4

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF THE OLDEST MELON SEEDS FOUND IN WESTERN MEDITERRANEAN

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF THE OLDEST MELON SEEDS FOUND IN WESTERN MEDITERRANEAN

Diego Sabato¹, Belén Picó², Oscar Grillo³, Cristina Esteras², Leonor Peña-Chocarro^{4,5}, Gianluigi Bacchetta¹

¹ *Centro Conservazione Biodiversità (CCB), Dip. di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Italy*

² *Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Universitat Politècnica de València, Spain.*

³ *Stazione Consorziale Sperimentale di Granicoltura per la Sicilia (SSGS), Caltagirone, Italy.*

⁴ *Escuela Española de Historia y Arqueología en Roma-CSIC, Rome, Italy*

⁵ *GI Arqueobiología, Instituto de Historia, CCHS-CSIC, Madrid, Spain*

Abstract

During a rescue archaeology campaign at Sa Osa, Sardinia (Italy), an intact Late Bronze Age well was discovered. The structure yielded a large amount of waterlogged plant remains from which a group of *Cucumis melo* seeds were one of the most remarkable. These remains are the earliest record of this *taxon* in the Western Mediterranean and one of the oldest ever recorded. Plant remains were preserved by permanent waterlogging in anoxic conditions making the *Cucumis* seeds good candidates for molecular and morphometric characterization.

DNA extraction from archaeological samples followed a special procedure to avoid contamination. Archaeological seeds were molecular and morphologically compared with a set of 172 accessions of traditional melon landraces from Europe, Africa and Asia. Genotyping was performed using 211 polymorphic SNPs and the genotyping technology iPLEX® Gold MassARRAY Sequenom. A total of 95 morphometric parameters, acquired by an automatic image analysis system, were specifically designed to evaluate seed size and shape.

Both molecular and morphological analyses confirmed that these archaeoseeds did not belong to a wild species but to a cultivated melon, likely to be an intermediate form between the two melon subspecies, *melo* and *agrestis*. This primitive melon could be attributed to a group of ancestral non-sweet or low sugar forms of *chate*, *flexuosus* and *ameri* varieties, showing similarities with African and Central Asia accessions. *Chate* and *flexuosus* are cucumber-like forms which played a central role in early melon selection while *ameri*, 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*.

Key words: *Cucumis melo*, ancient DNA, seed morphology, archaeobotany, image analysis, molecular analysis.

4.1 Introduction

4.1.1 Melon

Cucumis melo L. has gone through an intense process of diversification, and today it shows great variation in morphological and physiological characters with different groups and varieties being reported (Naudin 1859, Munger and Robinson 1991). Although *C. melo* has been traditionally divided into two subspecies according to ovary hairiness, subspecies *melo* (long hairs) and subspecies *agrestis* (short hairs) (Jeffrey 1980, 2001, 2005), nowadays this classification is based mainly on molecular clustering (Esteras *et al.* 2012, 2013). The wild forms of melon (*C. melo* subsp. *agrestis* var. *agrestis*) show a great genetic variability and are distributed across the tropical and sub-tropical belt in Africa and Asia and including numerous previously overlooked species-level relatives from Australia and around the Indian Ocean (Sebastian *et al.* 2012). Pitrat *et al.* (2000) recognized 16 varieties: *cantalupensis* Naudin, *reticulatus* Ser. (cantaloupe, muskmelon), *inodorus* H. Jac. (winter melon, casaba melon), *flexuosus* L. (snake melon), *chate* Hasselq. (cucumber melon), *adana* Pangalo, *chandalak* Gabaev, *ameri* Pangalo (Asian melon), *chito* C.Morren (American melon), *dudaim* L. (pocket melon), *tibish* Mohamed, *acidulus* Naudin, *conomon* Thunb., *makuwa* Makino, *chinensis* Pangalo (pickling melon), and *momordica* Roxb. (snap melon); in later revisions, Pitrat (2008) merged together some of these varieties.

The knowledge about the origin and diffusion of the main cultivated plants (cereals, legumes, grape and fruit trees) has greatly increased in the last two decades, whereas very little is still known about vegetable crops such as *Cucumis melo* (Zohary *et al.* 2012). To some extent, this is due to problems related to preservation of archaeological material. The origin of domesticated melon is not fully understood yet. North Africa and Near East have been considered the traditional centre of origin of this species (Zohary *et al.* 2012). However, recent studies suggest that the melon origin-distribution centre includes the Australia-Malaysia area since melon appears closer to the Australian-Asian group than to the African species (Renner *et al.* 2007). In addition, due to the high level of variation found in Asia, especially in India, it is suggested that melon may have originated there spreading later to Africa (Renner *et al.* 2007, Sebastian *et al.* 2010). Other theories point to the possibility of two independent domestication events (Bates and Robinson 1995, Jeffrey 1980, Esquinas-Alcazar and Gulick 1983, Mallick and Mausi 1986, Tanaka *et al.* 2013). A genetic relationship between South African and East Asian melons was found by Nakata *et al.* (2005).

It is likely that this crop had a marginal role at least up to Medieval times, when sweet melon was introduced to the Iberian Peninsula during the Arab domination (Paris *et al.* 2012). The oldest known archaeological record of this *taxon* is a single seed of *Cucumis* sp. found in the Spirit Cave, Thailand, in a layer dated 7622± 300 BP (Gordman 1969, 1972). This area is part of the region of native distribution of wild melon (Sebastian *et al.* 2010) and, therefore, this find has been considered more related to a wild plant than to a cultivated form (Solheim 1972). In Asia early finds have been reported from several sites between the 3rd and 2nd millenium BC in China (Watson 1969), in Iran

(Costantini 1977) and in India and Pakistan (Fuller and Madella 2001). The first Mediterranean records are located in Egypt. Two melon seeds were found in a burial at *Hierakonpolis* 3700-3300 BC (Fahmy 2001, 2003), and some doubtful non-carbonised and semi-carbonised seeds from Neolithic levels in *Maadi* 3500-3350 BC (van Zeist *et al.* 2003a). One further seed was found in a kitchen area of *Tell Hammam et-Turkman* dated to the Early Bronze Age IV, 2500-2000 BC (van Zeist *et al.* 2003b). A single pollen grain of *Cucumis* sp. was found in a core in Crete, at a level dated to ca. 2300 BC (Bottema and Sarpaki 2003). In Greece, three carbonized seeds have been reported in the Late Bronze Age *Tirynth* (Kroll 1982), a few others in the Iron Age *Kastanas* (Kroll 1983, 1984, Megaloudi 2006) and several in the Sanctuary of Hera in the island of Samos, dated to the 7th century BC (Kučan 1995, Megaloudi 2006). A single melon seed have been found in a Punic channel of Carthage (van Zeist and van der Veen 2001). During Roman period, several seeds have been found in Central and North-Western Europe, where the presence of melon is considered a sign of “Romanization” (Livarda 2011, Bakels and Jacomet 2002, Wiethold 2003). In Italy, several records have been reported in the North of the peninsula (Castelletti *et al.* 2001, Rinaldi *et al.* 2013), Pompeii (Murphy *et al.* 2013) and in the last phases of the Trajan Harbour, in Rome (Pepe *et al.* 2013, Sadori *et al.* 2014).

Rescue excavations carried out during 2008 and 2009 in Sa Osa, Central-West Sardinia, revealed a Nuragic settlement composed of numerous wells and pits related to living spaces. These structures were dug by local communities between the Early Copper and the Iron Age, with emphasis during the Middle and Late Bronze Age. The most remarkable structure was the so-called Well-N (Fig.1c) dated to the Late Bronze Age (Usai 2011). Sabato *et al.* (2015a) [Chapter 1] described the discovery of melon seeds in Well-N, which is the oldest known record of this *taxon* in the Western Mediterranean and one of the oldest in the world. Despite the archaeological context was undisturbed and that two grape seeds were already dated producing Bronze Age dates (Ucchesu *et al.* 2014a), waterlogged seeds can not be easily differentiated from modern material. For this reason, to avoid any doubt, fragments of these remains have been radiocarbon dated by AMS within a time range between 1310-1120 cal BC 2σ (Sabato *et al.* 2015 [Chapter 1]).

Details of earliest melon records, including artistic representations, written data and archaeological finds, listed in Table 1 and represented in Figure 1, although chronology is approximated since none of previous archaeological records has been directly radiocarbon dated. The anoxic conditions of the silt, the continuous supply of water and the constant temperature allowed maintaining the material in a exceptionally state of preservation making these seeds good candidates for ancient DNA preservation and morphological characterization.

	Age	Country	Place	Reference	
1	6272-5072 BC	A*	Thailand	Spirit Cave	Gordman 1969 ⁴ , 1972
2	3750-3300 BC	A	Egypt	Hierakonpolis	Fahmy 2001 ¹ , 2003
3	3500-3350 BC	A ⁺	Egypt	Maadi	van Zeist <i>et al.</i> 2003a ¹
4	ca. 3000 BC	A	China	Zhejieang	Watson 1969 ⁴
5	3000-2000 BC	A	Oman	Hili	Tengberg 2003
6	2686-2181 BC	G	Egypt	several	Keimer 1924 ^{1,2}
7	2500-2000 BC	A	Syria	Tell Hammam	van Zeist <i>et al.</i> 2003b ¹
8	ca. 2350 BC	P*	Greece	Kournas, Crete	Bottema and Sarpaki 2003
9	ca. 2000 BC	A	Iran	Shahr-I Sokhta	Costantini 1977 ¹
10	ca. 2000 BC	A	China	Shaanxi	Watson 1969 ⁴
11	2000-1700 BC	A*	India	Rojd	Weber 1991 ⁶
12	1700-1500 BC	A	India	Inamgaon	Kajale 1988 ⁶
13	1700-1300 BC	A*	India	Balathal	Kajale 1986 ⁶
14	1700-1300 BC	A*	Pakistan	Loebanr 3	Costantini 1987 ⁶
15	1550-1300 BC	G	Egypt	Theban Necropolis	Manniche 1989 ²
16	1517-1192 BC	G	Egypt	Theban Necropolis	Darby <i>et al.</i> 1977 ²
17	1310-1120 BC	A	Italy	Sa Osa, Sardinia	Sabato <i>et al.</i> 2015 [Chapter 1]
18	1200-1000 BC	A	Greece	Tirynt	Kroll 1982 ^{1,5}
19	1050-900 BC	A	Greece	Kastanas	Kroll 1983 ⁵ , 1984
20	1000-500 BC	W	China	-	Confucius (attributed) Keng 1974 ⁴
21	700-600 BC	A	Greece	Heraion, Samos	Kučan 1995 ^{1,5}
22	ca. 350 BC	A	Tunisia	Carthage	van Zeist and van der Veen 2001
23	ca. 168 BC	A	China	Hunan	Yu 1977 ⁴
24	100 BC-500 AD	A	NW Europe	several	Livarda 2011
25	10-0 BC	A ⁺	Switzerland	Vindonissa	Jacomet <i>et al.</i> 2002
26	15-40 AD	A	Italy	Mutina	Rinaldi <i>et al.</i> 2013
27	50-200 AD	A	Egypt	Mons Porphyrites	van der Veen and Tabinor 2007
28	50-250 AD	A	C Europe	several	Bakels and Jacomet 2002
29	ca. 64 AD	W	Italy	-	Columella ^{2,3}
30	ca. 77 AD	W	Italy	-	Gaius Plinius Secundus ^{2,3}
31	79 AD	A	Italy	Pompei	Murphy <i>et al.</i> 2013
32	100-200 AD	A*	Egypt	Mons Claudianus	van der Veen 1996, 2001
33	100-300 AD	G	Tunisia	several	Balmelle 1990, Blanchard-Lemé <i>et al.</i> 1995, Yacoub 1995 ²
34	180-250 AD	A	France	Alesia	Wiethold 2003
35	220-651 AD	A	Turkmenistan	Merv Oasis	Nesbitt and O'Hara 2000
36	ca. 250 AD	G	Greece	Thessaloniki	Pazaras 1981 ²
37	ca. 260 AD	W	Italy	-	Quintus Gargilius Martialis ³
38	300-400 AD	G	Spain	Mérida	Álvarez Martínez <i>et al.</i> 2000 ²
39	ca. 400 AD	W	Italy	-	Palladius ³
40	ca. 400 AD	W	Italy	-	Apicius ³
41	500-600 AD	G	Lebanon	-	Baratte 1978 ² , Balmelle <i>et al.</i> 1990 ²
42	500-600 AD	A	Italy	Portus	Pepe <i>et al.</i> 2013
43	550-600 AD	A	Tunisia	Carthage	van Zeist and van der Veen 2001
44	500-700 AD	W	China	several	Li 1970
45	533-544 AD	W	China	-	Jia Sixie (attributed) Li 1969 ⁴

A = Archaeological finds
G = Graphical representations
W=Written sources
P = Pollen record
* identified as *Cucumis* or *C. melo/sativus*
⁺ doubtful find or cf. *C. melo*

¹ References reported in Zohary *et al.* 2012
² Pictures reported in Janick *et al.* 2007
³ Sources reported in Paris *et al.* 2012
⁴ References reported in Walters 1989
⁵ References reported in Megaloudi 2006
⁶ References reported in Fuller and Madella 2001

Table 1. Records of melon in chronological order from the earliest identifications until the 6th century AD, including graphical representations, written sources and archaeological finds. Chronology is approximative since, apart from Sa Osa, none of archaeological remains has been directly radiocarbon dated.

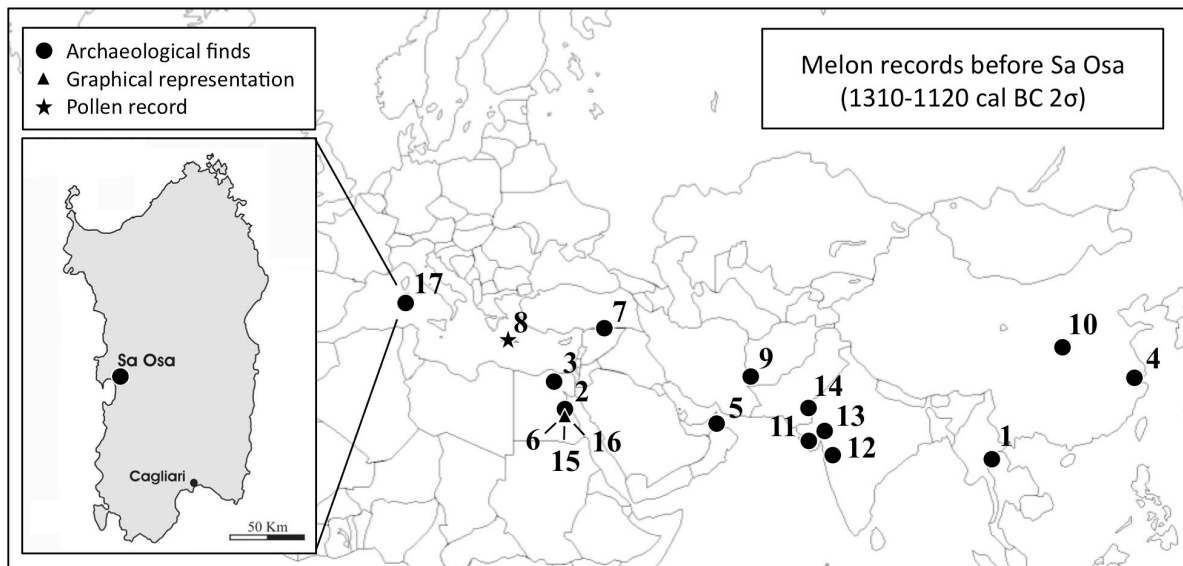


Figure 1. Melon records earlier than melon seeds found in Sa Osa. For reference numbers see Tab. 1.

4.1.2 Introduction to the analyses

Cold, dry and/or low oxygen environments are beneficial for DNA survival, where preservation by freezing and desiccation is the best way for ancient DNA preservation (Oliveira *et al.* 2012). Despite waterlogging does not favour DNA preservation as hydrolysis is one of the major decay reactions (Schlumbaum *et al.* 2008), a PCR (Polymerase Chain Reaction) designed to target small fragments, as lengths of 50-500b, may be successful to amplify ancient DNA (Pääbo *et al.* 2004, Speirs *et al.* 2009). Waterlogged plant remains have been used as source of ancient DNA (Manen *et al.* 2003, Elbaum *et al.* 2005; Pollmann *et al.* 2005, Gyulai *et al.* 2008, Speirs *et al.* 2009). Archaeogenetic studies have strongly increased over the last two decades and an extensive literature on the subject is available in Palmer *et al.* (2012).

Morpho-colorimetric evaluations are commonly used as a tool to assess shape, size and colour of objects, in order to relate quantitative physical characters and qualitative aspects. Several studies have focused on the application of image analysis to the diaspores of spontaneous flora, providing excellent results when taxonomic units very close to infra-generic and infra-specific levels are used (Bacchetta *et al.* 2008, Grillo *et al.* 2010). A previous research focused on grape varieties correlated molecular and seed morphology (Orrú *et al.* 2013a) and two further works correlated seed shape of Sardinian grape landraces and archaeological pips found in the same context of Sa Osa (Lovicu *et al.* 2011, Orrú *et al.* 2013b, Uccesu *et al.* 2014b).

Melon seed length has been already correlated to genetic and geographical differentiation among melon groups (Fujishita 1983). Tanka *et al.* 2013 indicated that large-seed melon is mainly cultivated in USA, Europe, West and Central Asia and northern Africa, whereas India both large- and small-seed melon are equally frequent, while small-seed are more common in southern Africa, South and East Asia melon. In Sabato *et al.* (in press) [Chapter 3] a strong correlation between molecular and seed characters of melon has been also shown.

Basing on this wide research background, the aims of the present work are:

- Extracting and genotyping the ancient DNA of Late Bronze Age melon seeds from Sa Osa;
- Acquiring the morphological features of the same remains by an image analysis system;
- Comparing molecular and morphological data with modern worldwide melon landraces

4.2 Materials and methods

4.2.1 Seed lots detail

The starting material was a core collection of 212 melon accessions built within the framework of a previous project (MELRIP 2007-2010, Esteras *et al.* 2012, 2013). These accessions, representative of all melon varieties from Europe, Africa and Asia, have genotyped with AFLP (Amplified Fragment Length Polymorphism) and SNP (Single Nucleotide Polymorphism) markers, and extensively phenotyped for plant and fruit traits. Only accessions belonging to traditional landraces were considered for the present research for a total of 172 seed lots (145 and 115 for molecular and morphological analysis respectively) from 44 different countries from Europe, Africa and Asia [**Annex C1**]. Seed lots have been mainly provided by USDA (United States Department of Agriculture) and COMAV (Conservación y Mejora de la Agrodiversidad Valenciana) Germplasm Banks. Four accession from Cyprus have been provided by the Cyprus Germplasm Bank and ten Sardinian landraces, mostly decrypted in Attene and Rodriguez (2008), have been supplied by the Agriculture Department of University of Sassari and one by a local farmer.

Archaeological seeds have been preserved in waterlogged condition and stored in distilled water at +5°C temperature (Sabato *et al.* 2015 [**Chapter 1**]). Identification of melon seeds followed indication of Frank and Stika (1988) apart from using a wide range of modern reference material. Only 15 full preserved seeds have been selected for morphological analysis (Fig. 2), while broken seeds were used for radiocarbon dating and DNA extraction.



Figure 2. Three waterlogged Late Bronze Age seeds from Sa Osa (Cabras, Sardinia) before molecular and morphological analyses.

4.2.2 *Molecular analysis*

DNA extraction of archaeological samples followed a special procedure to avoid contamination. DNeasy Plant Mini Kit (Qiagen) was used and its protocol was followed with minor changes (more time with the initial buffer). All the tools involved (tubes, gloves, blades, etc.) have been UV irradiated for 12 hours and previously autoclaved if not disposable (like pliers and steel beads). The archaeological material was never manipulated in rooms where modern cucurbits were being treated and extractions were carried out in a sterile flow-hood chamber. Seed surface were cleaned out with a solution of 10% $\text{Ca}(\text{OCl})_2$ w/v for one minute. Six samples were selected, four with a single seed, and two with three seeds. 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). Unfortunately, only a sample yielded a minimum amount of DNA for quantifying and genotyping.

A total of 123 SNPs 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 total of 38,587 SNPs that were in silico identified in two previous re-sequencing analysis (Blanca *et al.* 2011, Blanca *et al.* 2012). The most important one (Blanca *et al.* 2012) re-

sequenced 67 genotypes, grouped into 8 pools that represent all the botanical groups of the species, for mining for SNPs. These polymorphisms, identified by the alignment of the sequences to the reference transcriptome, represent the largest collection existing for melon. This transcriptome, the most complete version to date, was created by Blanca *et al.* (2012) using a combination of expressed sequence tags (ESTs) from classical Sanger sequencing and the next generation sequencing methods, e.g. 454 (Roche) and SOLID (Life Technologies Inc.).

Information about the SNPs used is available in **Annex 1** and detailed information for each SNP marker, such as sequence, allele variation and location is available in the Melogene database. Most SNPs used in this study were employed in previous mapping experiments and their position in the genetic map is known (Esteras *et al.* 2013). Genotyping with this set of markers was performed using iPLEX® Gold MassARRAY Sequenom technology, carried out at the epigenetic and genotyping unit of the University of Valencia (Unitat Central d'Investigació en Medicina UCIM). This genotyping technology relies on Single Base Extension (SBE) using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest to generate different allelic products. Using the MALDI-TOF mass spectrometry, the distinct mass of the extended primer identifies the SNP allele (Gabriel *et al.* 2009). The genotyping results were employed to perform a cluster analysis using the PowerMarker software (Liu and Muse 2005). Nei's genetic distance (Nei *et al.* 1983) was used, and the support values for the degree of confidence at the nodes of the dendrogram were analysed by bootstrap re-sampling 1,000 times. Phylip 3.69 software (Felsenstein 1997) was employed to construct the consensus tree and TreeView32 (Page 1996) to visualize it. The Principal Coordinate Analysis (PCoA) was performed using GenAlEx 6.501. In addition, 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).

4.2.3 *Morpho-metric seed analysis*

The process follows the same protocol described in Sabato *et al.* (in press) [**Chapter 3**] and [**Chapter 4**]. Images were acquired using a flatbed scanner, with a resolution of 400 dpi, 24 bit-depth and a scanning area not exceeding 1024×1024 pixel. As suggested by Venora *et al.* (2007), before image acquisition the scanner was calibrated for colour matching, following the protocol of Shahin and Symons (2003). Digital images of seeds, randomly disposed on the flatbed tray, were acquired and used for the analysis. Two images were acquired for each sample of seeds, with black and white background. Digital images of seeds were analyzed using the software package KS-400 V. 3.0 (Carl Zeiss, Vision, Oberkochen, Germany). The accuracy and speed of measurements was maximized by running an automated macro, specifically developed for the characterization of seeds (Bacchetta *et al.* 2008; Grillo *et al.* 2010). Considering that seed colour is altered in the archaeological seeds, colour and texture have been not considered in this research, but in order to increase the number of

discriminant parameters, the Elliptic Fourier Descriptors (EFDs) were also computed, as described by Orrù *et al.* (2013a), to accurately describe the shape of the analysed seeds. A total of 95 parameters, describing of seed size and shape, were computed (Tab. 1). Data were statistically elaborated applying the stepwise LDA (Linear Discriminant Analysis).

We selected five main groups of varieties to compare the archaeological seeds: the Sweet melon group (= SWG), all sweet melon varieties of subspecies *melo*: var. *ameri*, *inodorus*, *cantalupensis*, *reticulatus* and indeterminate landraces of subsp. *melo*; the Intermediate group (= ING), non sweet melons with intermediate characteristics between the two melon subspecies: var. *dudaim*, *chate*, *flexuosus* and *momordica*; the African agrestis group (= AFG), African *acidulus*, *tibish* and the two African indeterminate landraces of subsp. *agrestis*; the Conomon group (= COG), all sweet and semi-sweet melons of subspecies *agrestis* diffuse in Far East: var. *conomon*, *chinensis*, *makuwa* and Asian *acidulus*: and the Wild types group (= WTG), wild and semi-wild melons: var. *agrestis* and *chito*.

Shape parameters	
A	Area (mm ²)
P	Perimeter (mm)
Pconv	Convex Perimeter (mm)
PCrof	Crofton's Perimeter (mm)
Pconv/PCrof	Ratio between convex and Crofton's perimeters
Dmax	Maximum diameter of the seed (mm)
Dmin	Minimum diameter of the seed (mm)
Dmin/Dmax	Ratio between minimum and maximum diameters
Sf	Shape Factor = $(4 \times \pi \times \text{area}) / \text{Perimeter}^2$ (normalized value)
Rf	Roundness Factor = $(4 \times \pi \times \text{area}) / \text{max diameter}^2$ (norm. value)
Ecd	Diameter of a circle with an area equivalent to the seed (mm)
EAmx	Maximum axis of an ellipse with equivalent area (mm)
EAmn	Minimum axis of an ellipse with equivalent area (mm)
Cpt	Compact grade = $(\sqrt{2} (4/\pi) \times \text{area}) / \text{Dmax}$
C	Curl = ratio between maximum diameters and Fiber lengths
Fl	Fiber length (mm)
Cvx	Convexity = ratio of Crofton's Perimeters and real Perimeters
78 EFDs	Elliptic Fourier Descriptors

Table 2. List of characters analyzed in the morphological analyses.

4.3 Results

4.3.1 Molecular analysis

In **Annex C1** genotyping data are presented. Seventeen *loci* were not amplified in the archaeological material, probably due to ancient DNA degradation. Seventy-one SNPs were homozygous for one allele, a trait frequently found in the Eastern European, Western Asian and North African accessions belonging to *chate*, *flexuosus* and *ameri* varieties. Often (in approximately a 30% of cases) the same allele of the archaeological seeds was present in African melons (*acidulus* and *tibish*), but rarely found in Far Eastern Asian melons (*conomon*). Furthermore, ancient DNA shares more alleles with *inodorus* and *cantalupensis* varieties, which seem more related to the Far Eastern *conomon*. A high number of *loci* (35) were heterozygous, with one of the highest heterozygosity level. Both alleles of these heterozygous *loci*, are frequent in *melo* cultivars/African *agrestis* and in those shared by *conomon* and *agrestis*. One of the alleles of some of the heterozygous *loci* is quite rare in modern genotypes and it is only present in some North African, Indian or Far eastern accessions.

The PCoA carried out using the genotyping results of 123 polymorphic SNPs is shown in Figure 3a (coordinate 1 VS 2) and Figure 3b (coordinate 1 VS 3). In both representation the archaeological seeds were grouped in the left section of the graph according to the first axe (these explain the 49.05% of the total variation) together with accessions of subspecies *melo* and more distant from those of subspecies *agrestis*. These results clearly show that the Bronze Age melon seeds belong to cultivated melons, likely the subspecies *melo*, quite distant to modern landraces. Closest accessions were two *ameri* from Russia (AmRU42) and Morocco (AmMA37), two *chate* melons from South Italy (ChIT27 and ChIT122) and two indeterminate landraces from Mali (LaML35) and Italy (LaIT00) (Fig. 3c). ChIT27, ChIT122 and AmMA37 (Brix degree 4 to 6) can be considered non-sweet forms whereas AmRU42 and LaML35 can be considered a low sugar accession (Brix degree 5 to 8).

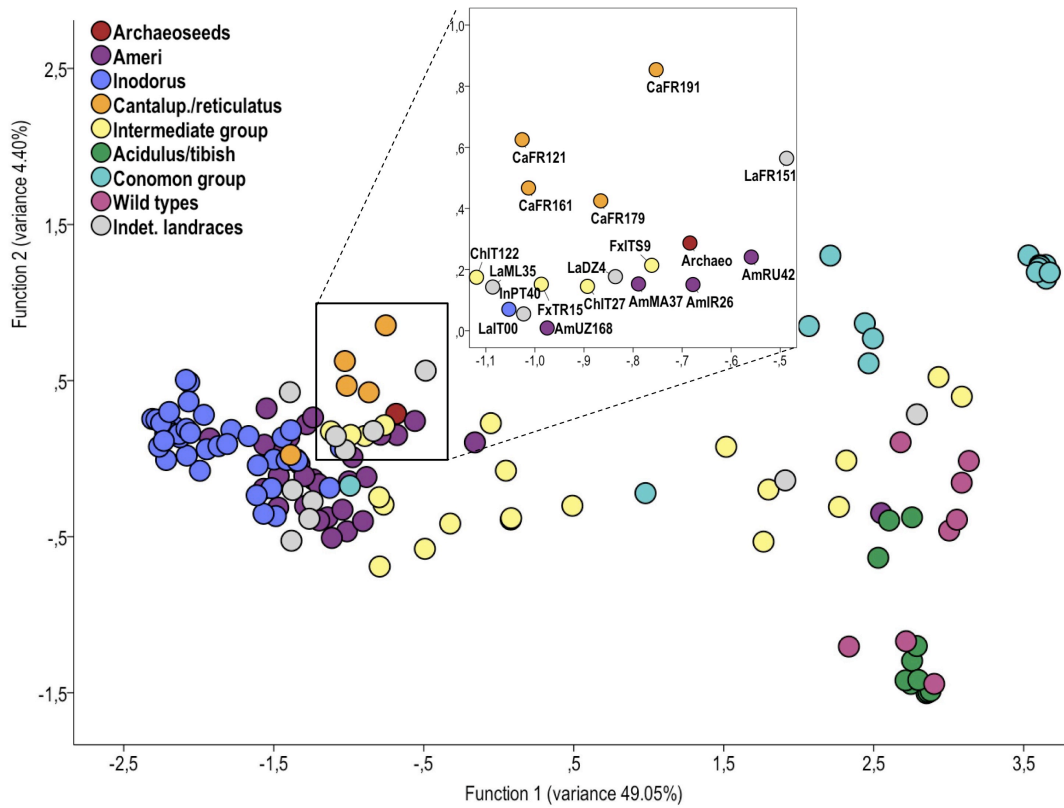


Figure 3a. PCoA analysis showing the molecular results from the archaeological seeds and the modern melon collection. The graph shows functions 1 and 2 corresponding to axis X and Y respectively.

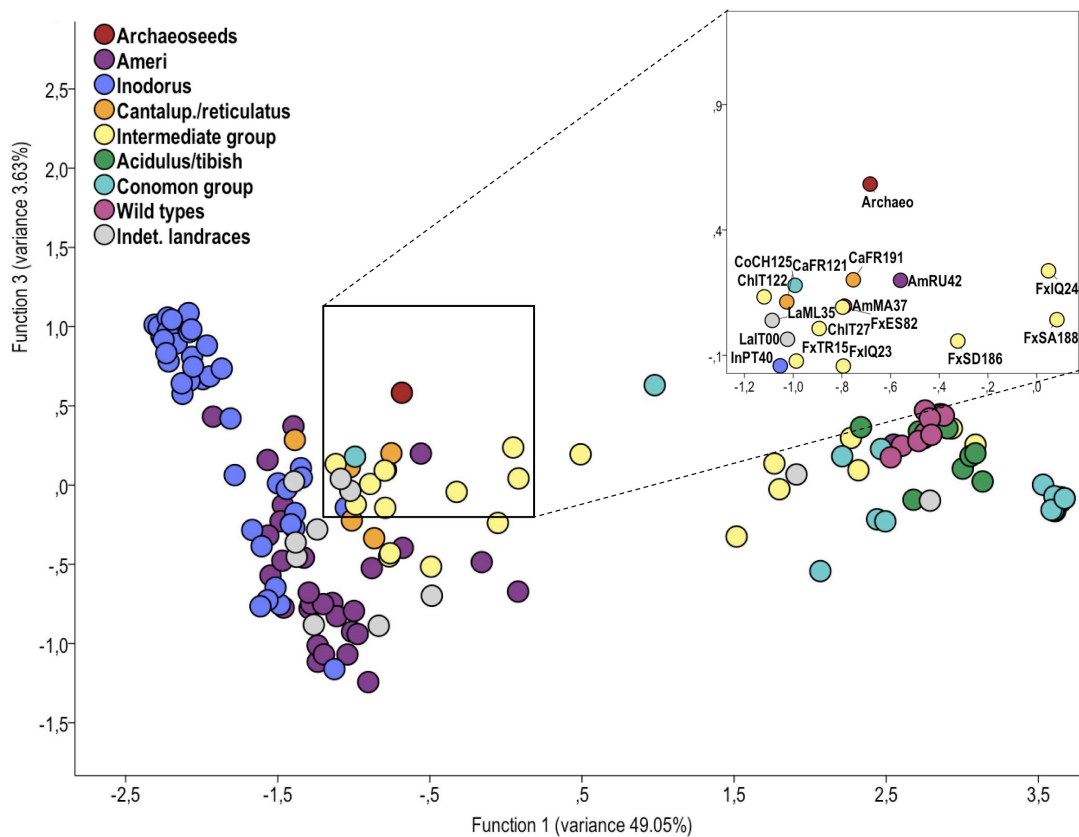


Figure 3b. PCoA analysis showing the molecular results of the archaeological seeds and the modern melon collection. The graph shows functions 1 and 3 corresponding to axis X and Y respectively.

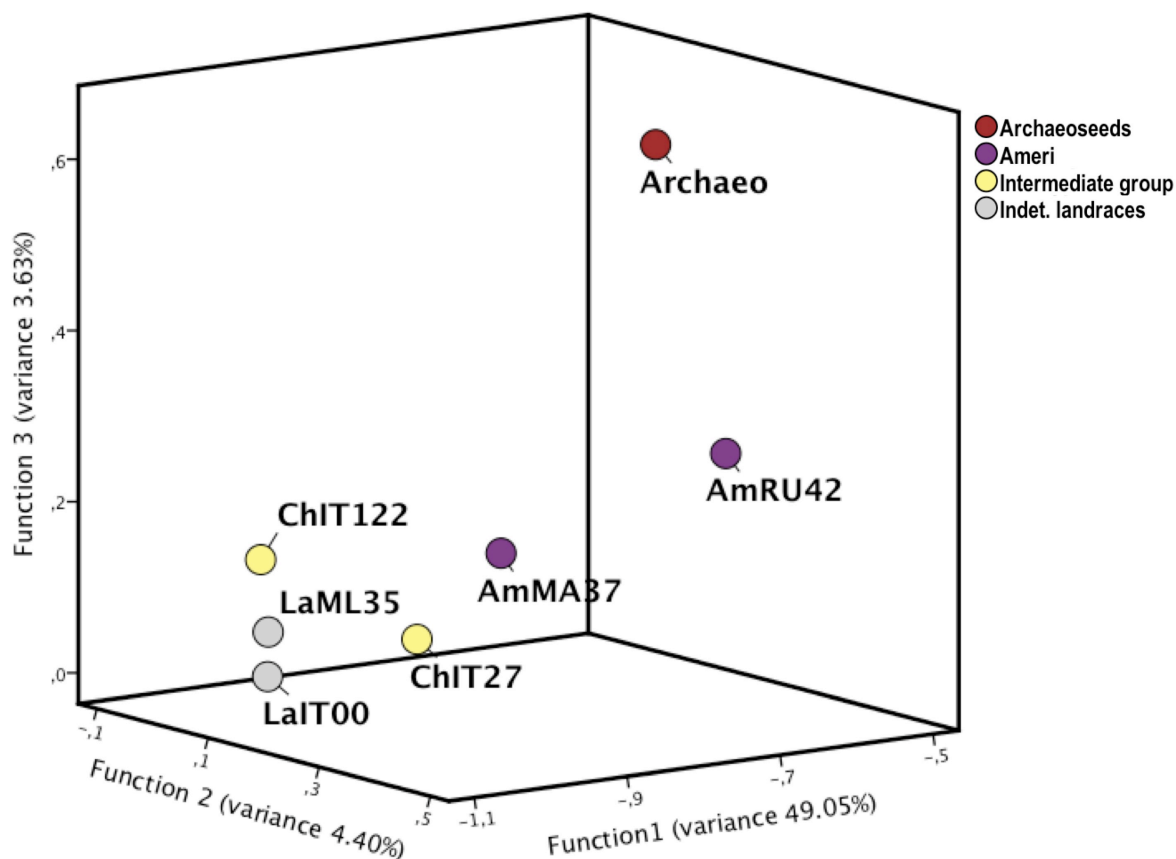


Figure 3c. PCoA analysis showing the molecular results of the archaeological seeds and the modern melon collection. The graph only focuses on closest accession. Functions 1, 2 and 3 corresponding to axis X, Y and Z respectively.

4.3.2 Morphological analysis

A first morphological comparison was carried out between the 15 archaeological seeds, the two melon subspecies (*melo* and *agrestis*) and wild melons (Table 3). The analysis, based on 11,374 seeds, gave an overall correct identification of 92.6% while misclassification between wild and cultivated melon was fairly close to zero. Correct classification of subspecies *melo* and wild melon was high (98.9% and 95.3% respectively), although subspecies *agrestis* overlapped 21.6% with subspecies *melo*. None of the ancient seeds have been classified as wild melons, being mostly classified as cultivated *agrestis* (80%, 12 seeds) and some as cultivated *melo* (20%, 3 seeds), suggesting their intermediate position between the two subspecies.

	subsp. <i>melo</i>		subsp. <i>agrestis</i>		wild melon		total	
	%	n°	%	n°	%	n°	%	n°
<i>C. melo</i> subsp. <i>melo</i>	98.9	8072	1.2	96	-	-	100.0	8168
<i>C. melo</i> subsp. <i>agrestis</i>	21.1	539	71.8	1830	7.1	181	100.0	2550
wild melon	-	-	4.6	30	95.4	625	100.0	656
Archaeoseeds	20.0	3	80.0	12	-	-	100.0	15

- 92.6% overall classification

Table 3. Results of the morphological comparison between archaeological seeds and cultivated melon subspecies and wild melon.

The comparison among the five main groups described in “Materials and methods” and the archaeological seeds is reported in table 4. Most macro-groups were classified correctly, with percentages of overall correct identification of 81.3%. SWG was correctly identified in the 93.4% of cases, although ING overlapped with this group in the 55.1% of cases, confirming the high similarity of these ecotypes. None of the archaeological seeds were classified as SWG or WTG. Nine of them (60.0%) have been classified as ING while three seeds (20.0%) have been associated to AFG. The remaining three seeds have been classified as COG (20.0%). Table 5 shows a list of 28 parameters that contributed to discrimination according to the *F-to-remove* value, which indicate the weight of a single parameter in the statistical analysis. The most important characters of discrimination were related to seed dimension, as the area, the diameter value of a circle with an equivalent area, the minimum axis value of an ellipse with equivalent area and the perimeter (A, Ecd, EAmin, P). In minor amount also features that describe seed shape contributed to discrimination, as the compact grade value, the ratio between minimum and maximum diameters and several Elliptic Fourier Descriptors (Cpt, Dmin/Dmax and FDs).

	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	757
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	627
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
ARC	-	-	60.0	9	20.0	3	20.0	3	-	-	100.0	15

- 81.3% overall classification

Table 4. Results from LDA analysis comparing the archaeological seeds (ARC) to variety groups with similar morphological characteristics. For code details see “Materials and methods”

LDA analysis has been also applied considering each accession as independent group. Scatterplot graph in Figure 4a and 4b show archaeological seed and accession distributions, the latter only represented by the mean of respective position of all seed coordinate directions (centroids). Closest accessions were a *conomon-chinensis* from Asia (CnPL169), indeterminate landraces of from Africa (LaMG202, LaZA47, LaET11), South African *acidulus* (AcZA98, AcZW99, AcZW100), a *dudaim* from Georgia (DuGE296) and a *flexuosus* from Iraq (FxiQ23). Further accessions that show similar features were several *ameri*, *conomon* and *flexuosus* from Central and East Asia. All of these closest accessions are non-sweet or low sweet cultivated landraces.

	Parameter	F-to-remove	Tolerance	Wilks' lambda
1	A	253.072	0.001	0.059
2	Ecd	193.983	0.001	0.057
3	EAMin	141.611	0.005	0.056
4	Cpt	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	Dmin/Dmax	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

Tab. 5. Factors used for discrimination among species in order of decreasing *F-to-remove*, that describes the power of each variable in the model. The *Tolerance* indicates the proportion of a variable variance not accounted by other independent variables in the equation. *Wilks' lambda* is a direct measure of the proportion of variance in the combination of dependent variables that is unaccounted for by the independent variable.

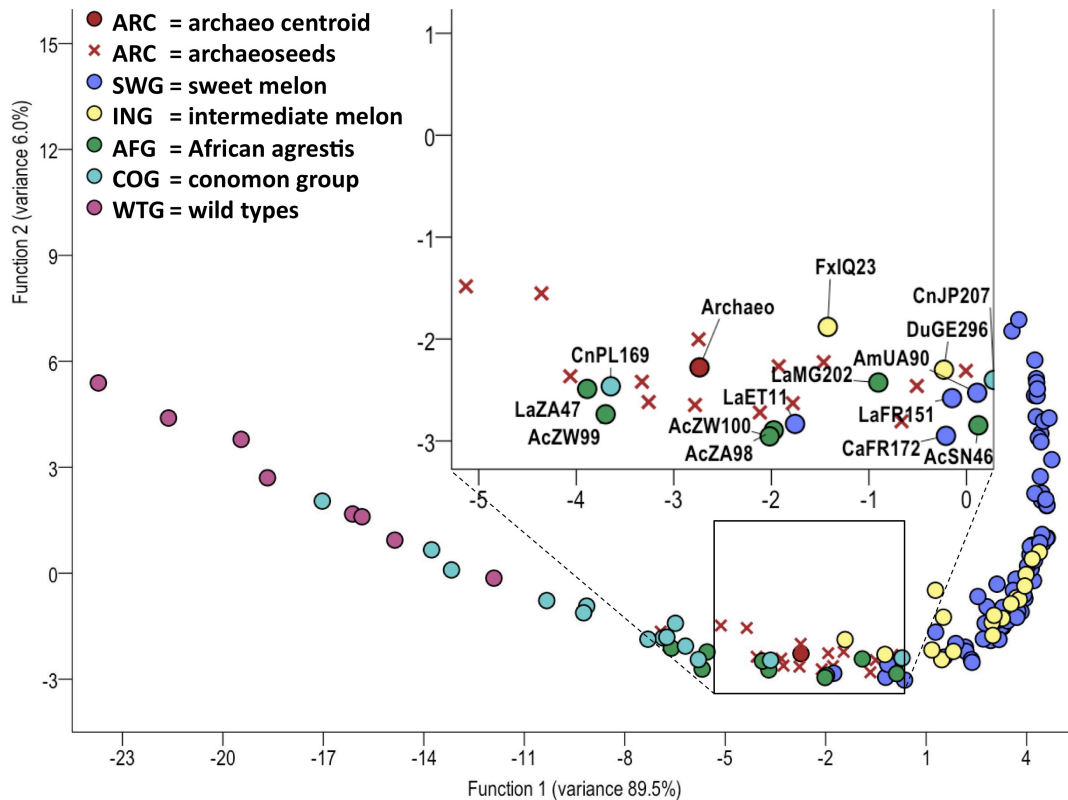


Figure 4a. LDA analysis results of morphological comparison between the archaeological melon seeds and the modern collection. Only accession centroids are represented. The graph shows functions 1 and 2 corresponding to axis X and Y respectively. For code details see “Materials and methods”

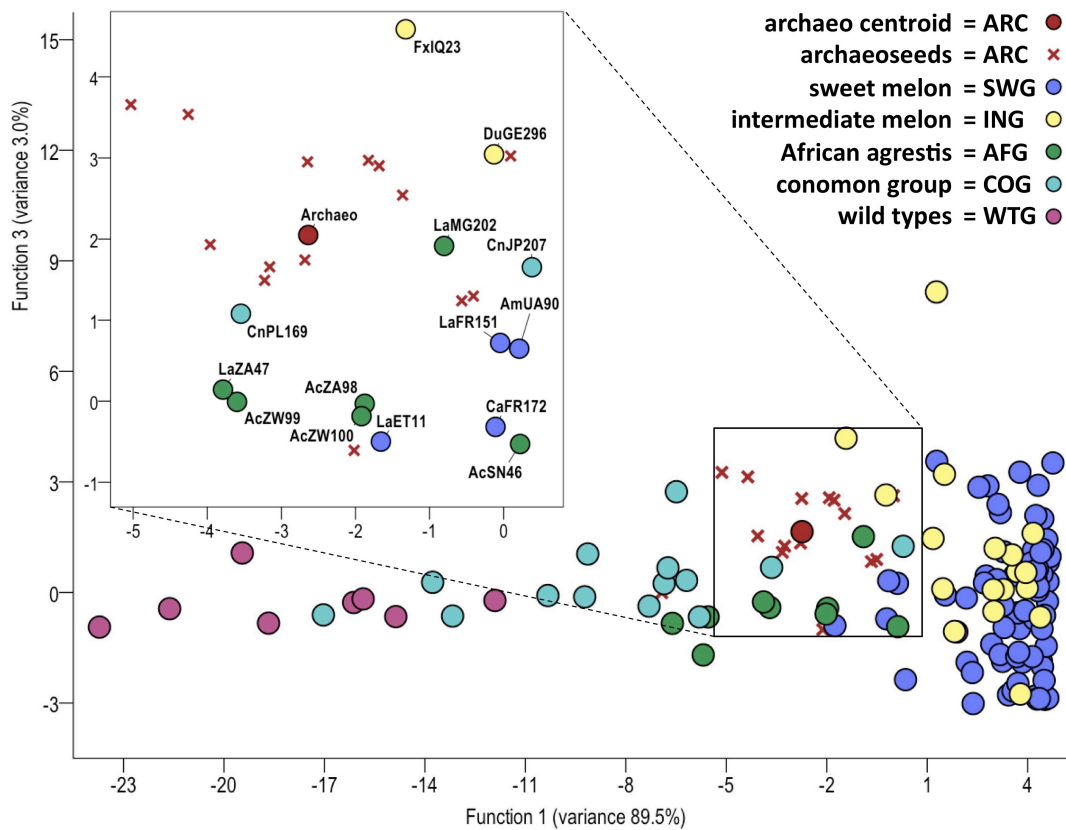


Figure 4b. LDA analysis results of morphological comparison between the archaeological melon seeds and the modern collection. Only accession centroids are represented. The graph shows functions 1 and 3 corresponding to axis X and Y respectively. For code details see “Materials and methods”

3.3 Discussion

The archaeological seeds from Sa Osa are chronologically situated in one of the most prosperous prehistoric Sardinian phase, the Nuragic period. This civilization began during the Middle Bronze Age when human communities started to mark their territories by means of monumental tombs, called *Tombe dei giganti*, and cyclopean towers, *Nuraghi* (Lilliu 1985). In this period Sardinia played a significant role as part of several interconnected trade routes between Western and Eastern Mediterranean (Lo Schiavo 2003, Bernardini and Perra 2012). The Nuragic societies developed commercial relations mainly with the island of Cyprus, which are documented by several, copper oxhide ingots and tools for metal working (Begemann *et al.* 2001, Lo Schiavo *et al.* 2009). The early presence of melon during the Late Bronze Age in Sardinia may suggest that its introduction could have followed these trade routes (Sabato *et al.* 2015a [Chapter 1]). The integrated analysis of morphological and molecular characterization represents a unique opportunity to study the history of the earliest spread of melon in the Mediterranean Basin and Europe.

Molecular results seem to demonstrate that this Late Bronze Age melon was quite distant from modern ecotypes and probably it is today extinct. In any way it was clearly distant to wild melons and more related to cultivated melons from the subspecies *melo*. Closest accessions were *ameri*, *chate*, *flexuosus* and hybrids nowadays mostly diffused in Italy, Near east, Central Asia and Africa. Admixture with *cantalupensis* and *inodorus* it is also shown. A high number of alleles of the subspecies *agrestis*

were also present indicating a closer relation to the African and Indian material than to the Far Eastern melons. Some *loci* of the archaeological material could not be amplified due, perhaps, to DNA degradation or to additional mutations in the flanking regions that prevent amplification. These mutations could have disappeared in the current analyzed germplasm collection. The higher level of heterozygosity found in the archaeological accession may reflect either crossing in open pollination or a mixture of different cultivars, which could point to the growing of different types of melons at the time. Several genomic regions have been related to sugar content in melon. Leida *et al.* (in press) associated the marker SNP711 located in LGI (46,cM) to sugar content. This marker has resulted heterozygous C/T in the archaeological material, while the allele C found in homozygous condition in most of the non-sweet or low sugar genotypes (*ameri*, *flexuosus*, *acidulous*, *momordica*, *dudaim* and *agrestis*) is absent in the sweet genotypes (*cantalupensis* and *inodorus*). Another interesting region is located in LGIX (22,4-33,6cM), marker SNP1035; Dai *et al.* (2011) demonstrated that this region maps the acid invertase 2, AIN2, a gene involved in sugar accumulation in melon fruits. The archaeological accession in this region is heterozygous as *chate* melon. It is also heterozygous for marker SNP144 being the allele G more frequent in non-sweet genotypes.

Also morphological analyses indicated that ancient material did not belong to wild melons, but more likely to an intermediate cultivated form between the subspecies *melo* and subspecies *agrestis*. Seed morphology related these seeds with *acidulus*, *flexuosus*, *dudaim* accessions from Near East and Africa, and *conomon* from Far Asia. The interpretation of morphological data has to take into account that seed size was one of the first parameters of discrimination. After more than three millennia of constant selection with the objective of increasing fruit size, current melon landraces are likely to produce bigger seeds than the archaeological forms. Seed and fruit size increase through human selection has been already demonstrated for other cultivated plants (Fuller 2007). Similar trend has been found in other cucurbits (Paris and Nerson 2003) and a strong statistical correlation has been found between melon seed and fruit size (Sabato *et al.* in press [Chapter 3]). Akashi *et al.* (2002) reconized two melon groups based on seed length, large-seed and small-seed groups, and Tanka *et al.* (2013) correlated the small-seed group with Southern African and Far Asia accessions. Considering that African landraces suffered less breeding processes compared to European and Central Asian accessions, seeds may have remained smaller than their European relatives, and this can explain the closer relation with the archaeological seeds.

Ameri landraces from the Near East and Central Asia are considered to be the first step of diversification of sweet melons (Pitrat *et al.* 2000); in fact, they are sweet, but with a lower sugar content than the modern *inodorus* and *cantalupensis* cultivars. Esteras *et al.* (2013) and Leida *et al.* (in press.) have highlighted that these accessions showed a molecular admixture of several subpopulations, including modern Charentais, *reticulatus*, a group of *inodorus* (including singular Spanish landraces still used in local markets and *inodorus* from Northern Africa, Eastern Europe and Western Asia), and highly variable *ameri* landraces from Egypt, Israel, Turkey, Russia, Central Asia

and Middle East. Therefore non-sweet varieties of *chate* and *flexuosus* played a central role in primitive crop selection. Janick *et al.* (2007) collected several ancient representations of melon, mostly found in North Africa, which belong to these cucumber-like forms. The oldest representations, in ancient Egypt, were found in several tombs from the Old Kingdom (2686-2181 BC) (Keimer 1924), followed by others from the New Kingdom (1550-1300 BC) (Manniche 1989) and some wooden models almost coeval (1517-1192 BC) (Darby *et al.* 1977). The cultivation of these varieties, consumed unripe in salad, is mentioned by several ancient authors, as *Columella* (ca. 64 AD) and *Plinius the Elder* (ca. 77 AD). Although for long time these representations were considered as cucumbers (*Cucumis sativus*), they have recently identified as *flexuosus* types (Janick *et al.* 2007). *Chate* melon, unknown to the international market, has nowadays a limited diffusion in the Mediterranean basin. In Italy, it is traditionally cultivated only in a small area in the South of Apulia, where it is known as *Carosello*, *Meloncella* and *Cummarazzo* (Laghetti *et al.* 2008). Also in China written sources report the knowledge of melons at least since the 10th century BC. Melons are mentioned in *Shih-Ching* (Book of songs), editing attributed to *Confucius* (551–470 BC), which includes 305 traditional songs and poems of the Western Zhou dynasty (1046–771 BC) composed approximately between 1000 and 500 BC (Keng 1974). The melons mentioned in this work have been associated to sweet forms of *conomon* for which China is a secondary centre of diversification (Keng 1974, Walters 1989).

3.4 Conclusion

Both molecular and morphological analyses confirmed that Sardinian Late Bronze Age melon seeds from Sa Osa belong to a domesticated plant. This extinct primitive melon was probably an intermediate form between the two melon subspecies, *melo* and *agrestis*, likely close to varieties of *chate*, *flexuosus* and *ameri*. Specific genomic regions indicated non-sugar content of this fruit, which agree with the theory that non-sweet cucumber-like forms of *chate* and *flexuosus* melon played a central role in early selection. *Ameri*, 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 showed as well some affinity with the ancient materials. Both analyses suggested a relation of archaeological seeds with African landraces, which probably suffered less breeding process than European and Central Asian relatives, while a strong contribution of Near East and Central European ecotypes is also evident.

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Annex 1. 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 markers were experimentally validated and further information is available.

Marker name	LG	cM	Major Allele Frequency	Genotype n°	Allele n°	Gene Diversity	Heterozygosity	PIC
AI_09-F07	1	0	0,8185	3	2	0	0,021	0,253
CMPSNP1095	1	3,2	0,8390	3	2	0,270	0,089	0,234
CMPSNP83	1	18,1	0,5034	3	2	0,500	0,069	0,375
AI_17-E07	1	45,2	0,6233	3	2	0,470	0,068	0,359
CMPSNP711	1	46,8	0,5479	3	2	0,495	0,041	0,373
CMPSNP410	1	59,6	0,5034	3	2	0,500	0,069	0,375
F116	1	69,2	0,6301	3	2	0,466	0,068	0,357
AI_05-G01	1	72,4	0,8322	3	2	0,279	0,007	0,240
CMPSNP731	1	80,4	0,6918	3	2	0,426	0,055	0,336
CMPSNP204	1	86,8	0,7911	3	2	0,331	0,021	0,276
CMPSNP774	2	0	0,5274	3	2	0,498	0,041	0,374
CMPSNP431	2	4,8	0,5068	3	2	0,500	0,055	0,375
CMPSNP502	2	32,6	0,6276	3	2	0,467	0,041	0,358
CMPSNP1057	2	37,4	0,8288	3	2	0,284	0,027	0,244
AI_14-H05	2	40,6	0,6541	3	2	0,453	0,089	0,350
CMPSNP128	2	50,2	0,8356	3	2	0,275	0,027	0,237
CMPSNP246	2	53,4	0,7448	3	2	0,380	0,041	0,308
CMPSNP1003	2	58,2	0,7945	3	2	0,327	0,041	0,273
CMPSNP886	2	63	0,5925	3	2	0,483	0,062	0,366
CMPSNP658	2	77,9	0,6336	3	2	0,464	0,021	0,357
CMPSNP566	2	86	0,6301	3	2	0,466	0,027	0,357
CMPSNP94	2	90,9	0,7379	3	2	0,387	0,041	0,312
AI_18-E05	3	3,2	0,6448	3	2	0,458	0,076	0,353
CMPSNP275	3	4,8	0,6096	3	2	0,476	0,027	0,363
CMPSNP540	3	8	0,6507	3	2	0,455	0,041	0,351
CMPSNP165	3	24,3	0,5171	4	3	0,506	0,075	0,385
CMPSNP769	3	42,8	0,6575	3	2	0,450	0,027	0,349
CMPSNP164	3	46,2	0,6781	3	2	0,437	0,041	0,341
CMPSNP998	3	56,3	0,6370	3	2	0,462	0,041	0,356
CMPSNP595	3	61,1	0,7774	3	2	0,346	0,048	0,286
CMPSNP712	3	62,7	0,5308	3	2	0,498	0,062	0,374
CMPSNP480	4	0	0,6250	3	2	0,469	0,056	0,359
CMPSNP787	4	6,4	0,7363	3	2	0,388	0,062	0,313
CMPSNP1132	4	11,2	0,5377	3	2	0,497	0,048	0,374
PS_34-C02	4	17,6	0,6884	3	2	0,429	0,062	0,337
CMPSNP907	4	20,8	0,5959	3	2	0,482	0,041	0,366
CMPSNP264	4	32,1	0,5514	3	2	0,495	0,062	0,372
CMPSNP147	4	48,3	0,5548	3	2	0,494	0,082	0,372
AI_03-F03	4	53,1	0,6414	3	2	0,460	0,110	0,354
CMPSNP352	4	54,7	0,6610	3	2	0,448	0,062	0,348
CMPSNP852	4	62,7	0,6952	3	2	0,424	0,034	0,334
CMPSNP607	4	69,1	0,7671	3	2	0,357	0,027	0,293
CMPSNP677	4	77,1	0,6849	3	2	0,432	0,041	0,338
CMPSNP24	4	86,8	0,6586	3	2	0,450	0,062	0,349
PS_07-E07	4	101,5	0,5308	3	2	0,498	0,075	0,374
SC51-3375	4	114,6	0,8527	3	2	0,251	0,021	0,220
CMPSNP898	5	0	0,6130	3	2	0,474	0,103	0,362
CMPSNP387	5	18,5	0,6541	3	2	0,453	0,048	0,350
CMPSNP437	5	26,5	0,6747	3	2	0,439	0,034	0,343
CMPSNP726	5	41,2	0,8664	3	2	0,231	0,034	0,205
CMPSNP788	5	50,9	0,6336	3	2	0,464	0,021	0,357
SSH9G15	5	52,5	0,7324	3	2	0,392	0,056	0,315
60k41.243	5	73,4	0,5753	3	2	0,489	0,055	0,369
CMPSNP1155	5	79,8	0,6655	3	2	0,445	0,076	0,346
AI_13-H12	5	89,4	0,6747	3	2	0,439	0,048	0,343
CMPSNP735	5	94,2	0,7500	3	2	0,375	0,048	0,305
CMPSNP925	6	1,6	0,5799	3	2	0,487	0,063	0,369
CMPSNP218	6	8	0,6370	3	2	0,462	0,068	0,356
CMPSNP571	6	20,8	0,5345	3	2	0,498	0,076	0,374
CMPSNP1167	6	25,6	0,8459	3	2	0,261	0,048	0,227
CMPSNP433	6	32	0,7808	3	2	0,342	0,068	0,284
CMPSNP3	6	43,2	0,6690	3	2	0,443	0,041	0,345

CMPSNP292	6	49,6	0,5856	3	2	0,485	0,048	0,368
CMPSNP295	6	49,6	0,8241	3	2	0,290	0,090	0,248
CMPSNP1021	6	57,6	0,5034	3	2	0,500	0,075	0,375
CMPSNP1038	6	57,6	0,8507	3	2	0,254	0,035	0,222
A_38-F04	6	70,7	0,5690	3	2	0,490	0,062	0,370
AI_13-F02	6	85,3	0,5207	3	2	0,499	0,048	0,375
CMPSNP378	6	86,9	0,6747	3	2	0,439	0,021	0,343
AI_05-F11	7	4,9	0,5000	3	2	0,500	0,048	0,375
CMPSNP249	7	11,3	0,6575	3	2	0,450	0,068	0,349
CMPSNP262	7	30,5	0,6815	3	2	0,434	0,048	0,340
CMPSNP579	7	30,5	0,5137	3	2	0,500	0,055	0,375
CMPSNP1009	7	32,1	0,8931	3	2	0,191	0,048	0,173
CMPSNP287	7	35,3	0,7034	3	2	0,417	0,028	0,330
CMPSNP56	7	43,3	0,6610	3	2	0,448	0,075	0,348
CMPSNP465	7	59,4	0,6918	3	2	0,426	0,027	0,336
CMPSNP415	7	72,2	0,6404	3	2	0,461	0,021	0,355
CMPSNP12	8	0	0,6379	3	2	0,462	0,062	0,355
CMPSNP766	8	4,8	0,6610	3	2	0,448	0,034	0,348
CMPSNP718	8	11,2	0,6207	3	2	0,471	0,041	0,360
CMPSNP97	8	19,2	0,6438	3	2	0,459	0,397	0,353
CMPSNP44	8	22,4	0,7295	3	2	0,395	0,034	0,317
AI_21-D08	8	28,8	0,5149	3	2	0,500	0,015	0,375
CMPSNP181	8	35,2	0,6146	3	2	0,474	0,007	0,362
F013	8	48,1	0,6852	3	2	0,431	0,052	0,338
PSI_25-H03	5/8	59,4	0,5582	3	2	0,493	0,034	0,372
CMPSNP1066	8	79,2	0,6389	2	2	0,461	0,000	0,355
CMPSNP553	9	0	0,5274	3	2	0,498	0,068	0,374
CMPSNP173	9	3,2	0,5479	3	2	0,495	0,055	0,373
P5.64	9	8	0,6541	3	2	0,453	0,075	0,350
CMPSNP1077	9	19,2	0,6336	3	2	0,464	0,007	0,357
CMPSNP320	9	20,8	0,7637	3	2	0,361	0,048	0,296
CMPSNP144	9	22,4	0,6438	3	2	0,459	0,027	0,353
CMPSNP1035	9	33,6	0,6438	3	2	0,459	0,055	0,353
CMPSNP159	9	36,8	0,7207	3	2	0,403	0,034	0,322
CMPSNP1133	9	59,2	0,5616	3	2	0,492	0,096	0,371
CMPSNP890	9	64	0,5420	3	2	0,496	0,063	0,373
psi36-10864	10	0	0,6268	3	2	0,468	0,070	0,358
psi36-839	10	0	0,6172	3	2	0,473	0,048	0,361
CMPSNP172	10	1,6	0,7705	3	2	0,354	0,034	0,291
CMPSNP528	10	8	0,5959	3	2	0,482	0,068	0,366
CMPSNP65	10	14,4	0,7808	3	2	0,342	0,055	0,284
CMPSNP762	10	23,9	0,6910	3	2	0,427	0,049	0,336
CMPSNP671	10	28,8	0,5724	3	2	0,490	0,124	0,370
CMPSNP550	10	38,5	0,6747	3	2	0,439	0,349	0,343
CMPSNP426	11	0	0,6541	3	2	0,453	0,034	0,350
HS_35-E11	11	16,4	0,7363	3	2	0,388	0,075	0,313
PSI_41-B07	11	27,6	0,7089	3	2	0,413	0,021	0,328
CMPSNP389	11	47,7	0,6138	3	2	0,474	0,207	0,362
CMPSNP30	11	66	0,6517	3	2	0,454	0,062	0,351
CMPSNP315	11	90,9	0,7188	3	2	0,404	0,035	0,323
CMPSNP475	11	98,9	0,6418	3	2	0,460	0,035	0,354
CMPSNP122	11	100,5	0,6473	3	2	0,457	0,034	0,352
CMPSNP385	12	4,8	0,8000	3	2	0,320	0,041	0,269
CMPSNP310	12	9,8	0,5171	3	2	0,499	0,034	0,375
AI_35-A08	12	16,4	0,5034	3	2	0,500	0,021	0,375
AI_09-G07	12	18,1	0,8028	3	2	0,317	0,127	0,266
CMPSNP285	12	21,4	0,9212	3	2	0,145	0,034	0,135
CMPSNP361	12	37	0,5414	3	2	0,497	0,062	0,373
CMPSNP5	12	58,1	0,7655	3	2	0,359	0,028	0,295
FR14F22	12	67,7	0,5655	3	2	0,491	0,041	0,371
P02.03	12	69,3	0,6267	3	2	0,468	0,075	0,358

CHAPTER 5

MEDIEVAL MELON AND WATERMELON SEEDS FROM SASSARI (ITALY): MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION

MEDIEVAL MELON AND WATERMELON SEEDS FROM SASSARI (ITALY):

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION

**Diego Sabato¹, Belén Picó², Oscar Grillo^{1,3}, Cristina Esteras², Leonor Peña-Chocarro^{4,5},
Giovanna Bosi⁶, Gianluigi Bacchetta¹**

¹ *Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Italy.*

² *Centro de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Universitat Politècnica de València, Spain.*

³ *Stazione Consorziale Sperimentale di Granicoltura per la Sicilia (SSGS), Caltagirone, Italy.*

⁴ *Escuela Española de Historia y Arqueología en Roma-CSIC, Rome, Italy*

⁵ *GI Arqueobiología, Instituto de Historia, CCHS-CSIC, Madrid, Spain.*

⁶ *Laboratorio di Palinologia e Paleobotanica, Dip. di Scienze della Vita, Università di Modena e Reggio Emilia, Modena, Italy.*

Abstract

In 2007, during an archaeological excavation in the city centre of Sassari (Italy), a well dated 1330-1350/60 AD was discovered. The fill of this context yielded a huge assemblage of ceramics, zoological and plant remains. Among the 117 plant *taxa* identified, a consistent presence of melon and watermelon seeds was recorded. Their exceptional state of preservation allowed the application of specific analyses for molecular and morphological characterization.

Sets of 172 accessions of melon and 36 watermelon landraces from Europe, Asia and Africa, included Sardinian traditional cultivars, were selected for molecular and morphological comparison to the archaeological seeds. Genotyping was performed using 211 polymorphic SNPs and the genotyping technology iPLEX® Gold MassARRAY Sequenom. A total of 95 morphometric parameters, acquired by an automatic image analysis system, were specifically designed to evaluate seeds size and shape.

Results from the molecular and morphological analyses showed a good match between the archaeological seeds and domesticated melons with both sweet and non-sweet landraces. The analyses suggested that several types of melon were already cultivated in the Medieval period although sugary melons became probably widely diffused only in later phases. The morphological characterization of watermelon evidenced a close relation with Sardinian, Spanish and Asian landraces, suggesting that this fruit was already close to modern varieties.

Key words: *Cucumis melo*, *Citrullus lanatus*, ancient DNA, morphological analysis, molecular analysis, archaeobotany

5.1 Introduction

5.1.1 Historical and archaeological context

The development of the city of Sassari, today the second largest city in Sardinia (Italy), took place during the 12th and 13th century AD, when it became the main centre for inland and foreign trade of a wide variety of products (Rovina and Flori 2013). In 1272, the city fell under the control of the Republic of Pisa and later, in 1294, under that of the Republic of Genoa (Rovina and Flori 2013). In 1323, citizens spontaneously decided to become part of the Aragon kingdom of King James II (Porcu Gaias 1996). This alliance did not last long, since a few years later, between 1330 and 1331 AD, the continuous insurrections of the city led the Aragonese to the expulsion of some local citizens which were soon replaced by colonists from Catalonia, Aragon, Valencia and Tarragona (Pala 1980).

During the renovation of via Satta road, in 2007, in the core of the city-centre of Sassari, a Medieval well belonging to this period was discovered. It was originally part of an open area or domestic courtyard which has been dated between 1330-1350/60 AD according to the typology of majolica fragments from Pisa, Savona, and Valencia widely diffused in this period (Biccone 2013). The sediment appeared very rich in waterlogged plant remains. Wood remains were studied at the University of Sassari (Becca *et al.* 2013) while seeds and fruits were studied by the Laboratorio di Palinologia e Paleobotanica of University of Modena and Reggio Emilia. A total of 880,000 items, mainly fruits of 117 *taxa* have been identified (Bertacci 2011/2012; Bosi and Bandini Mazzanti 2013). A significant number of melon and watermelon seeds were recovered (respectively 1964 and 116 seeds); their excellent degree of preservation made of many of these remains perfect candidates for molecular and morphological analysis.

5.1.2 Melon

Cucumis melo L. has gone through an intense process of diversification and several groups and varieties have been reported (Naudin 1859, Munger and Robinson 1991, Pitrat *et al.* 2000, Pitrat 2008). This *taxon* diverges in two subspecies, *melo* and *agrestis*, and the main varieties are: *cantalupensis* Naudin, *reticulatus* Ser. (cantaloupe, muskmelon), *inodorus* H. Jac. (winter melon, casaba melon), *flexuosus* L. (snake melon), *chate* Hasselq. (cucumber melon), *ameri* Pangalo, *dudaim* L. (pocket melon) to subsp. *melo*; and *agrestis* Naudin, *chito* C.Morren, *tibish* Mohamed, *acidulus* Naudin, *conomon* Thunb., *makuwa* Makino, *chinensis* Pangalo (pickling melon), and *momordica* Roxb. (snap melon) within the subsp. *agrestis*. Nowadays, classification is mainly based on molecular clustering (Esteras *et al.* 2012, 2013). Wild melons (*C. melo* subsp. *agrestis* var. *agrestis*) are distributed on the tropical and sub-tropical belt from Africa and Asia and show a great genetic variability. There are numerous previously overlooked species-level relatives in Australia and around the Indian Ocean (Sebastian *et al.* 2012). Renner *et al.* (2007) suggested that their origin-distribution centre include the Australia-Malaysia area as melon is closer to the Australian–Asian group than to the

African species. Due to the high level of variation found in Asia, especially in India, melon could have originated in this area and then reached Africa (Renner *et al.*, 2007; Sebastian *et al.*, 2010). Other theories suggest that African and Asian melons may have diversified independently (Bates and Robinson 1995, Jeffrey 1980, Esquinas-Alcázar and Gulick 1983, Mallick and Mausi 1986, Tanaka *et al.* 2013). A genetic relationship between South African and East Asian melons was found by Nakata *et al.* (2005).

One of the oldest archaeological records of melon in Europe has been found in Sardinia, being likely a cucumber-like type (Sabato *et al.* 2015 [**Chapter 1**] and [**Chapter 4**]). Non-sweet forms of melon, often confused with *Cucumis sativus* (cucumber), were probably the first melons known in the Near East, North Africa and the South-West Mediterranean (Zohary *et al.* 2012, Janick *et al.* 2007), while modern market sweet melons became widely diffused in Europe only in the Renaissance period (Paris *et al.* 2012). Sugary melon was probably introduced into Italy and neighbouring countries in the late 15th century (Pitrat *et al.* 2000, Jeffrey 2001, Goldman 2002), and its cultivation rapidly expanded to large parts of Europe and then to the Americas, becoming very popular as it is suggested by the representation of cantaloupes and muskmelons in painted festoons of the luxury palace of Villa Farnesina (1515-1518 AD) near Rome (Janick and Paris 2006). In Ferrara (North Italy), melon seeds were found in several sites (Bandini Mazzanti *et al.* 2005, 2009), including a pit dated to the second half of 15th century located in the Ducal Palace; these remains showed a significant bigger size compared to other late 14th century seeds discovered in the same area, pointing out a different crop typology (Bosi *et al.* 2009).

5.1.3 *Watermelon*

Citrullus lanatus (Thunb.) Matsum & Nakai (watermelon) is divided into three subspecies: subsp. *vulgaris* (Schrad. ex Eckl. et Zeyh.), subsp. *mucospermus* Fursa, and subsp. *lanatus*, which includes the var. *caffer* (Schrad.) Mansf. ex Fursa, only spread in the Kalahari desert, and var. *citroides* (Bailey) Mansf. ex Greb. (Jeffrey 2001). Although some authors considered *C. colocynthis* (L.) Schrad., or *C. lanatus* var. *caffer* (Jeffrey 2001, Navot and Zamir 1987) or var. *citroides* (Maynard 2001) the wild ancestors of modern watermelon, genetic analysis showed that watermelon and the citron type (var. *citroides*) diverged into separate lineages which independently evolved from a common ancestor, possibly *C. ecirrhosus* (Dane and Liu 2007). *C. colocynthis* is a perennial (rarely annual) wild species growing on sandy habitats in desert and semi-desert areas of North Africa, the Near East and South-West Asia as far as India (Jeffrey 2001).

It is generally accepted that watermelon originated in Africa in the Kalahari Desert (Esquinas-Alcázar and Gulick 1983), where it was growing at least since the beginnings of the fourth millennium BP (Zohary *et al.* 2012). The earliest records of watermelon were found in Egypt during the second millennium BC (Hepper 1990) and in Sudan (van Zeist 1983). However, Wasylikowa and Van der Veen (2004) suggest that some of the seeds from Egyptian sites may have been erroneously identified

as different *Citrullus* species being most likely *C. lanatus* seeds. Cox and Van der Veen (2008) reported further records from the first millennium BC such as those from South Arabia, Greece and Israel. In Europe, watermelon seeds have been found in several Punic [Chapter 2], and Roman sites (Castelletti *et al.* 2001, Rinaldi *et al.* 2013) and are widely recorded during Medieval times in Italy (Bosi *et al.* 2009), Hungary (Gyulai *et al.* 2011, 2012) and in Islamic contexts of the Iberian Peninsula, (López Garí and Marlasca 2009).

5.1.4 Introduction to the analyses

Archaeogenetics have strongly developed in the last two decades (Palmer *et al.* 2012). As hydrolysis is one of the major decay reactions (Pääbo *et al.* 2004, Schlumbaum *et al.* 2008, Oliveira *et al.* 2012), waterlogging does not appear to favour DNA preservation. However, waterlogged plant remains have been already used as a good source of ancient DNA (Schlumbaum *et al.* 1998, Manen *et al.* 2003, Elbaum *et al.* 2005, Pollmann *et al.* 2005, Gyulai *et al.* 2008, Speirs *et al.* 2009).

Morpho-colorimetric evaluation has been already carried out to the diaspores of several spontaneous plants, providing excellent results to characterize very close *taxa* at infra-generic and infra-specific levels (Bacchetta *et al.* 2008, Grillo *et al.* 2010). A previous research focused on grape varieties correlated molecular and seed morphology (Orrú *et al.* 2013a) and two further works correlated seed shape of Sardinian grape landraces and archaeological pips found in the same context of Sa Osa (Ucchesu *et al.* 2014, Orrú *et al.* 2013b).

Melon seed length has been already correlated to genetic and geographical differentiation among melon groups (Fujishita 1983). Tanaka *et al.* 2013 indicated that large-seed melon is mainly cultivated in USA, Europe, West and Central Asia and northern Africa, whereas in India both large- and small-seed melon are equally frequent, while small-seed is more common in southern Africa, South and East Asia melon. In Sabato *et al.* (in press) [Chapter 3] a strong correlation between molecular and seed characters of melon has been shown, and in [Chapter 4] ancient DNA extraction has been already successfully carried out on waterlogged Late Bronze Age melon seeds from Sardinia, together with morphological seed characterization.

Basing on this wide research background, the aims of present work are:

- Extracting and genotyping ancient DNA of Medieval melon seeds from Sassari;
- Acquiring morphological features of melon seeds by an image analysis system;
- Acquiring morphological features of watermelon seeds from the same context;
- Comparing molecular and morphological data with modern worldwide melon and watermelon landraces.

5.2 Materials and methods

5.2.1 Seed lots details

The starting material was a core collection of 212 melon accessions established on the framework of a previous project (MELRIP 2007-2010, Esteras *et al.* 2012, 2013), genotyped with AFLP and SNPs markers, and extensively phenotyped for plant and fruit traits. Only accessions belonging to traditional landraces were considered for the present research for a total of 172 seed lots (145 for molecular and 115 for morphological analysis) from 44 different countries [Annex C1]. Further 36 watermelon and colocynth accessions have been used only for morphological analysis [Annex 1].

Accessions have been mainly provided by USDA (United States Department of Agriculture) and COMAV (Centro de la Conservación y Mejora de la Agrodiversidad Valenciana) germplasm bank, and, to a lesser extent by Cyprus and Bari germplasm banks, and the Cagliari Botanical Gardens. Sardinian landraces, mostly described in Attene and Rodriguez (2008), have been supplied by the Agriculture Department of University of Sassari, AGRIS (Agenzia per la Ricerca in Agricoltura della regione Sardegna) and local farmers.

Archaeological seeds have been identified and dried at the Laboratorio di Palinologia e Paleobotanica of University of Modena and Reggio Emilia. A total of 196 full preserved melon (Fig. 1a) and 70 watermelon seeds (Fig. 1b) have been selected for morphological analysis. Broken or hard-distorted samples were used for DNA extraction.

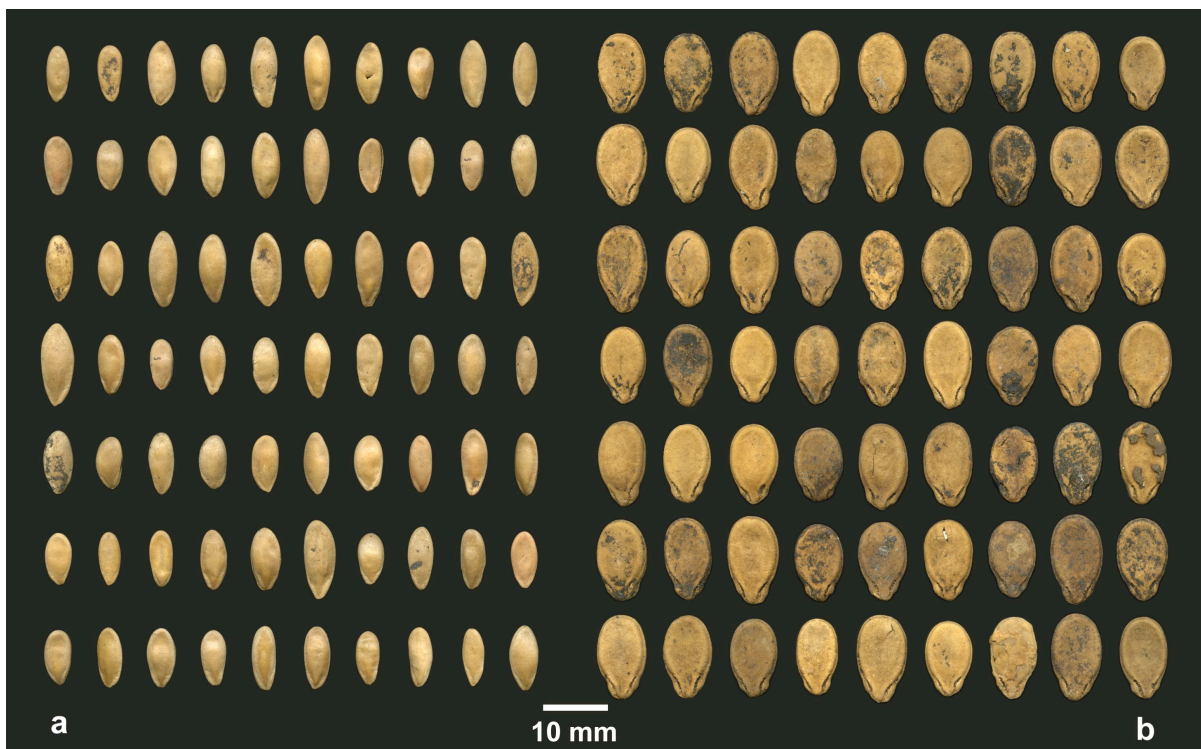


Figure 1. Medieval seeds from Sassari: (a) melon, *Cucumis melo*; (b) watermelon, *Citrullus lanatus*.

5.2.2 *Molecular analysis*

DNA extraction from archaeological samples followed a special procedure to avoid contamination. DNeasy Plant Mini Kit (Qiagen) was used and its protocol was followed with minor changes (more time with the initial buffer). All the tools involved (tubes, gloves, blades, etc.) have been UV irradiated for 12 hours and previously autoclaved if not disposable (like pliers and steel beads). The archaeological material was never manipulated in rooms where modern cucurbits were being treated and extractions were carried out in a sterile flow-hood chamber. Seed surfaces were cleaned out with a solution of 10% Ca(OCl)₂ w/v for one minute. Five samples were selected, three with a single seed, one with three seeds and a third with seed fragments. 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). Only two samples yielded a minimum amount of DNA for quantifying and genotyping, the sample with three seeds, named Archaeo1, and the one with seed fragments, named Archaeo2.

A total of 123 SNPs 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 total of 38,587 SNPs that were in silico identified in two previous re-sequencing analysis (Blanca *et al.* 2011, Blanca *et al.* 2012). The most important one (Blanca *et al.* 2012) re-sequenced 67 genotypes, grouped into 8 pools that represent all the botanical groups of the species, for mining for SNPs. These polymorphisms, identified by the alignment of the sequences to the reference transcriptome, represent the largest collection existing for melon. This transcriptome, the most complete version to date, was created by Blanca *et al.* (2012) using a combination of expressed sequence tags (ESTs) from classical Sanger sequencing and the next generation sequencing (NGS) methods, e.g. 454 (Roche) and SOLID (Life Technologies Inc.).

Information about the SNPs used is available in **Annex 2** and detailed information for each SNP marker, such as sequence, allele variation and location is available in the Melogene database. Most SNPs used in this study were employed in previous mapping experiments and their position in the genetic map is known (Esteras *et al.* 2013). Genotyping with this set of markers was performed using iPLEX® Gold MassARRAY Sequenom technology, carried out at the epigenetic and genotyping unit of the University of Valencia (Unitat Central d'Investigació en Medicina UCIM). This genotyping technology relies on Single Base Extension (SBE) using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest to generate different allelic products. Using the MALDI-TOF mass spectrometry, the distinct mass of the extended primer identifies the SNP allele (Gabriel *et al.* 2009). The genotyping results were employed to perform a cluster analysis using the PowerMarker software (Liu and Muse 2005). Nei's genetic distance (Nei *et al.* 1983) was used, and the support values for the degree of confidence at the nodes of the dendrogram were analysed by bootstrap re-sampling 1,000 times. Phylip 3.69 software (Felsenstein 1997) was employed to construct the consensus tree and

TreeView32 (Page 1996) to visualize it. The Principal Coordinate Analysis (PCoA) was performed using GenAlEx 6.501. In addition, 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).

5.2.3 Morphological analysis

The process follows the same protocol described in Sabato *et al.* (in press) [Chapter 3] and [Chapter 4]. Images were acquired using a flatbed scanner, with a resolution of 400 dpi, 24 bit-depth and a scanning area not exceeding 1024×1024 pixel. As suggested by Venora *et al.* (2007), before acquiring the image, the scanner was calibrated for colour matching, following the protocol of Shahin and Symons (2003). Digital images of seeds, randomly disposed on the flatbed tray, were acquired and used for the analysis. Two images were acquired for each sample of seeds, with black and white background. Digital images of seeds were analyzed using the software package KS-400 V. 3.0 (Carl Zeiss, Vision, Oberkochen, Germany). The accuracy and speed of measurements was maximized by running an automated macro, specifically developed for the characterization of seeds (Bacchetta *et al.* 2008; Grillo *et al.* 2010). Considering that seed colour is altered in the archaeological seeds, colour and texture have been not considered in this research, but in order to increase the number of discriminant parameters, the Elliptic Fourier Descriptors (EFDs) were also computed, as described by Orrù *et al.* (2013a), to accurately describe the shape of the analysed seeds. A total of 95 parameters, describing seed size and shape, were computed (Tab. 1). Data were statistically elaborated applying the stepwise LDA (Linear Discriminant Analysis).

Shape parameters	
A	Area (mm ²)
P	Perimeter (mm)
Pconv	Convex Perimeter (mm)
PCrof	Crofton's Perimeter (mm)
Pconv/PCrof	Ratio between convex and Crofton's perimeters
Dmax	Maximum diameter of the seed (mm)
Dmin	Minimum diameter of the seed (mm)
Dmin/Dmax	Ratio between minimum and maximum diameters
Sf	Shape Factor = $(4 \times \pi \times \text{area}) / \text{Perimeter}^2$ (normalized value)
Rf	Roundness Factor = $(4 \times \pi \times \text{area}) / \text{max diameter}^2$ (norm. value)
Ecd	Diameter of a circle with an area equivalent to the seed (mm)
EAmx	Maximum axis of an ellipse with equivalent area (mm)
EAmn	Minimum axis of an ellipse with equivalent area (mm)
Cpt	Compact grade = $(\sqrt{2} (4/\pi) \times \text{area}) / \text{Dmax}$
C	Curl = ratio between maximum diameters and Fiber lengths
Fl	Fiber length (mm)
Cvx	Convexity = ratio of Crofton's Perimeters and real Perimeters
78 EFDs	Elliptic Fourier Descriptors

Table 2. List of characters analyzed in morphological analyses.

We selected five main groups of varieties to compare the archaeological seeds: the Sweet melon group (= SWG), all sweet melon varieties of subspecies *melo*: var. *ameri*, *inodorus*, *cantalupensis*, *reticulatus* and indeterminate landraces of subsp. *melo*; the Intermediate group (= ING), non sweet melons with intermediate characteristics between the two melon subspecies: var. *dudaim*, *chate*, *flexuosus* and *momordica*; the African *agrestis* group (= AFG), African *acidulus*, *tibish* and the two African indeterminate landraces of subsp. *agrestis*; the Conomon group (= COG), all sweet and semi-sweet melons of subspecies *agrestis* diffuse in Far East: var. *conomon*, *chinensis*, *makuwa* and Asian *acidulus*: and the Wild types group (= WTG), wild and semi-wild melons: var. *agrestis* and *chito*.

5.3 Results

5.3.1 Melon molecular analysis

In **Annex C2** genotyping data are presented. In Archaeo1 seven *loci* were not amplified (CMPSNP83, CMPSNP731, CMPSNP1066, CMPSNP144, CMPSNP30, CMPSNP5 and FR14F22), while the number of non amplified *loci* in Archaeo2 (48) were rather high due probably to the low amount of DNA of this sample. A high number of *loci* in both samples (56 in Archaeo1 and 25 in Archaeo2) were heterozygous.

PCoA analysis is shown in Figure 2a (functions 1 and 2), Figure 2b (functions 1 and 3) and Figure 2c (3D representation). In all graphs, the archaeological seeds were grouped together with accessions of the subspecies *melo* and at some distance from those of the subspecies *agrestis*. These results clearly show that the medieval seeds belong to a cultivated melon of subspecies *melo* to a certain distance to modern landraces in Archaeo1 and closer in Archaeo2. The closest accessions to Archaeo1 were two *ameri* accessions, one sugary melon from Russia (AmRU42) and another low-sweet type from Morocco (AmMA37). Several snake-melons (var. *flexuosus*) from India, Near East and North-East Africa (FxIN20, FxIQ24, FxSA188, FXSD186), and a cucumber-melon (var. *chate*) from Italy (ChIT27, ChIT122) were also close. Archaeo2 sample was close to the above-mentioned accessions plus further snake-melons from Turkey (FxTR86), Spain (FxES82) and Italy (FxITS9) and several sweet *ameri*, as AmTN86, AmIR26, AmIR183 and AmITS10, from Tunisia, Iran and Italy. Other accessions, as *conomon* from China (CoCH125), *cantalupensis* from France (CaFR121, CaFR191), *inodorus* from Portugal (InPT40) and two indeterminate landraces from Italy and Mali (LaIT00, LaML35) belonged to the same area.

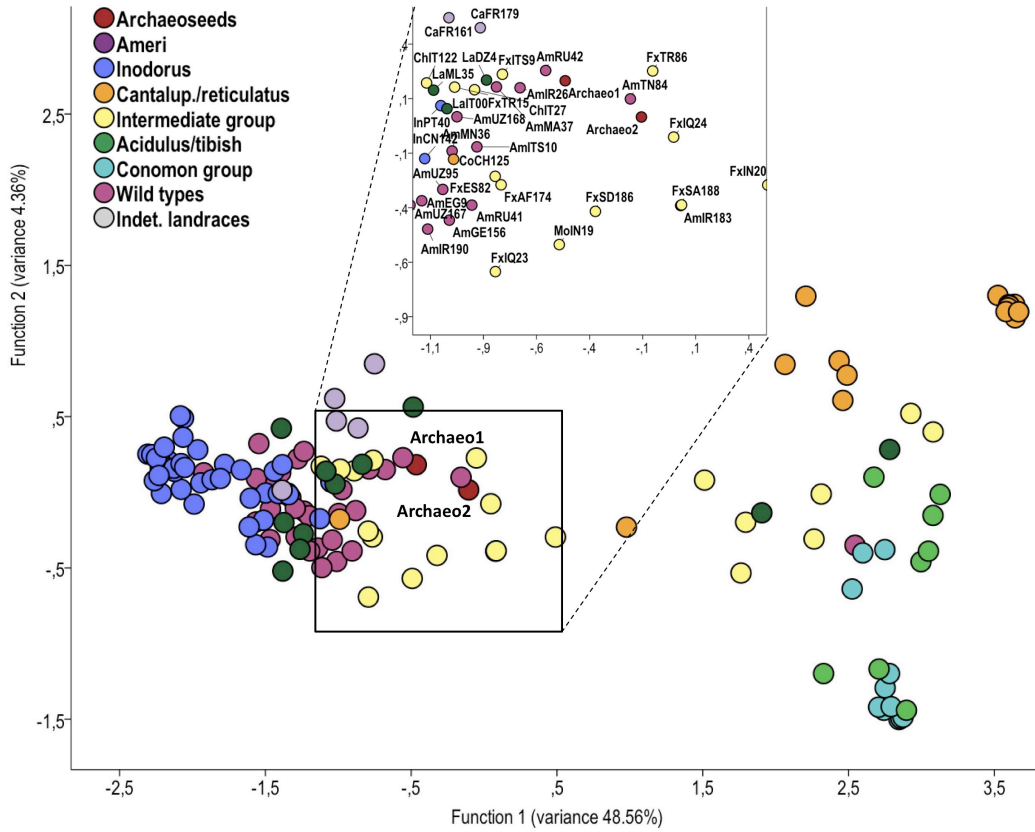


Figure 2a. PCoA analysis of the molecular comparison of archaeological seeds and the modern melon collection. The graph shows functions 1 and 2 corresponding to axis X and Y respectively.

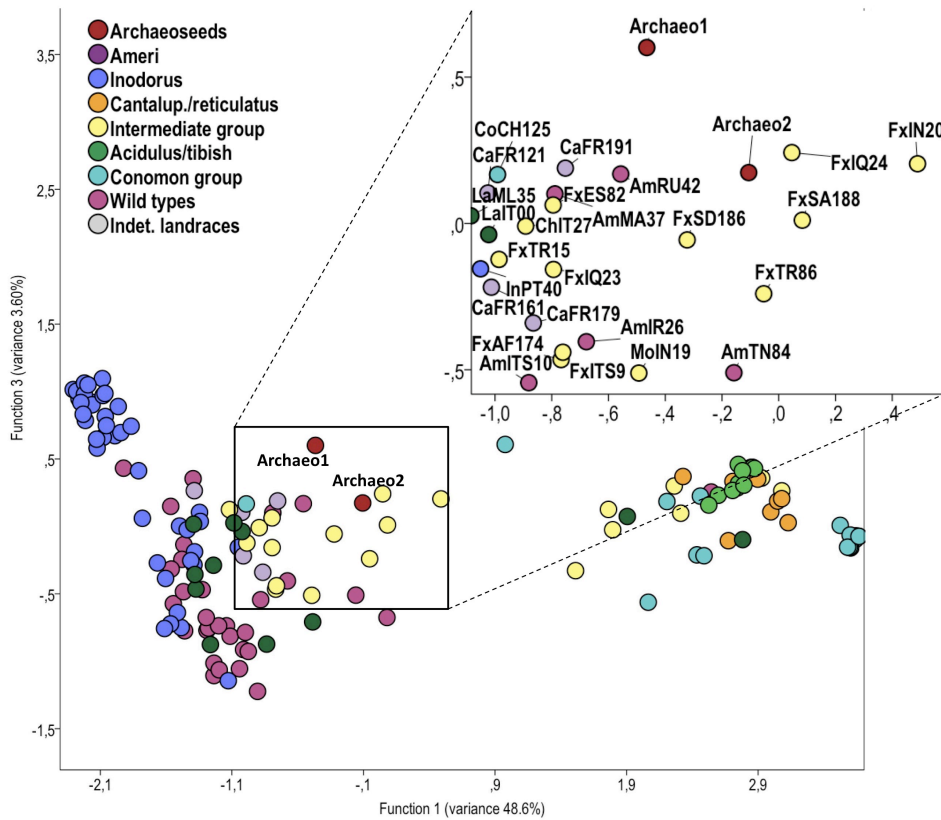


Figure 2b. PCoA analysis of the molecular comparison of archaeological seeds and the modern melon collection. The graph shows functions 1 and 3 corresponding to axis X and Y respectively.

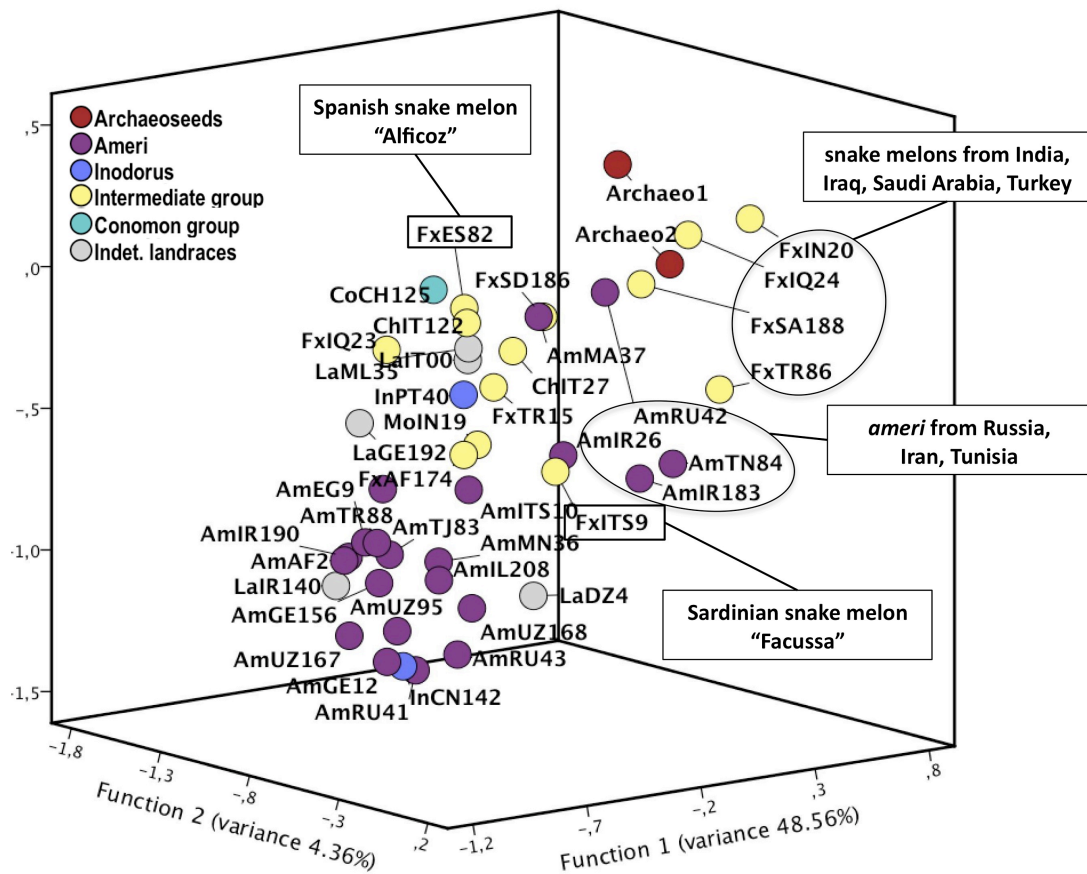


Figure 2c. PCoA analysis of the molecular comparison of archaeological seeds and the modern melon collection. The graph shows 3D representation of the closer accessions to the archaeological seeds.

5.3.2 Melon morphological analysis

A first morphological comparison between the 196 archaeological melon seeds, considered as an unknown group, the two cultivated melon subspecies and the wild melons was carried out (Tab. 2). Among the total of 11,374 seeds, 92.6% was correctly classified. The subsp. *melo* and the wild melons were correctly classified in 98.8% and 95.4% of the cases respectively, while subsp. *agrestis* overlapped in 21.1% of the cases with subsp. *melo*. None of the archaeological seeds was grouped with the wild melons, while 169 seeds (86.2%) were classified as subsp. *melo* and 27 seeds (13.8%) were grouped with the cultivated subsp. *agrestis*.

	subsp. <i>melo</i>		subsp. <i>agrestis</i>		wild melon		total	
	%	n°	%	n°	%	n°	%	n°
subsp. <i>melo</i>	98.8	8072	1.2	96	-	-	100.0	8168
subsp. <i>agrestis</i>	21.1	539	71.8	1830	7.1	181	100.0	2550
wild melon	-	-	4.6	30	95.4	626	100.0	656
Archaeo	86.2	169	13.8	27	-	-	100.0	196

- 92.6% overall classification

Table 2. LDA analysis results comparing the archaeological seeds with the two melon subspecies and the wild melon.

The comparison among the five main groups described in the *Materials and methods* section and the archaeological seeds is reported in table 3. Overall correct classification reached the 81.3% with picks of 93.4% and 88.2% for the SWG and WTG. Most of the Medieval seeds have been classified as belonging to the ING (102 seeds, 52.0%) and SWG (72 seeds, 36.7%), but it should be considered that more than half of the seeds of ING (930 seeds, 55.1%) were classified as belonging to SWG. The few remaining archaeological seeds have been assigned to AFG (19 seeds, 9.7%) and COG (3 seeds, 1.5%). None of the archaeological seeds has been classified as wild melon.

	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	757
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	627
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
ARC	36.7	72	52.0	102	9.7	19	1.5	3	-	-	100.0	196

- 81.3% overall classification

Table 3. Results from the LDA analysis comparing the archaeological seeds (ARC) to variety groups with similar morphological characteristics. For code details see *Materials and methods*.

The scatter-plot graphs of Figure 3a and 3b show accession distribution considering each accession as an independent group. All archaeological seeds and the mean of accessions coordinate (centroids) are represented. Archaeological seeds showed rather large distribution, indicating high variability. Closest accessions to archaeoseeds centroid were in most cases accessions of subspecies *melo* both sweet and non-sweet: *flexuosus* from Turkey and Italy (FxTR21, FxTR86, FxITS9), *dudaim* from Georgia and Afghanistan (DuGE296, DuAF1), *ameri* from Ukraine and Germany (AmUA90, AmDE166), *reticulatus* from Libya, *cantalupensis* from France (CaFR172), *inodorus* from Spain and Turkey (InES80, InES55, InES61, InTR104). Some accessions belonging to subspecies *agrestis* (*chinensis* from Japan, CnJP207; *acidulus* from Senegal, AcSN46) together with some indeterminate landrace of the two subspecies (LaFR151, LaMG202) were also close to the average of medieval seed features.

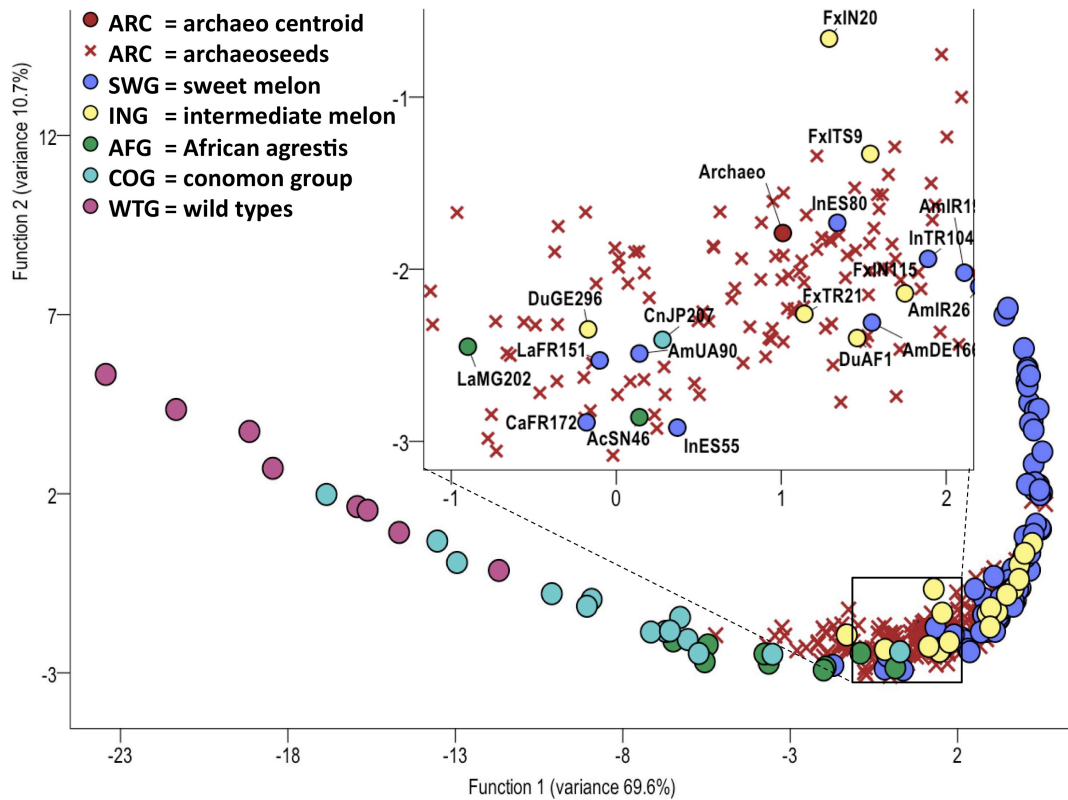


Figure 3a. LDA analysis results from morphological comparison between the archaeological melon seeds and the modern collection. Only accession centroids are represented. The graph shows functions 1 and 2 corresponding to axis X and Y respectively. For code details see *Materials and methods*.

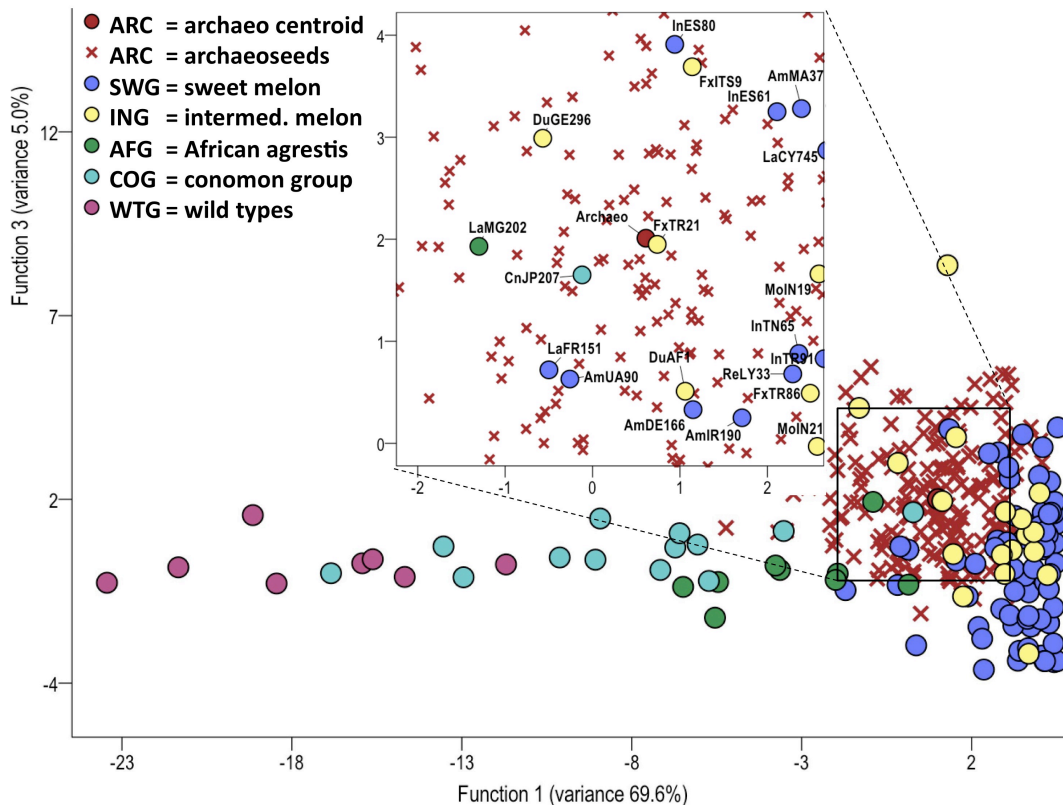


Figure 3b. LDA analysis results of the morphological comparison between the archaeological melon seeds and the modern collection. Only accession centroids are represented. The graph shows functions 1 and 3 corresponding to axis X and Y respectively. For code details see *Materials and methods*.

5.3.3 Watermelon morphological analysis

The 70 archaeological watermelon seeds were compared to 1,199 seeds of modern *Citrullus lanatus* landraces (1,712 of *C. lanatus* var. *lanatus* and 487 of *C. lanatus* var. *citroides*) and 1,039 seeds of *Citrullus colocynthis* (Table 4). Correct classification of these three groups was very high with a 93.8% of correct identification. Misclassification among groups never exceeded 6.4%. None of the archaeological seeds has been classified as *Citrullus colocynthis*, while 67 seeds (95.7%) were classified as var. *lanatus* and only 3 seeds (4.4%) as var. *citroides*.

	<i>Citrullus colocynthis</i>		<i>C. lanatus</i> var. <i>lanatus</i>		<i>C. lanatus</i> var. <i>citroides</i>		total	
	%	n°	%	n°	%	n°	%	n°
<i>Citrullus colocynthis</i>	89.1	926	8	83	2.9	30	100.0	1039
<i>C. lanatus</i> var. <i>lanatus</i>	0.7	12	97.3	1665	2.0	35	100.0	1712
<i>C. lanatus</i> var. <i>citroides</i>	1.8	9	6.4	31	91.8	447	100.0	487
Archaeo	-	-	95.7	67	4.3	3	100.0	70

- 93.8% overall classification

Table 4. LDA analysis results comparing archaeological seeds with watermelon landraces and wild colocynthis.

Figure 4a and 4b show distribution of accessions considering each as an independent group. Traditional Sardinian landraces showed high affinity with the archaeological seeds, mainly accessions LnITS3 (a yellow flash watermelon from the southern island of Sant'Antioco), LnITS1 and LnITS5 (both from Benetutti, near Sassari) and LnITS2 (from Gonnosfanadiga, SW Sardinia). Also, accessions from southern Spain (LnES65), central Asia, Uzbekistan (LnUZ78) and Kyrgyzstan (LnKZ81) were close to the medieval seeds.

5.4 Discussion

5.4.1 Melon

Melons have been recorded in Sardinia since the Late Bronze Age (Sabato *et al.* 2015 [Chapter 1]) being probably a non-sweet type [Chapter 3]. Janick *et al.* (2007) have highlighted the lack of clear indications of the presence of sugary melons comparable in quality to the modern sweet melon in the Mediterranean basin during classical times. Pitrat *et al.* (2000) hypothesized that a primitive *ameri* melon, diffused from the Near East and Central Asia, was the ancestor of modern sugary varieties. Paris *et al.* (2012) reported that the earliest clear evidence of sweet melon comes from the 9th century from Central Asia from the Khorasan region, an area extending across Turkmenistan, Uzbekistan, Afghanistan, Tajikistan, Iran and eastern Iraq. Several accessions from this area showed molecular and morphological affinity with the medieval seeds. In addition, Paris *et al.* (2012), suggest that the first description of sugary melons in Europe, most probably a *casaba* type (var. *inodorous*), comes from Andalusia (southern Spain) and it is dated to the late 11th century during the Islamic domination of Spain. They also indicate that the spread of sweet melons from Central Asia to Western Europe was the consequence of the Islamic conquest, a period of intense trade and agricultural development. Archaeological *Cucumis melo/sativus* seeds were found abundant in the Islamic contexts (8th-12th century AD) of the city of Lleida, Catalonia (Alonso Martínez 2005).

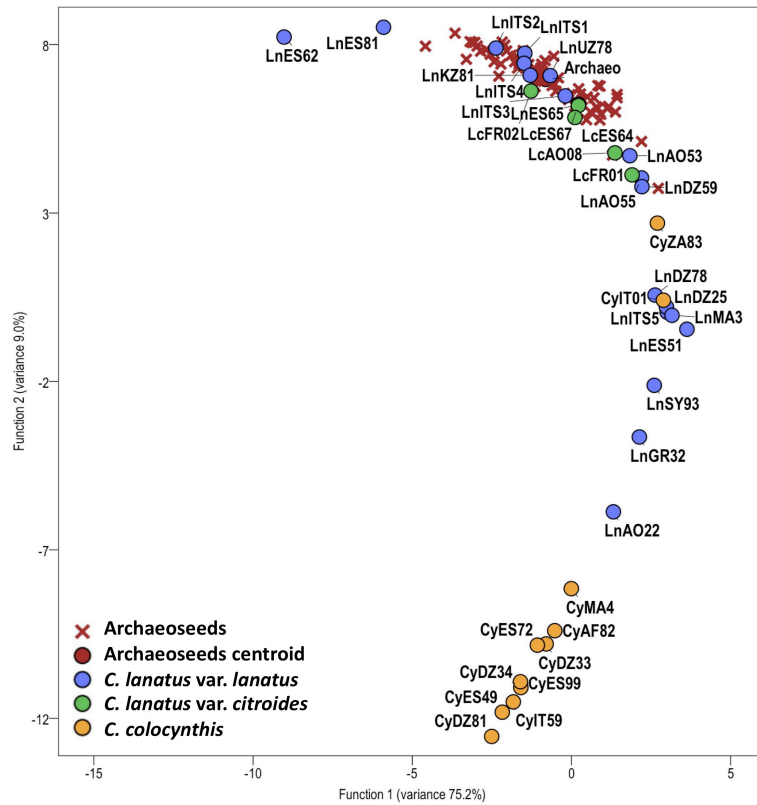


Figure 4a. LDA results from the morphological comparison between archaeological watermelon seeds and the modern collection. Only accession centroids are represented. Functions 1 and 2 correspond to axis X and Y respectively.

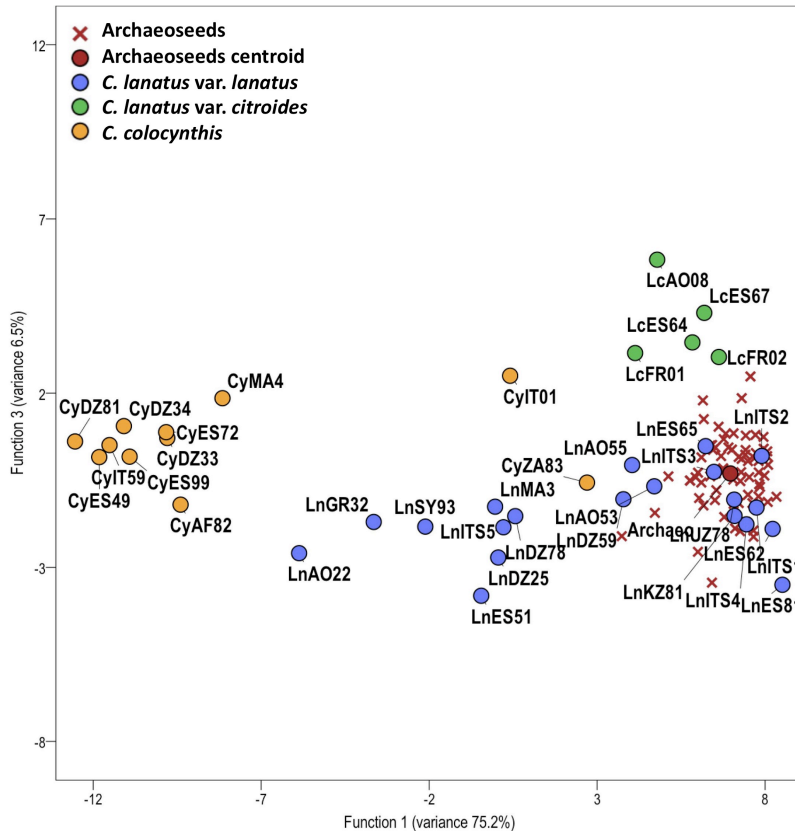


Figure 4b. LDA results from the morphological comparison between archaeological watermelon seeds and the modern collection. Only accession centroids are represented. Functions 1 and 3 correspond to axis X and Y respectively.

The spread of sweet melon during Medieval times may have been limited by farmer's lack of knowledge about its cultivation. Even if the cultivated genotypes have the potential for sugar accumulation, growing sweet melons is hard in soils with excessive humidity and under low temperatures (Pitrat 2008). This may be one of the reasons because melons from Central Asia moved first to regions of similar hot and semi-arid climate as Andalucía, but had more difficulties to spread in Central Europe. Cantaloupes and muskmelons depicted in Villa Farnesina (Roma, 1515-1518 AD) show the typical features of over-watering effects, being cracked and puckered, suggesting that by the 15th century local farmers did not yet fully controlled melon cultivation (Janick and Paris 2006). Modern breeding-lines resistant to over watering, higher sweetness, lower temperature, lower solar exposure and to the most common pathogens have been selected only during the last few decades (Fernández-Trujillo *et al.* 2011). Different sweet melon varieties were probably introduced gradually in Europe in several occasions, following central European routes (Paris *et al.* 2012), as it is suggested by the similarity of some Hungarian and Turkish varieties (Szamosi *et al.* 2010).

Molecular analysis of the medieval melon seeds showed some affinity with modern landraces, but they are still distant in their main characters. The closest accessions to the archaeological seeds were both sweet varieties, as *ameri*, *inodorus* and *cantalupensis*, and non-sweet varieties of *flexuosus* and *chate* coming from Central Asia, Near East, North Africa and Europe. Several genomic regions have been related to sugar content in melon; Leida *et al.* (in press) associated the marker CMSNP711 located in LGI (46.0cM) to sugar content, this marker is heterozygous C/T in both archaeological samples, as it happens in a non sweet melon; in addition, the allele C found in homozygous condition in most of the non-sweet or low sugar genotypes is absent in most of the sweet genotypes. Another interesting region is located in LGIX (22.4-33.6cM) marker CNSNP1035; Dai *et al.* (2011) demonstrated that this region maps the acid invertase 2, AIN2, a gene that is involved in sugar accumulation in melon fruits. In Archaeo1 this region is homozygous as most sweet accessions of *cantalupensis*, *ameri* and *inodorus* melons, while heterozygous in Archaeo2, which is also heterozygous for marker SNP144 having the allele G more frequent in non-sweet genotypes. In any case, the coexistence of two or more cultivar cannot be ruled out, since the high heterogeneity of Medieval seeds, one of the highest within the whole melon collection can reflect a mixture of typologies. The fact that some *loci* could not be amplified in the archaeological material can be explained as result of DNA degradation or by the fact that additional mutations can exist in the flanking regions that prevent amplification. These mutations could have disappeared in the current germplasm collection.

Morphological analysis showed as well a high variability in seed shape, due probably to the coexistence of a mixture of different melon types. In our reference collection several non-sweet accessions have morphological seed features similar to sweet forms, being located in an intermediate position across the two melon subspecies (mostly corresponding to *flexuosus*, *chate*, *dudaim* and *momordica* varieties). Two Sardinian accessions showed some similarity by both molecular and

morphologically analyses, mainly FxITS9, a snake-melon diffused in Sant'Antioco, in the South, known as *Facussa* (Attene and Rodriguez 2008), and AmITS10 a sugary *ameri* collected in Villamar, in the centre of the island. Although these accessions belong to quite different varieties they showed to be part to the same genetic pool.

Some accession with molecular and morphological affinity to the ancient materials were the Iberian landraces, as FxES82, a snake-melon called *Alficoz* which is a traditional crop mostly diffused today in the area of Valencia and Catalonia. A representation of a melon plant (with branches, leaves and round fruits) appears in a palace as a symbol and crest of the Meloni family, dated to the Sassari Aragonese period, between the 14th -15th centuries (Porcu Gaias 1996, Atzei 2009). At the time of the finds (1330-1350/60 AD) the city of Sassari was part of the Kingdom of Aragon. Historical sources reported that colonists from this area moved into the city between 1330 and 1331 AD and this is also demonstrated by the finds of Valencian pottery, together with ceramics from Pisa and Savona, in the same context of the seeds (Biccone 2013). The Arab domination of the territory of Valencia lasted until 1238 AD, but also after this event the Arabs continued living in this region (Coscollá Sanz 2003). Sugary melons may have arrived into Sardinia through Aragonese commercial trades which probably knew this crop from the Arabs.

5.4.2 *Watermelon*

It is believed that the area of domestication of *Citrullus lanatus* is located in Africa where it was growing at least since the beginnings of the 4th millennium BC (Zohary *et al.* 2012). In Sardinia, it has been recorded in Phoenician-Punic contexts in the western coast of the island [**Chapter 2**]. Romans knew this crop, although it probably had a marginal role (Janick *et al.* 2007). Apart from the fruit, watermelon seeds were also consumed as “snack food” in Egypt (Cox and Van der Veen 2008) at least since the Islamic period. This practice has survived today in the area and also in other regions of Africa (Jensen *et al.* 2011); a similar use is recorded in Italy in the Renaissance period (Castelvetro 1614). The fruit is commonly mentioned in the Islamic literature during the 12th-13th centuries AD and, as for melon, it seems that watermelon was introduced by the Arab conquerors in Spain delaying its cultivation in other parts of Europe, where the cultivation of this crop started much later due the non favourable climatic conditions (Zohary *et al.* 2012). Bates and Robinson (1995) noticed that before the 16th century AD, written sources rarely mention watermelons. The two main varieties of watermelon (*citroides* and *lanatus*) were probably cultivated in early periods. Citron melon (*C. lanatus* var. *citroides*) is an old and neglected crop, quite rare nowadays in Europe that was used mainly as fodder, since their raw fruits are inedible for humans. In Corse, Laghetti and Hammer (2007) reported the traditional use of this variety (in the present it is represented by accessions LcFR01 and LcFR02) only in the North of the island where it is used for making jams. In Sardinia, there is no evidence of similar use.

Morphological analysis confirmed that the Medieval watermelon seeds from Sassari most likely belonged to the proper sugary watermelon instead of to the wild colocynth or citron melon. All Sardinian traditional cultivars as well as some Spanish and Central Asian accessions, showed high similarity with the archaeological seeds pointing out, perhaps, to a common ancestor. The closest local accession is a yellow flesh watermelon from the South of the island but the number of samples was not enough to suggest that the archaeological seeds belonged to this form. The flesh colour of watermelons varies from red to yellow, although the latter is more uncommon nowadays, and both are still well represented in traditional landraces (Szamosi *et al.* 2009). Ancient DNA analysis on Medieval and Renaissance watermelon seeds have demonstrated the presence of both red-flesh and yellow-flesh watermelons in Hungary (Gyulai *et al.* 2011, 2012).

5.5 Conclusions

The integrated analysis of molecular and morphological characterization of medieval melon and watermelon seeds from Sassari offered the opportunity to understand the diffusion of these crops in Europe.

Both analyses indicated that different varieties of melon were cultivated in Sardinia during the Medieval period. These varieties likely included sweet and non-sweet forms, and the high genetic admixture suggested that phenotypes were still not fixed, making these fruits still distant to present melon typologies. Two Sardinian landraces and several accession from Central Asia, the Near East, Africa and Spain, showed molecular and morphological affinity with the archaeological material. This is in agreement to the previous idea that the first selection of sugary melons may occurred in Central Asia and then, through the Arabs, they reached the Western Mediterranean, while the wide diffusion in Europe only occurred later as a consequence of independent introductions between the late Medieval and Renaissance periods (Paris *et al.* 2012).

According to seed features, watermelon seeds also showed a close relation with traditional Sardinian, Spanish and Central Asia landraces, suggesting that the cultivation of this crop was already in advanced stage.

Sardinian commercial and political connections with the Kingdom of Aragon at the time of the findings may suggest a relation with the introduction of sugary melon and watermelon, which probably hybridized with pre-existing local types, which are known since the Late Bronze Age (for melon) and the Punic period (for watermelon).

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Annex 1. *Citrullus lanatus* and *Citrullus Colocynthis* seed lots detail.

CODE		species	variety	Country	Place	local name	seed n°
CyAF82	C	<i>colocynthis</i>	<i>colocynthis</i>	Afghanistan			89
CyDZ33	C	<i>colocynthis</i>	<i>colocynthis</i>	Algeria	Béchar		100
CyDZ34	C	<i>colocynthis</i>	<i>colocynthis</i>	Algeria	Béchar		91
CyDZ81	C	<i>colocynthis</i>	<i>colocynthis</i>	Algeria	Béchar		110
CyES49	C	<i>colocynthis</i>	<i>colocynthis</i>	Spain	Murcia	Citruyus	95
CyES72	C	<i>colocynthis</i>	<i>colocynthis</i>	Spain	Almería		91
CyES99	C	<i>colocynthis</i>	<i>colocynthis</i>	Spain	Tenerife	Sandía	86
CyIT01	O	<i>colocynthis</i>	<i>colocynthis</i>	Italy			100
CyIT59	C	<i>colocynthis</i>	<i>colocynthis</i>	Italy		Coloquintida	99
CyMA4	C	<i>colocynthis</i>	<i>colocynthis</i>	Morocco	Tinghir		106
CyZA83	C	<i>colocynthis</i>	<i>colocynthis</i>	South Africa			72
LnAO22	C	<i>lanatus</i>	<i>lanatus</i>	Angola	Kanbongue		104
LnAO53	C	<i>lanatus</i>	<i>lanatus</i>	Angola	Luanda		75
LnAO55	C	<i>lanatus</i>	<i>lanatus</i>	Angola	Luanda		68
LnDZ25	C	<i>lanatus</i>	<i>lanatus</i>	Algeria	Mostaganem		85
LnDZ59	C	<i>lanatus</i>	<i>lanatus</i>	Algeria	Béchar		94
LnDZ78	C	<i>lanatus</i>	<i>lanatus</i>	Algeria	Brahim		78
LnES51	C	<i>lanatus</i>	<i>lanatus</i>	Spain	Cádiz	Sandía de Rota	89
LnES62	C	<i>lanatus</i>	<i>lanatus</i>	Spain	Granada	Sandía inverniza	78
LnES65	C	<i>lanatus</i>	<i>lanatus</i>	Spain	Huelva	Sandía de verano	74
LnES81	C	<i>lanatus</i>	<i>lanatus</i>	Spain	Mallorca	de pinyol blanc	65
LnGR32	C	<i>lanatus</i>	<i>lanatus</i>	Greece	Argholidha		97
LnITS1	S	<i>lanatus</i>	<i>lanatus</i>	Italy	Sardinia	Bianca di Benetutti	100
LnITS2	A	<i>lanatus</i>	<i>lanatus</i>	Italy	Sardinia	Sindria di Carloforte	100
LnITS3	S	<i>lanatus</i>	<i>lanatus</i>	Italy	Sardinia	Gialla di Sant'Antioco	100
LnITS4	A	<i>lanatus</i>	<i>lanatus</i>	Italy	Sardinia	Sindria di Gonnos	100
LnITS5	S	<i>lanatus</i>	<i>lanatus</i>	Italy	Sardinia	Niedda di Benetutti	100
LnKZ81	C	<i>lanatus</i>	<i>lanatus</i>	Kyrgyzstan	Lenin Dzho		89
LnMA3	C	<i>lanatus</i>	<i>lanatus</i>	Morocco	Khmelat		91
LnSY93	C	<i>lanatus</i>	<i>lanatus</i>	Syria	Damasco		45
LnUZ78	C	<i>lanatus</i>	<i>lanatus</i>	Uzbekistan	San Salar		80
LnAO08	C	<i>lanatus</i>	<i>citroides</i>	Angola	Huila		79
LcES64	C	<i>lanatus</i>	<i>citroides</i>	Spain	Valencia	para mermeladas	81
LcES67	C	<i>lanatus</i>	<i>citroides</i>	Spain	AValencia		86
LcFR01	B	<i>lanatus</i>	<i>citroides</i>	France	Corsica	Pastè que à confiture	120
LcFR02	B	<i>lanatus</i>	<i>citroides</i>	France	Corsica	Pastè que à confiture	121

C = COMAV Germoplasm Bank

S = Sassari University

O = Cagliari Botanical Gardens

A = Agris Sardegna

B = Bari Germplasm Bank

Annex 2. 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 markers were experimentally validated and further information is available.

Marker name	LG	cM	Major Allele Frequency	Genotype n°	Allele n°	Gene Diversity	Heterozygosity	PIC
AI_09-F07	1	0	0,8185	3	2	0	0,021	0,253
CMPSNP1095	1	3,2	0,8390	3	2	0,270	0,089	0,234
CMPSNP83	1	18,1	0,5034	3	2	0,500	0,069	0,375
AI_17-E07	1	45,2	0,6233	3	2	0,470	0,068	0,359
CMPSNP711	1	46,8	0,5479	3	2	0,495	0,041	0,373
CMPSNP410	1	59,6	0,5034	3	2	0,500	0,069	0,375
F116	1	69,2	0,6301	3	2	0,466	0,068	0,357
AI_05-G01	1	72,4	0,8322	3	2	0,279	0,007	0,240
CMPSNP731	1	80,4	0,6918	3	2	0,426	0,055	0,336
CMPSNP204	1	86,8	0,7911	3	2	0,331	0,021	0,276
CMPSNP774	2	0	0,5274	3	2	0,498	0,041	0,374
CMPSNP431	2	4,8	0,5068	3	2	0,500	0,055	0,375
CMPSNP502	2	32,6	0,6276	3	2	0,467	0,041	0,358
CMPSNP1057	2	37,4	0,8288	3	2	0,284	0,027	0,244
AI_14-H05	2	40,6	0,6541	3	2	0,453	0,089	0,350
CMPSNP128	2	50,2	0,8356	3	2	0,275	0,027	0,237
CMPSNP246	2	53,4	0,7448	3	2	0,380	0,041	0,308
CMPSNP1003	2	58,2	0,7945	3	2	0,327	0,041	0,273
CMPSNP886	2	63	0,5925	3	2	0,483	0,062	0,366
CMPSNP658	2	77,9	0,6336	3	2	0,464	0,021	0,357
CMPSNP566	2	86	0,6301	3	2	0,466	0,027	0,357
CMPSNP94	2	90,9	0,7379	3	2	0,387	0,041	0,312
AI_18-E05	3	3,2	0,6448	3	2	0,458	0,076	0,353
CMPSNP275	3	4,8	0,6096	3	2	0,476	0,027	0,363
CMPSNP540	3	8	0,6507	3	2	0,455	0,041	0,351
CMPSNP165	3	24,3	0,5171	4	3	0,506	0,075	0,385
CMPSNP769	3	42,8	0,6575	3	2	0,450	0,027	0,349
CMPSNP164	3	46,2	0,6781	3	2	0,437	0,041	0,341
CMPSNP998	3	56,3	0,6370	3	2	0,462	0,041	0,356
CMPSNP595	3	61,1	0,7774	3	2	0,346	0,048	0,286
CMPSNP712	3	62,7	0,5308	3	2	0,498	0,062	0,374
CMPSNP480	4	0	0,6250	3	2	0,469	0,056	0,359
CMPSNP787	4	6,4	0,7363	3	2	0,388	0,062	0,313
CMPSNP1132	4	11,2	0,5377	3	2	0,497	0,048	0,374
PS_34-C02	4	17,6	0,6884	3	2	0,429	0,062	0,337
CMPSNP907	4	20,8	0,5959	3	2	0,482	0,041	0,366
CMPSNP264	4	32,1	0,5514	3	2	0,495	0,062	0,372
CMPSNP147	4	48,3	0,5548	3	2	0,494	0,082	0,372
AI_03-F03	4	53,1	0,6414	3	2	0,460	0,110	0,354
CMPSNP352	4	54,7	0,6610	3	2	0,448	0,062	0,348
CMPSNP852	4	62,7	0,6952	3	2	0,424	0,034	0,334
CMPSNP607	4	69,1	0,7671	3	2	0,357	0,027	0,293
CMPSNP677	4	77,1	0,6849	3	2	0,432	0,041	0,338
CMPSNP24	4	86,8	0,6586	3	2	0,450	0,062	0,349
PS_07-E07	4	101,5	0,5308	3	2	0,498	0,075	0,374
SC51-3375	4	114,6	0,8527	3	2	0,251	0,021	0,220
CMPSNP898	5	0	0,6130	3	2	0,474	0,103	0,362
CMPSNP387	5	18,5	0,6541	3	2	0,453	0,048	0,350
CMPSNP437	5	26,5	0,6747	3	2	0,439	0,034	0,343
CMPSNP726	5	41,2	0,8664	3	2	0,231	0,034	0,205
CMPSNP788	5	50,9	0,6336	3	2	0,464	0,021	0,357
SSH9G15	5	52,5	0,7324	3	2	0,392	0,056	0,315
60k41.243	5	73,4	0,5753	3	2	0,489	0,055	0,369
CMPSNP1155	5	79,8	0,6655	3	2	0,445	0,076	0,346
AI_13-H12	5	89,4	0,6747	3	2	0,439	0,048	0,343
CMPSNP735	5	94,2	0,7500	3	2	0,375	0,048	0,305
CMPSNP925	6	1,6	0,5799	3	2	0,487	0,063	0,369
CMPSNP218	6	8	0,6370	3	2	0,462	0,068	0,356
CMPSNP571	6	20,8	0,5345	3	2	0,498	0,076	0,374
CMPSNP1167	6	25,6	0,8459	3	2	0,261	0,048	0,227
CMPSNP433	6	32	0,7808	3	2	0,342	0,068	0,284
CMPSNP3	6	43,2	0,6690	3	2	0,443	0,041	0,345

CMPSNP292	6	49,6	0,5856	3	2	0,485	0,048	0,368
CMPSNP295	6	49,6	0,8241	3	2	0,290	0,090	0,248
CMPSNP1021	6	57,6	0,5034	3	2	0,500	0,075	0,375
CMPSNP1038	6	57,6	0,8507	3	2	0,254	0,035	0,222
A_38-F04	6	70,7	0,5690	3	2	0,490	0,062	0,370
AI_13-F02	6	85,3	0,5207	3	2	0,499	0,048	0,375
CMPSNP378	6	86,9	0,6747	3	2	0,439	0,021	0,343
AI_05-F11	7	4,9	0,5000	3	2	0,500	0,048	0,375
CMPSNP249	7	11,3	0,6575	3	2	0,450	0,068	0,349
CMPSNP262	7	30,5	0,6815	3	2	0,434	0,048	0,340
CMPSNP579	7	30,5	0,5137	3	2	0,500	0,055	0,375
CMPSNP1009	7	32,1	0,8931	3	2	0,191	0,048	0,173
CMPSNP287	7	35,3	0,7034	3	2	0,417	0,028	0,330
CMPSNP56	7	43,3	0,6610	3	2	0,448	0,075	0,348
CMPSNP465	7	59,4	0,6918	3	2	0,426	0,027	0,336
CMPSNP415	7	72,2	0,6404	3	2	0,461	0,021	0,355
CMPSNP12	8	0	0,6379	3	2	0,462	0,062	0,355
CMPSNP766	8	4,8	0,6610	3	2	0,448	0,034	0,348
CMPSNP718	8	11,2	0,6207	3	2	0,471	0,041	0,360
CMPSNP97	8	19,2	0,6438	3	2	0,459	0,397	0,353
CMPSNP44	8	22,4	0,7295	3	2	0,395	0,034	0,317
AI_21-D08	8	28,8	0,5149	3	2	0,500	0,015	0,375
CMPSNP181	8	35,2	0,6146	3	2	0,474	0,007	0,362
F013	8	48,1	0,6852	3	2	0,431	0,052	0,338
PSI_25-H03	5/8	59,4	0,5582	3	2	0,493	0,034	0,372
CMPSNP1066	8	79,2	0,6389	2	2	0,461	0,000	0,355
CMPSNP553	9	0	0,5274	3	2	0,498	0,068	0,374
CMPSNP173	9	3,2	0,5479	3	2	0,495	0,055	0,373
P5.64	9	8	0,6541	3	2	0,453	0,075	0,350
CMPSNP1077	9	19,2	0,6336	3	2	0,464	0,007	0,357
CMPSNP320	9	20,8	0,7637	3	2	0,361	0,048	0,296
CMPSNP144	9	22,4	0,6438	3	2	0,459	0,027	0,353
CMPSNP1035	9	33,6	0,6438	3	2	0,459	0,055	0,353
CMPSNP159	9	36,8	0,7207	3	2	0,403	0,034	0,322
CMPSNP1133	9	59,2	0,5616	3	2	0,492	0,096	0,371
CMPSNP890	9	64	0,5420	3	2	0,496	0,063	0,373
psi36-10864	10	0	0,6268	3	2	0,468	0,070	0,358
psi36-839	10	0	0,6172	3	2	0,473	0,048	0,361
CMPSNP172	10	1,6	0,7705	3	2	0,354	0,034	0,291
CMPSNP528	10	8	0,5959	3	2	0,482	0,068	0,366
CMPSNP65	10	14,4	0,7808	3	2	0,342	0,055	0,284
CMPSNP762	10	23,9	0,6910	3	2	0,427	0,049	0,336
CMPSNP671	10	28,8	0,5724	3	2	0,490	0,124	0,370
CMPSNP550	10	38,5	0,6747	3	2	0,439	0,349	0,343
CMPSNP426	11	0	0,6541	3	2	0,453	0,034	0,350
HS_35-E11	11	16,4	0,7363	3	2	0,388	0,075	0,313
PSI_41-B07	11	27,6	0,7089	3	2	0,413	0,021	0,328
CMPSNP389	11	47,7	0,6138	3	2	0,474	0,207	0,362
CMPSNP30	11	66	0,6517	3	2	0,454	0,062	0,351
CMPSNP315	11	90,9	0,7188	3	2	0,404	0,035	0,323
CMPSNP475	11	98,9	0,6418	3	2	0,460	0,035	0,354
CMPSNP122	11	100,5	0,6473	3	2	0,457	0,034	0,352
CMPSNP385	12	4,8	0,8000	3	2	0,320	0,041	0,269
CMPSNP310	12	9,8	0,5171	3	2	0,499	0,034	0,375
AI_35-A08	12	16,4	0,5034	3	2	0,500	0,021	0,375
AI_09-G07	12	18,1	0,8028	3	2	0,317	0,127	0,266
CMPSNP285	12	21,4	0,9212	3	2	0,145	0,034	0,135
CMPSNP361	12	37	0,5414	3	2	0,497	0,062	0,373
CMPSNP5	12	58,1	0,7655	3	2	0,359	0,028	0,295
FR14F22	12	67,7	0,5655	3	2	0,491	0,041	0,371
P02.03	12	69,3	0,6267	3	2	0,468	0,075	0,358

Annex C1: Seed lots detail *Cucumis melo*

CODE	CODE 2	Molec. Analy.	Morph. Analy.	subsp.	variety	Country	local name	seed n°
AcIN193	Ac-SVIInd	C	X		<i>agrestis acidulus</i>	India		
AcLK148		M		X	<i>agrestis acidulus</i>	Sri Lanka	kekiri	97
AcLK187	Ac-SRKSLan	C	X		<i>agrestis acidulus</i>	Sri Lanka		
AcSN45	Ac-G22843Se	U	X	X	<i>agrestis acidulus</i>	Senegal		100
AcSN46		U		X	<i>agrestis acidulus</i>	Senegal		96
AcZA98	Ac-5384Zamb	U	X	X	<i>agrestis acidulus</i>	Zambia		86
AcZW100	Ac-TGR1551Zimb	U	X	X	<i>agrestis acidulus</i>	Zimbabwe		93
AcZW99	Ac-TGR1843Zimb	U	X	X	<i>agrestis acidulus</i>	Zimbabwe		96
AgCM195	Ag-TayCam	C	X		<i>agrestis agrestis</i>	Cameroon	tayer	
AgGH14	Ag-15591Gha	U	X	X	<i>agrestis agrestis</i>	Ghana		98
AgIN128	Ag-CallInd	C	X	X	<i>agrestis agrestis</i>	India	callosus	97
AgIN204	Ag-WChInd	C	X	X	<i>agrestis agrestis</i>	India	wild chibbar	96
AgNG38	Ag-Co38Nig	C	X	X	<i>agrestis agrestis</i>	Nigeria		91
AgSN133	Ag-FadSud	M	X	X	<i>agrestis agrestis</i>	Sudan	fadasi	95
AgSN144	Ag-Hsd2446Sud	C	X		<i>agrestis agrestis</i>	Sudan		
AgSN145	Ag-Hsd93Sud	C	X		<i>agrestis agrestis</i>	Sudan		
AgSN146	Ag-HumSud	M	X	X	<i>agrestis agrestis</i>	Sudan	humaid	91
AgSN147	Ag-Hum93Sud	C	X		<i>agrestis agrestis</i>	Sudan		
AgSN197	Ag-TendSud	M	X	X	<i>agrestis agrestis</i>	Sudan	tendelti	88
AmAF109		C		X	<i>melo ameri</i>	Afganistan		98
AmAF2	Am-3584Afg	U	X	X	<i>melo ameri</i>	Afganistan		98
AmDE166	Am-OpalGer	M	X	X	<i>melo ameri</i>	Germany		95
AmEG113	Am-AnaDokEgy	C	X		<i>melo ameri</i>	Egypt	ananas dokki	
AmEG9	Am-KafEgy	U	X	X	<i>melo ameri</i>	Egypt	kafr hakim	100
AmES51	Am-LaVGSsp	C	X		<i>melo ameri</i>	Spain	verde gordo	
AmGE12	Am-NanaGeorg	C	X	X	<i>melo ameri</i>	Georgia	nanari	100
AmGE13	Am-KolGeor	U	X	X	<i>melo ameri</i>	Georgia	koljonitza	85
AmGE156	Am-NesviGeor	M	X	X	<i>melo ameri</i>	Georgia	mucha nesvi	91
AmIL208	Am-YokIs	C	X		<i>melo ameri</i>	Israel	yokneam	
AmIR149	Am-KhaIran	M	X	X	<i>melo ameri</i>	Iran	Khatoni	74
AmIR183	Am-SarakIran	C	X		<i>melo ameri</i>	Iran		
AmIR190	La-SousIran	M	X	X	<i>melo ameri</i>	Iran	souski	98
AmIR26	Am-6053Iran	U	X	X	<i>melo ameri</i>	Iran		99
AmITS10		L	X	X	<i>melo ameri</i>	Italy	Villamar	100
AmKZ106		C		X	<i>melo ameri</i>	Kazakhstan		92
AmKZ31	Am-MestKaz	C	X		<i>melo ameri</i>	Kazakhstan	mestnaia	
AmMA189	Am-SouiMor	C	X		<i>melo ameri</i>	Morocco	souilah	
AmMA37	Am-Afr1Mor	C	X	X	<i>melo ameri</i>	Morocco		92
AmMN36	Am-ChandMon	C	X	X	<i>melo ameri</i>	Mongolia		79
AmRU41	Am-KorcaRus	C	X	X	<i>melo ameri</i>	Russia	korça	99
AmRU42	Am-ApelRus	C	X	X	<i>melo ameri</i>	Russia	apelsinaja	92
AmRU43	Am-ChandAfg	C	X		<i>melo ameri</i>	Afganistan	chandalak	
AmRU44	Am-KuvRus	U	X	X	<i>melo ameri</i>	Russia	kuvinska	99
AmTJ83	Am-TokTaj	C	X	X	<i>melo ameri</i>	Tajikistan	tokash	98
AmTN84	Am-BattiTun	C	X	X	<i>melo ameri</i>	Tunisia	battikh	90
AmTR88	Am-AltimTur	U	X	X	<i>melo ameri</i>	Turkey	al timbas	88
AmTR89	Am-HassanTur	U	X	X	<i>melo ameri</i>	Turkey	hassanbey	97
AmTU137	Am-GalaTun	C	X	X	<i>melo ameri</i>	Tunisia	galaoui	98
AmUA119	Am-BirUkr	M	X	X	<i>melo ameri</i>	Ukraine	birjucecutskaja	99
AmUA90		U		X	<i>melo ameri</i>	Ukraine		100
AmUZ167	Am-OuzUzb	C	X	X	<i>melo ameri</i>	Uzbekistan		84

AmUZ168	Am-OuzUzb2	C	X		<i>melo</i>	<i>ameri</i>	Uzbekistan		
AmUZ95	Am-KokUzb	C	X	X	<i>melo</i>	<i>ameri</i>	Uzbekistan	kokcha	98
AmUZ96		C		X	<i>melo</i>	<i>ameri</i>	Uzbekistan	kizil-uruk	81
CaFR121	Can-CAFran	C	X		<i>melo</i>	<i>cantalupensis</i>	France	cantalup d'Alger	
CaFR161	Can-NCFran	C	X	X	<i>melo</i>	<i>cantalupensis</i>	France	noir des carmes	98
CaFR172	Can-PGFran	C	X	X	<i>melo</i>	<i>cantalupensis</i>	France	petit gris de Rennes	100
CaFR179	Can-PresFran	C	X	X	<i>melo</i>	<i>cantalupensis</i>	France	prescott fond blanc	100
CaFR191	Can-SucrFran	C	X		<i>melo</i>	<i>cantalupensis</i>	France	sucrin de tours	
CaHU18		C		X	<i>melo</i>	<i>cantalupensis</i>	Hungary	ezüst ananasz	96
CaITS8		S		X	<i>melo</i>	<i>cantalupensis</i>	Italy	zeurrosu di Gesico	63
ChIT122	Chate-CarIta	C	X		<i>melo</i>	<i>chate</i>	Italy	carosello	
ChIT27	Chate-CarBIta	C	X	X	<i>melo</i>	<i>chate</i>	Italy	carosello barese	98
CnCH105	Con-GouChi	C	X	X	<i>agrestis</i>	<i>chinensis</i>	China	gogua	96
CnCH6	Con-Co6Chi	C	X	X	<i>agrestis</i>	<i>chinensis</i>	China		98
CnJP207	Con-YapuJa	C	X	X	<i>agrestis</i>	<i>chinensis</i>	Japan	yamato purinsu	96
CnKR173	Con-SCKo	C	X	X	<i>agrestis</i>	<i>chinensis</i>	Korea		95
CnKR32	Con-Pat81Ko	C	X	X	<i>agrestis</i>	<i>chinensis</i>	Korea		100
CnPL169	Con-PaulPol	M	X	X	<i>agrestis</i>	<i>chinensis</i>	Polonia	paul	100
CoCH117	Con-BaishChi	C	X		<i>agrestis</i>	<i>conomon</i>	China	baishami	
CoCH125	Con-Chi51Chi	C	X		<i>agrestis</i>	<i>conomon</i>	China		
CoCH154	Con-MielChi	M	X	X	<i>agrestis</i>	<i>conomon</i>	China	miel blanc	98
CoCH164	Con-OgonChi	C	X		<i>agrestis</i>	<i>conomon</i>	China	ogon	
CoCH206	Con-XiaoChi	C	X		<i>agrestis</i>	<i>conomon</i>	China	Xiaobai	
CoJP136		C		X	<i>agrestis</i>	<i>conomon</i>	Japan	freeman's cucumber	99
CoJP185	Con-ShiroJa	C	X	X	<i>agrestis</i>	<i>conomon</i>	Japan	shiro uri okayama	99
CoJP200	Con-MamJa	C	X		<i>agrestis</i>	<i>conomon</i>	Japan	Tokio mammoth	
CoPH138	Con-GapPhi	C	X		<i>agrestis</i>	<i>conomon</i>	Philippines	gapan	
CoPH182	Con-SanIIPhi	C	X		<i>agrestis</i>	<i>conomon</i>	Philippines	san Ildefonso	
CtIN22	Chi-VellInd	C	X	X	<i>agrestis</i>	<i>chito</i>	India	velleri	90
DuAF1		C		X	<i>melo</i>	<i>dudaim</i>	Afganistan		100
DuAF180	Dud-QPMAfg	C	X	X	<i>melo</i>	<i>dudaim</i>	Afganistan	queen's pocket melon	97
DuGE296		C		X	<i>melo</i>	<i>dudaim</i>	Georgia		98
FxAF174	Flex-TarehAfg	C	X	X	<i>melo</i>	<i>flexuosus</i>	Afganistan		40
FxES82	Flex-AlficozSp	C	X	X	<i>melo</i>	<i>flexuosus</i>	Spain	alficoz	100
FxIN115	Flex-AryaInd	C	X	X	<i>melo</i>	<i>flexuosus</i>	India	arya	99
FxIN20	Flex-Co20Ind	C	X	X	<i>melo</i>	<i>flexuosus</i>	India		96
FxIQ23	Flex-KhiIrak	C	X	X	<i>melo</i>	<i>flexuosus</i>	Irak	khiaar taaruzy	98
FxIQ24	Flex-Co24Irak	C	X		<i>melo</i>	<i>flexuosus</i>	Irak		
FxITS9		S	X	X	<i>melo</i>	<i>flexuosus</i>	Italy	facussa	100
FxSA188	Flex-SnakeSA	C	X		<i>melo</i>	<i>flexuosus</i>	Saudi Arabia	snake melon	
FxSD186	Flex-SilkaSud	M	X	X	<i>melo</i>	<i>flexuosus</i>	Sudan	silka	100
FxTR15		C	X		<i>melo</i>	<i>flexuosus</i>	Turkey		
FxTR16		C		X	<i>melo</i>	<i>flexuosus</i>	Turkey		96
FxTR2		C			<i>melo</i>	<i>flexuosus</i>	Turkey		
FxTR21		C	X	X	<i>melo</i>	<i>flexuosus</i>	Turkey		95
FxTR4		C		X	<i>melo</i>	<i>flexuosus</i>	Turkey		96
FxTR54		C		X	<i>melo</i>	<i>flexuosus</i>	Turkey	adsiz	98
FxTR86	Flex-AcukTur	U	X	X	<i>melo</i>	<i>flexuosus</i>	Turkey	acuk	89
FxTR9		C	X		<i>melo</i>	<i>flexuosus</i>	Turkey		
InCN142	In-HamiChi	C	X	X	<i>melo</i>	<i>inodorus</i>	China	hami	96
InES48	In-AmCañSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	caña dulce	
InES49	In-LaCocaSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	coca	
InES50	In-LaEscrSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	escrito oloroso	
InES52	In-BBescrSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	blanco escriturado	

InES53	In-TeNinvSp2	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	tendral negro de inv.	95
InES54	In-BTempSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	tempranillo	81
InES55	In-LaHCCSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	hilo carrete	95
InES56	In-BLisSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	blanco liso	81
InES58	La-MadASp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	madura amarilla	
InES59	In-TeMollSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	mollerusa	
InES61	In-PsPiñSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	piel de sapo (piñonet)	97
InES62	In-BBlargSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	blanco	
InES63	In-TeNinvSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	negro de invierno	
InES64	In-PsPipaSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	pipa de oro	
InES66	In-PsPiñonSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	piñoncillo	
InES67	In-BBredSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	amarillo	
InES68	In-RoMoch1Sp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	mochuelo	
InES69	In-TeLVillSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	largo de villaconejos	
InES70	In-LaMelAmaSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	amarilla	
InES71	In-LaCalSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	calamonte	
InES72	In-HCECSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	hilo Carrete	
InES73	In-PsVPintSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	verde pinto	
InES74	In-LaAmanSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	amarillo manchado	
InES75	La-ErizoSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	eriçó mallorquin	98
InES76	La-ComunSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	común	
InES77	In-TeNinvSp	C	X		<i>melo</i>	<i>inodorus</i>	Spain	de invierno	
InES78	In-LaBolasSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	bolas	85
InES79	In-AmAoroSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	amarillo oro	95
InES80	In-RoSp	C	X	X	<i>melo</i>	<i>inodorus</i>	Spain	rochet	99
InGR118	In-BaskGreece	M	X	X	<i>melo</i>	<i>inodorus</i>	Greece	baskavas	98
InHU17	In-MusHung	C	X	X	<i>melo</i>	<i>inodorus</i>	Hungary	cukordinnye	99
InIT28	In-CucumIta	C	X	X	<i>melo</i>	<i>inodorus</i>	Italy	cucummarazzo	99
InITS1		S		X	<i>melo</i>	<i>inodorus</i>	Italy	fattitu di Bosa	75
InITS2		S		X	<i>melo</i>	<i>inodorus</i>	Italy	di Orroli	98
InITS3		S		X	<i>melo</i>	<i>inodorus</i>	Italy	de jerru	68
InITS4		S		X	<i>melo</i>	<i>inodorus</i>	Italy	biancu	96
InITS5		S		X	<i>melo</i>	<i>inodorus</i>	Italy	di Villagrande	94
InITS6		S		X	<i>melo</i>	<i>inodorus</i>	Italy	bou di Gesico	100
InITS7		S		X	<i>melo</i>	<i>inodorus</i>	Italy	muscadeddu	99
InPT120		M		X	<i>melo</i>	<i>inodorus</i>	Portugal	branco de ribeteja	69
InPT126	In-CraPor	M	X	X	<i>melo</i>	<i>inodorus</i>	Portugal	crabranco	82
InPT39	La-MelaoPor	C	X	X	<i>melo</i>	<i>inodorus</i>	Portugal		48
InPT40	La-CascaPor	C	X	X	<i>melo</i>	<i>inodorus</i>	Portugal	casca de carvalho	98
InTN65	In-AsliTun	C	X	X	<i>melo</i>	<i>inodorus</i>	Tunisia	asli	98
InTN85	In-MaazTun	C	X	X	<i>melo</i>	<i>inodorus</i>	Tunisia	maazoun	98
InTR104		U		X	<i>melo</i>	<i>inodorus</i>	Turkey		98
InTR150	In-KirkTur	C	X		<i>melo</i>	<i>inodorus</i>	Turkey	kirkagac	
InTR209	In-YuTur	C	X		<i>melo</i>	<i>inodorus</i>	Turkey	yuva	
InTR91		C		X	<i>melo</i>	<i>inodorus</i>	Turkey		100
LaBG163	La-OgenBul	C	X		<i>melo</i>	indet.	Bulgaria	ogen	
LaBG177	La-BanBul	C	X	X	<i>melo</i>	indet.	Bulgaria	plovdider banane	99
LaDZ4	La-MalacAlg	C	X		<i>melo</i>	indet.	Algeria	malacara	
LaET11		C		X	<i>melo</i>	indet.	Etiopia	popone	111
LaFR151	La-KroFran	M	X	X	<i>melo</i>	indet.	France	Kroumir	96
LaGE192	La-SusaGeo	C	X		<i>melo</i>	indet.	Georgia	susakitri	
LaIR140	La-GorgIran	C	X		<i>agrestis</i>	indet.	Iran	gorgab	
LaIT00	Can-PopIta	C	X	X	<i>melo</i>	indet.	Italy	popone d'oro	98
LaIT210	La-ZatIta	C	X		<i>melo</i>	indet.	Italy	zatta	

LaMG202	La-VoaMad	M	X	X	<i>agrestis</i>	indet.	Madagascar	voatango	81
LaML35	La-KankMali	U	X	X	<i>melo</i>	indet.	Mali	kamkani	72
LaZA47	La-TransSAfr	U	X	X	<i>agrestis</i>	indet.	South Africa		99
MkCH158	Con-NanChi	M	X	X	<i>agrestis</i>	<i>makuwa</i>	China	nanbukin	96
MkCH7	Con-LongtChi	U	X	X	<i>agrestis</i>	<i>makuwa</i>	China	longtian	96
MkJP188		C		X	<i>agrestis</i>	<i>makuwa</i>	Japan	Omaru Gin makuwa	100
MkJP30	Con-GMJa	U	X	X	<i>agrestis</i>	<i>makuwa</i>	Japan	ginsen makuwa	96
MoIN135	Mom-FPInd	M	X	X	<i>agrestis</i>	<i>momordica</i>	India	faizabadi phoont	95
MoIN176	Mom-MR1Ind	C	X		<i>agrestis</i>	<i>momordica</i>	India		
MoIN19	Mom-KhaInd	C	X	X	<i>agrestis</i>	<i>momordica</i>	India	kharbuja	99
MoIN21	Mom-PI124Ind	U	X	X	<i>agrestis</i>	<i>momordica</i>	India		93
TiSN198	Tibish-DSud	M	X	X	<i>agrestis</i>	<i>tibish</i>	Sudan		98
TiSN199	Tibish-KSud	M	X	X	<i>agrestis</i>	<i>tibish</i>	Sudan	tibish khurtagat	58

U = NPGS (USDA)

C = COMAV Germoplasm Bank

M = MELRIP project

Y = Germoplasm Bank of Cyprus

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CODE	variety	Country	AT_09-F07	CMFNS1095	CMFNSP83	AL_17-E07	CMFNSP711	CMFNSP410	F116	AL_05-G01	CMFNSP731	CMFNSP204	CMFNSP774	CMFNSP431	CMFNSP502	CMFNSP1057	AL_14-H05	CMFNSP128	CMFNSP246	CMFNSP1003	CMFNSP658	CMFNSP566	CMFNSP94	AL_18-E05	CMFNSP275	CMFNSP540	CMFNSP165	CMFNSP769	CMFNSP164	CMFNSP998	CMFNSP246	CMFNSP712	CMFNSP480	CMFNSP787	CMFNSP1132	PS_34-C02	CMFNSP907	CMFNSP264	CMFNSP147	AL_03-F03	CMFNSP352	CMFNSP852	CMFNSP607			
FXTR21	<i>flexuosus</i>	Turkey	T/T	G/G	A/A	A/A	C/C	C/C	C/T	T/T	G/G	G/G	T/T	C/C	G/G	C/C	T/T	G/G	T/T	A/A	G/T	G/T	C/C	C/C	C/C	C/C	T/T	C/C	T/T	G/G	T/T	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	T/C	G/G	C/C	C/C		
FXTR86	<i>flexuosus</i>	Turkey	T/T	G/A	A/G	G/A	C/T	T/T	C/T	T/T	G/G	G/G	T/T	C/C	G/G	C/C	T/T	G/G	T/T	A/A	G/T	G/T	C/C	C/C	C/C	C/C	C/C	T/T	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/T	A/G	C/T	C/C	
FXTR9	<i>flexuosus</i>	Turkey	T/T	G/G	G/G	G/G	T/T	C/C	C/T	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	C/C	G/G	A/A	T/T	C/C	
CHIT22	<i>chate</i>	Italy	T/T	G/G	A/G	G/G	C/T	C/C	C/T	T/T	G/G	T/T	T/T	C/C	G/G	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	C/C	G/T	A/G	T/T	T/T	
CHIT27	<i>chate</i>	Italy	T/T	G/G	A/G	G/G	C/T	C/C	C/T	T/T	G/G	T/T	T/T	C/C	G/G	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	C/C	G/T	A/G	T/T	T/T	
Sa Osa	Archaeo	Italy	T/T	G/G	??	G/G	C/T	T/T	T/T	??	T/T	G/A	T/T	T/T	G/A	T/T	C/C	G/G	T/T	A/A	G/T	C/C	G/G	T/T	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/T	A/A	C/T	T/T	
Sassari	Archaeo2	Italy	T/T	G/G	??	G/A	C/T	C/T	??	T/T	T/T	G/G	T/T	T/T	G/G	T/T	C/C	G/G	T/T	A/A	G/T	C/C	G/G	T/T	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/T	A/A	C/T	T/T
LaLR140	indel landrace	Inn	T/T	G/G	G/G	G/G	T/T	T/T	C/C	T/T	G/G	A/A	C/C	T/T	G/G	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaMG202	indel landrace	Madagascar	T/T	G/A	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	G/G	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/T	A/G	C/C	C/C
LaZ4A7	indel landrace	South Africa	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	G/G	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/T	A/G	C/C	T/C
LaBG163	indel landrace	Bulgaria	T/T	G/G	A/A	G/G	T/T	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaBG177	indel landrace	Bulgaria	T/T	G/G	G/G	G/G	C/C	T/T	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaDZ4	indel landrace	Algeria	T/T	G/G	G/G	G/G	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/T	C/C	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaFR151	indel landrace	France	T/T	G/G	G/G	G/G	T/T	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/T	C/C	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaGE192	indel landrace	Georgia	C/C	G/A	A/A	G/G	T/T	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/T	C/C	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaIT00	indel landrace	Italy	T/T	G/G	A/A	G/G	C/C	T/T	T/T	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaIT210	indel landrace	Italy	T/T	G/G	A/A	G/G	T/T	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
LaML35	indel landrace	Mali	T/T	G/G	G/G	G/G	T/T	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/T	C/C	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/T	T/C	G/G	A/A	T/T	T/T
MoLN135	<i>momorica</i>	India	T/T	G/A	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
MoLN176	<i>momorica</i>	India	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
MoLN19	<i>momorica</i>	India	T/T	G/G	G/G	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
MoLN21	<i>momorica</i>	India	T/T	G/G	G/G	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
TISN198	<i>tibish</i>	Sudan	T/T	G/A	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
TISN199	<i>tibish</i>	Sudan	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AcLN193	<i>acidalis</i>	India	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AcLN187	<i>acidalis</i>	Sri Lanka	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AcSN45	<i>acidalis</i>	Senegal	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AcZ9A8	<i>acidalis</i>	Zambia	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AcZWN100	<i>acidalis</i>	Zimbabwe	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AcZWN99	<i>acidalis</i>	Zimbabwe	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
CIN22	<i>chito</i>	India	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AgCM195	<i>agrestis</i>	Cameroon	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AgGH14	<i>agrestis</i>	Ghana	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AgLN128	<i>agrestis</i>	India	T/T	G/G	A/A	A/A	C/C	C/C	C/C	T/T	G/G	T/T	T/T	C/C	A/A	C/C	T/T	C/C	G/G	T/T	A/A	G/G	G/G	C/C	C/C	C/C	C/C	C/C	T/T	A/A	A/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/A	A/A	C/C	G/G	G/G	T/T	C/C
AgLN204	<i>agrestis</i>	India	T/T	G/G	A/A	A/A	C/C																																							

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CODE	variety	Country	AT_09-F07	CMPSN1095	CMPSN883	AL_17-E07	CMPSN711	CMPSN410	F116	AL_05-G01	CMPSN731	CMPSN204	CMPSN774	CMPSN431	CMPSN502	CMPSN1057	AL_14-H05	CMPSN128	CMPSN246	CMPSN1003	CMPSN886	CMPSN658	CMPSN566	CMPSN94	AL_18-E05	CMPSN275	CMPSN540	CMPSN165	CMPSN769	CMPSN164	CMPSN998	CMPSN595	CMPSN712	CMPSN480	CMPSN787	CMPSN1132	PS_34-C02	CMPSN907	CMPSN264	CMPSN147	AL_03-F03	CMPSN352	CMPSN852	CMPSN607					
CnCH105	<i>chinensis</i>	China	T/T	G/G	A/A	G/G	C/C	C/C	C/C	T/T	G/G	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	G/G	T/T	
CnCH6	<i>chinensis</i>	China	T/T	V/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnJ207	<i>chinensis</i>	Japan	T/T	G/G	G/G	G/G	T/T	T/T	T/T	T/T	C/C	A/A	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnKR173	<i>chinensis</i>	Korea	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnKR32	<i>chinensis</i>	Korea	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnPL169	<i>chinensis</i>	Polonia	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnCH117	<i>conomon</i>	China	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnCH125	<i>conomon</i>	China	T/T	G/A	G/G	G/G	T/T	T/T	T/T	T/T	G/G	A/A	C/C	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	C/C	G/G	G/G	T/T	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnCH154	<i>conomon</i>	China	T/T	G/A	A/A	A/A	C/C	C/C	C/C	T/T	G/G	A/A	C/C	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	C/C	G/G	G/G	T/T	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnCH164	<i>conomon</i>	China	T/T	V/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CnCH206	<i>conomon</i>	China	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CoJ185	<i>conomon</i>	Japan	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CoJ200	<i>conomon</i>	Japan	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CoPH138	<i>conomon</i>	Philippines	T/T	G/G	A/A	A/A	C/C	T/T	T/T	T/T	G/G	A/A	C/C	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	A/A	A/A	T/T	A/A	A/A	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
CoPH182	<i>conomon</i>	Philippines	T/T	G/A	A/A	A/A	C/C	T/T	T/T	T/T	G/G	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
MICH158	<i>makawa</i>	China	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
MICH17	<i>makawa</i>	China	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T
MkJP30	<i>makawa</i>	Japan	T/T	A/A	A/A	A/A	C/C	C/C	C/C	T/T	C/C	G/G	T/T	C/C	G/G	C/C	T/T	A/A	G/G	T/T	T/T	G/G	G/G	T/T	G/G	G/G	C/C	T/T	G/G	T/T	A/A	A/A	A/A	A/A	C/C	T/T	A/A	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T	A/A	C/C	T/T

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CODE	variety	Country	CMPSN181	F013	PS1_25-H03	CMPSN1066	CMPSN553	CMPSN173	PS64	CMPSN1077	CMPSN20	CMPSN144	CMPSN1035	CMPSN159	CMPSN1133	PS36-10864	PS46-839	CMPSN172	CMPSN528	CMPSN65	CMPSN762	CMPSN671	CMPSN550	CMPSN426	HS_35-E11	PS1_41-B07	CMPSN389	CMPSN90	CMPSN315	CMPSN475	CMPSN122	CMPSN385	CMPSN310	AL_35-A08	AL_09-G07	CMPSN285	CMPSN361	CMPSN5	FR14F22	P0203										
CnCH105	<i>chinensis</i>	China	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	G/G	V/A	V/A	V/A	T/T	A/A	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G								
CnCH6	<i>chinensis</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G						
CnJP207	<i>chinensis</i>	Japan	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G					
CnKR173	<i>chinensis</i>	Korea	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G				
CnKR32	<i>chinensis</i>	Korea	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G			
CnPL169	<i>chinensis</i>	Polonia	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G			
CnCH117	<i>conomon</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G			
CnCH125	<i>conomon</i>	China	C/C	G/G	C/C	G/G	G/G	C/C	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G			
CnCH154	<i>conomon</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G		
CnCH164	<i>conomon</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G		
CnCH206	<i>conomon</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	
CoJP185	<i>conomon</i>	Japan	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
CoJP200	<i>conomon</i>	Japan	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
CoPH138	<i>conomon</i>	Philippines	C/C	A/A	C/C	A/A	V/A	V/A	T/T	G/G	V/A	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
CoPH182	<i>conomon</i>	Philippines	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
MICH158	<i>makova</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
MICH17	<i>makova</i>	China	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
MkJP30	<i>makova</i>	Japan	T/T	V/A	G/G	A/A	V/A	V/A	T/T	G/G	G/G	G/G	V/A	V/A	V/A	G/G	G/G	T/T	C/C	G/G	T/T	A/A	C/T	G/G	G/G	G/G	A/T	T/T	T/T	G/G	A/A	G/G	A/A	A/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G

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