Survey of over 4, 500 monumental olive trees preserved on-farm in the northeast Iberian Peninsula, their genotyping and characterization

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Abstract

Inventorying, characterising and conserving on-farm ancient olive trees is a priority for safeguarding their genetic, natural and agricultural value and for protecting ancient genotypes threatened with extinction. In the "Taula del Sénia" (M-TdS) area (northeast Iberian Peninsula) a highly important cultural landscape has been preserved, in which the olive groves play an outstanding social and economic role: the ancient olive trees, sustained by many local farmers, constitute a living heritage and provide a clear example of High Nature Value (HNV). A total of 4,526 ancient productive olive trees, with a trunk circumference (PBH) larger than 3.5 m, were inventoried and their spatial localization and biometric measurements were collected. 41 olive trees have shown the highest category in monumentality (PBH>8.1 m). The outstanding trees might be 634-1082 years old. The endocarp morphology of a representative sample of the most

ancient trees from this settlement resulted in 14 different profiles. The ancient trees genotyped, through eight simple sequence repeat (SSR) markers, revealed 43 SSR profiles. The use of SSR enabled us to verify that most of the trees (98%) belong to the local cv. 'Farga', a male sterile variety with a rare chlorotype, only a few trees corresponded with other local varieties, 'Morrut', 'Canetera' and 'Sevillenca', and ten hitherto unidentified genotypes were distinguished, some with chloroplast lineages different from the 'Farga' type. The M-TdS area holds a unique living and exploitable heritage with the highest concentration of ancient olive trees worldwide. On-farm conservation of this germplasm by the community of local growers is enabling preservation of this important source of genetic variation, potentially holding traits of resilience and adaptation to adverse soil and climatic conditions, demonstrated by the survival of these trees over the centuries. Farmers have undertaken initiatives to valorize the olive oil deriving from these M-TdS trees.

Keywords: *Olea europaea*, ancient trees, Farga, local varieties, genetic variability

1. Introduction

The olive tree (*Olea europaea* L.) is a long-lived fruit tree species considered a reliable indicator of the Mediterranean environment (Moriondo et al. 2013; Vargas and Kadereit 2001). Recent studies have identified ancient olive trees, including both cultivated and wild forms, in several Mediterranean countries as Italy (Baldoni et al. 2006; Cicatelli et al. 2013; Erre et al. 2010; Salimonti et al. 2013), Greece (Cherubini et al. 2013; Maravelakis et al. 2013; Michelakis 2002), Montenegro (Lazović et al. 2016), Israel and the Palestinian territories (Barazani et al. 2014; Petruccelli et al. 2014), and even in

Western Asia (Iran) (Mousavi et al. 2014). These trees testify to the antiquity of olive growing throughout the Mediterranean region, as well as their long lifespan and ability to survive under adverse conditions (Baldoni et al. 2006).

There are archaeological evidences of olive cultivation in the Iberian Peninsula since Neolithic and Chalcolithic times (Buxó 1997; Terral et al. 2004) and of its extension during the Bronze Age. In Spain, the main expansion was during the Roman period (Buxó 2005; Rodríguez-Ariza and Montes 2010; Terral and Arnold-Simard 1996), as confirmed by the proliferation of oil extraction structures (Rodríguez-Ariza and Montes 2005).

Spain is currently the world´s major olive oil producer (Nations FAO 2014) and still preserves a rich olive genetic heritage, as shown by the large number of olive varieties cultivated in different regions (Belaj et al. 2004c; Belaj et al. 2010; Rallo et al. 2005). The presence of centennial trees (Díez et al. 2004; Díez et al. 2011) and wild olive forests (Belaj et al. 2007; Belaj et al. 2010; Belaj et al. 2011) have also been reported. The M-TdS area under investigation is one of the few zones which still has retained a large local olive patrimony, probably originating from the initial introduction of ancestral varieties, followed by their cross breeding and empirical selection (Barranco and Rallo 2000). Although Andalusia, in southern Spain, is the main olive producing region, with more than 1.45 Mha (MAGRAMA 2014), olive growing is also one of the most important agricultural activities in northeastern Spain (Catalonia, Valencia and Aragon regions), with seven protected denominations of origin (PDO) and a large number of local varieties. They are still cultivated and preserved, both in *ex situ* regional collections (Paz et al. 2005; Tous et al. 2005) and at the World Olive Collection in Córdoba (Belaj et al. 2012), attesting to the richness of this local germplasm (Belaj et al.

2002; Belaj et al. 2004c; Belaj et al. 2007; Fernández i Martí et al. 2015; Sanz-Cortés et al. 2001; Sanz-Cortés et al. 2003).

On-farm conservation (Meilleur and Hodgkin 2004) complements efforts to preserve the diversity of cultivated species (Altieri and Merrick 1987) and also makes it possible to maintain the microbial and wild herb communities associated with the trees (Aranda et al. 2011). Creating catalogues of ancient monumental olive trees may represent a good first step towards their protection (Díez et al. 2004; Díez et al. 2011). This is very relevant because, particularly in recent years, the patrimony of ancient olive trees has suffered serious spoliation, with cases of trees being removed from their original locations and planted in gardens for ornamental purposes, or due to the progressive transformation of traditional olive groves into modern, intensive orchards (Tous et al. 2011). In this regard, inventorying, characterizing and conserving ancient olive trees *in situ* should be considered a priority tasks. This is especially important given the observation that numerous ancient olive trees represent previously uncatalogued varieties and thereby constitute a hitherto unexploited reservoir of genetic diversity (Díez et al. 2011).

The "Taula del Sénia" association (M-TdS, www.tauladelsenia.org) is an entity representing 27 municipalities from three different regions, Valencia (15), Catalonia (9) and Aragon (3), covering an area of 2,000 km^2, and it represents a clear example of the participatory on-farm conservation of olive genetic resources. The M-TdS area has a cultural landscape in which the olive groves play an outstanding social and economic role, and the ancient olive trees conserved under cultivation by many local farmers constitute a living heritage. The high concentration of ancient productive olive trees with semi-natural patches, and the historical man-made field margins, stone walls and plots, preserving a wealth of environments, make the M-TdS olive trees a valuable

example of a High Nature Value (HNV) permanent crop system (Andersen et al. 2003). Olive is the main crop in the M-TdS area, covering 15.5% of the total cultivated area. Many of the trees show an exceptional trunk size, a parameter considered directly related to age (Pannelli et al. 2010) and widely used as an indicator of multi-centennial olives (Arnan et al. 2012). The M-TdS territory is also located along what used to be the Via Augusta, the ancient Roman road connecting the Iberian Peninsula to Rome. This further suggests the possibility that olive growing in this region may have had its origins in the Roman period (Buxó 2008).

The four major cultivars grown in the area are 'Farga', 'Morrut', 'Canetera' and 'Sevillenca', although there are also some less represented cultivars (Íñiguez et al. 2001; Tous et al. 2005). Recently, the genome of one 'Farga' ancient tree, from M-TdS territory, has been sequenced (Cruz et al. 2016).

The present study was carried out in the M-TdS area within the framework of a national project whose main objectives were to preserve the genetic heritage of ancient olive trees and to promote their exploitation by local farmers. The following activities were carried out: a) exploration of the territory and cataloguing of the ancient olives trees, b) estimation of tree age, and c) characterisation, identification and evaluation of the local genetic diversity, at both nuclear and plastidial level. SSR markers, considered the most appropriate tools for accurate and reliable discrimination and identification of fruit crop varieties (Aranzana et al. 2010; Belaj et al. 2004a; Boccacci et al. 2008; Díaz et al. 2006), were used for molecular analysis. In addition, chloroplast markers were used to elucidate the maternal inheritance of the collected genotypes.

2. Material and Methods

2.1. Plant material: inventorying, sampling and measuring

A systematic survey of the M-TdS area, carried out by "Associació Taula del Sénia", allowed us to locate, identify and measure 4,526 ancient olive trees. The survey was performed within a limited geographic area between latitudes 40º26'-40º46'N (UTM: 31TBF16-BE73) and longitudes 0º01'-0º36'E (UTM: 31TBF48-BF99), and at altitudes ranging from 10 to 430 m above sea level, in a mostly flat territory (Figure 1a). Each tree was spatially localized by GPS (Oregon 400t, Garmin, Kansas) and individually labelled. GPS coordinates were plotted on Google Earth and a map was produced showing the distribution of the ancient olive trees (Figure 1b). For each tree, the trunk circumference at soil level and at 1.30 m above the ground (perimeter breast height: PBH), canopy height and diameter were recorded. Based on previous criteria established for olive trees (Díez et al. 2004; Díez 2008), only those with a PBH greater than 3.5 m were considered ancient monumental olive trees (Figure 2). The ancient trees were then classified into six levels of monumentality: (M1), trees with a PBH between 3.5 m and 4.0 m; (M2), 4.1 – 5.0 m; (M3), 5.1 – 6.0 m; (M4), 6.1 – 7.0 m; (M5), 7.1 – 8.0 m; and (M6), trees with a PBH of over 8.1 m. A table showing the main historical and climatic events (Figure S1) was drawn up in order to also take into account how history and climate over a long time-lapse may have influenced the establishment of olive growing in the area of interest, as well as tree survival and growth rates. The tree age was estimated by means of the three most used algorithms in olive tree based on trunk size: (1) Radial growth rate 0.8-1.5 mm/year (Michelakis 2002). It should be noted that, considering the data on pollen dating and the main historical and climatic events, this calculation was based on the highest growth rate (1.5 mm/year) to avoid overestimations of the age; (2) $y=5.2983x + 54.431$, where y=years and x=radius at a height of 1.0 m in cm (Pannelli et al. 2010), and (3), $y=2.1125x + 88.925$, where y=years and x=diameter at a height of 1.3 m in cm (Arnan et al. 2012).

147 *2.2. Morphological characterisation*

148 A preliminary discrimination of the surveyed trees included in the M3 to M6 groups of
149 trees (with PBH values exceeding 5 m), for a total of 852 productive ancient olive trees
150 (Figure 3), was performed through a morphological description limited to endocarp
151 traits, given the ease of sample management, high level of stability and discrimination
152 capacity (Belaj et al. 2011; Cantini et al. 1999; Trujillo et al. 2014). This description
153 included eleven qualitative and quantitative traits: weight, shape, symmetry in positions
154 "A" (maximum symmetry) and "B" (at 90º with respect to position "A"), position of
155 maximum diameter in position "B", shape of the apex and base in position "A", surface
156 roughness, the presence of a mucron, and the distribution and number of grooves (Rallo
157 et al. 2005). A representative sample of 25 endocarps per tree was studied. The
158 morphological profile of each ancient tree was defined as the combination of its level of
159 expression for each one of the 11 endocarp traits under evaluation. In addition, the
160 morphological profiles obtained in the present study were confronted with endocarps of
161 reference from olive trees of IRTA-Mas de Bover Olive Germplasm Collection (Rallo et
162 al. 2005; Tous and Romero-Aroca 1993).

163 *2.3. Genotyping by SSR markers*

164 A subset of 293 samples from the morphologically characterized M3-M6 trees was used
165 for SSR analysis, selected based on their exceptional size (263), collected from sites
166 with different soils and orchard management systems for territorial representation of the
167 entire M-TdS area and considered a priori as belonging to the 'Farga' cultivar. The
168 remaining samples (30) included both the ancient trees with undistinguished by
169 morphological analysis (19) as well as those that shared the endocarp profile of the
170 well-known local cultivars of 'Canetera' (3), 'Morrut' (7) and 'Sevillenca' (1). Four
171 reference DNA controls ('Farga', 'Morrut', 'Sevillenca' and 'Canetera') from the

IFAPA World Olive Germplasm Collection at Cordoba were included in each PCR-SSR run.

Total DNA was extracted from young leaf tissue following the CTAB method based on Doyle and Doyle (1990) and then stored at -20°C for further analyses. Eight SSR markers were used: DCA3, DCA7, DCA8, DCA9, DCA10, DCA11, DCA16 and DCA18 (Sefc et al. 2000). The SSR regions were amplified to a final volume of 20 µL containing 20 ng template genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs and 0.25 µM for each primer and 1.5 U GoTaq (Promega). Forward primers were labelled with one of the four fluorescent dyes, 6FAM™ (DCA10, DCA18), VIC® (DCA8, DCA16), NED™ (DCA7, DCA11) and PET® (DCA3, DCA9). The following PCR conditions were used: an initial denaturing step at 94ºC (2 min), then 35 cycles of 94ºC (25 s), 52-65ºC (20 s) and 72ºC (1 min), followed by a final elongation step at 72ºC (5 min) (Illa et al. 2011). Amplified fragments were separated in an automated sequencer, ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using GeneScan™ –500 LIZ® as Size Standard (Applied Biosystems). Fragment analysis was using the GeneMapper v.4.0 software (Applied Biosystems). Analysis was performed with two independent replicates and to confirm genotypes that differed in a few alleles two additional replicates were done.

For complete genotype identification, the SSR profiles obtained were also compared with those available on the IRTA-Mas de Bover Olive Germplasm Collection database, which currently includes more than 150 olive cultivars (116 non-redundant genotypes) from 13 countries, and with the CNR-IBBR olive database which has more than 3,000 genotypes. The resulting different genotypes were given a label (OMG) and a consecutive number.

2.4. DNA chloroplast polymorphism

The most polymorphic chloroplast markers available to date in olive (Besnard et al. 2011; Hosseini-Mazinani et al. 2014; Mariotti et al. 2010) were used to study the different SSR genotypes found among the monumental trees, a total of 44 chloroplast markers (SSRs, SNPs or indels) (Table S2). The primers and techniques used were as reported in Hosseini-Mazinani et al. (2014). To discriminate between different lengths, a fluorescent tail was annealed to each forward primer using two-step PCR as follows: first, 31 cycles of regular amplification were performed at 60°C Tm, followed by 14 tail annealing cycles at 52°C. Negative controls (no template DNA) were included in all experiments. All other conditions, which are not specified here, were taken from the SSR amplification protocol. For SNP identification, the SNaPshot Multiplex System technique was used according to the manufacturer's instructions (Life Technologies). The first PCR was performed using the same amplification conditions as those used for the SSRs. After this step, pre-amplicons were purified to remove primers and unincorporated dNTPs using ExoSAPIT (GE* Healthcare ExoSAPIT* PCR Purification Kit), and the next cycle was performed at 37°C for 45 min with a final step at 75°C for 15 min. The cpDNA profiles obtained for the ancient trees analysed were also compared with previously published olive chlorotypes (Besnard et al. 2011; Besnard et al. 2013; Mariotti et al. 2010).

2.5 Data analysis

The number of observed alleles (N_a), number of effective alleles (N_e), Shannon's Information Index (I), observed (H_o), expected (H_e) and unbiased expected (uHe) heterozygosity values, and fixation index (F) were obtained by GenAlex 6.5 (Peakall and Smouse 2006). The estimated frequency of null alleles ($F_{(Null)}$) was calculated for each microsatellite locus using the CERVUS v.3.0 software (Marshall et al. 1998). A first round dendrogram was constructed based on the neighbor joining (NJ) method

using DARwin software 6.0.1 (Perrier et al. 2003) with 10,000 bootstrap replicates. The obtained tree was visualized with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/). A second dendrogram based only on polymorphic genotypes was constructed using the NJ method with MEGA6 software (Tamura et al. 2013).

3. Results

3.1. Prospecting survey and age estimation of ancient olive trees

A total of 4,526 ancient trees with trunk circumference of more than 3.5 m were inventoried in the M-TdS area (Figure 1a, Table 1) by the "Taula del Sénia" Association. Depending on the trunk circumference, the ancient olive trees were classified into six monumental categories, M1 to M6, from lowest to highest degree of monumentality. Most of them (73%) fell within the M1 and M2 categories (with PBH values between 3.5 and 5.0 m), whereas 1,187 (26%) were classified in the M3 to M5 categories (PBH values between 5.1 and 8.0 m) and 41 trees (0.9%) in M6, with PBH over 8 m, one exceeding 10 m in circumference (Table 1). These values were used to estimate the age of the trees with three algorithms. The results using the methods from Arnan et al. (2012) and Pannelli et al. (2010) were similar, with estimated ages ranging from 324 to 775 years and from 350 to 915 years, respectively. In contrast, the Michelakis method (Michelakis 2002) gave a higher age estimation, with values which ranged from 371 to 1,082 years. The oldest trees had probably been planted in the Middle Ages, during the Muslim occupation of the Iberian Peninsula or during the reconquest by Christians forces: this would have coincided with the Medieval Warm Period (MWP) (Figure S1). The geographical distribution of ancient olive trees shows the greatest density was in the valleys (Figure 1b). At some locations, more than 27 ancient olive trees/ha were found.

247 The further morphological and molecular analyses focused on the M3 to M6 groups of
248 trees (Figures 2 and 3).

249 *3.2. Morphological characterization*

250 The morphological characterisation of 852 productive ancient trees (PBH values > 5m)
251 by means of 11 endocarp traits, revealed variability in all but one trait (the mucron)
252 (Table 2). The number of observed states per trait ranged from 1 (mucron) to 3 (several
253 traits). The position of maximum endocarp diameter proved to be the most
254 discriminating trait, whereas symmetry and surface traits were intermediate states. No
255 ovoid or spherical stone shapes were found amongst the trees analysed. The 11
256 endocarp traits discriminated 14 different morphological profiles among the 852 ancient
257 trees under study (Table 2 and Figure 3). As expected, most of these trees (822)
258 consistently displayed the same endocarp morphology as the main local cultivar 'Farga'
259 (Rallo et al. 2005; Tous and Romero-Aroca 1993), while seven had endocarp traits
260 similar to 'Morrut', one to 'Sevillenca' and three to 'Canetera'. The endocarp profile
261 observed in the 19 remaining trees had ten different profiles, none coinciding with any
262 of the local cultivars. Nine of them were represented by only one olive tree while the
263 other profile was shared by ten olive trees.

264 *3.3. Nuclear (SSR) and chloroplast discrimination of ancient olive trees*

265 Eight SSR markers were used to genotype 293 ancient olive trees, identifying 73 alleles,
266 with an average of 9.1 alleles per locus (Table 3), ranging from six (DCA3 and DCA11)
267 to twelve (DCA8). Nearly half of these alleles were present at very low frequencies
268 (1%). At all the loci except DCA7, the observed heterozygosity (H_o) was always higher
269 than the expected heterozygosity (H_e), with H_o values ranging from 0.27 (DCA7) to
270 1.00 (DCA16), with an average of 0.89. The low heterozygosity observed for DCA7

may be due to the presence of null alleles at this locus or the large number of repeated samples of cv. Farga.

The eight SSR markers used showed a high capacity of discrimination, with a total of 43 different profiles (Table 4 and Figure 3). In general, a good level of concordance (more than 65%) was found between endocarp morphology and the SSR patterns for ancient trees. Similarly, three different SSR profiles were found for the eleven ancient trees classified as 'Canetera', 'Morrut' and 'Sevillenca'.

Eight out of ten genotypes, not corresponding to the endocarp profiles of local cultivars, exhibited a single SSR pattern and were confirmed as belonging to hitherto unidentified genotypes (OMG32-OMG39). Five of them shared at least one allele for each SSR locus with the cultivar 'Farga', while the remaining genotypes had two allelic differences with the cultivar 'Sevillenca' (OMG31) and three with 'Farga'(OMG30) (Table 4 and Figure 3). Allelic differences were also identified among 98 of the 263 ancient trees defined morphologically as 'Farga', resulting in 30 different SSR profiles (OMG01-OMG29). Differences of only one allele from the reference cv. 'Farga' were found in 87 trees, while the remaining 11 trees differed by two alleles. In many cases, these small differences were shared by more than one ancient tree (Table 4 and Table S1).

Among the 43 profiles previously discriminated by SSR markers, chloroplast polymorphisms revealed two chlorotypes (E3.1 and E1.1) (Table S2). The local cultivars 'Farga' 'and Canetera' shared the same cpDNA haplotype (E3.1), while the cultivars 'Morrut' and 'Sevillenca' had the haplotype E1.1. The haplotype E3.1 was also shared by all the ancient trees, with the same and/or very similar SSR profiles to 'Farga' (up to three different alleles) (Table 4 and Figure 3). Five trees, with unknown endocarp profiles but sharing one allele per locus with the cultivar 'Farga' (OMG33-

296 OMG36 and OMG38), had the chlorotype E3.1, highlighting a possible relationship as
297 seedling or parent of cv. Farga. The chlorotype E1.1 was detected in three genotypes
298 with unknown endocarp profiles and specific SSR patterns and it was also found in the
299 ancient tree with small SSR allelic differences (three alleles) compared to the reference
300 cv. 'Sevillenca'.

301 *3.4. Genetic relationships among the ancient trees*

302 To show the genetic relationships between the 293 ancient olive trees analysed by the
303 eight SSR loci, a Neighbour Joining dendrogram was constructed (Figure 4a). For all
304 analysed genotypes, 165 were identical to the cultivar 'Farga', while 98 olive trees
305 clustered close to this variety, differing only by one or two alleles. As expected, three
306 ancient olives clustered with cv. Canetera, while seven had the same genotype as
307 'Morrut' and only one was identical to 'Sevillenca' (Figure 4a). When the SSR profiles
308 of these 19 ancient trees (ten different genotypes) were compared with those of all
309 genotypes included in the IRTA-Mas de Bover Olive Germplasm Collection and with
310 the CNR-IBBR olive database, no identity with previously analysed varieties was
311 found. Due to the large number of redundant ancient olive trees which shared the same
312 genotype, only one tree per SSR profile was included in the Neighbor Joining tree
313 construction by MEGA6 (Figure 4b). The dendrogram showed three main clusters: one
314 related to the 'Farga' cultivar containing genotypes with few different alleles and its
315 possible seedlings, the second with 'Canetera' and 'Morrut' varieties, and the last
316 cluster related to the 'Sevillenca' cultivar. In regard the endocarp profiles (Figure 4c) it
317 was observed that all olive trees differing only by one or two alleles showed a unique
318 endocarp profile which correspond to 'Farga' endocarp profile. The rest of the ancient
319 olive trees showed a different endocarp profile.

320 **4. Discussion**

This survey represents the first attempt to characterize a large representative sample of a very large number of ancient olives (4,526), spread over the restricted M-TdS area and presumably planted within a fairly narrow period of time, sharing the same planting techniques, pedo-climatic conditions and common cultural practices. All the ancient trees had exceptional trunk size, with circumferences of over 3.5 m.

To the best of our knowledge, this is the most complete case-study of ancient cultivated olive trees at molecular and morphological levels. In previous studies, a variable number of trees with diverse tree size and estimated age have been considered (Cipriani et al. 2002; Erre et al. 2010; Michelakis 2002; Pannelli et al. 2010; Salimonti et al. 2013). The broadest study of such a type included 310 trees from 32 groves in Israel and Palestinian territories (Barazani et al. 2014), while in Southern Spain (Andalusia), in a larger area than that considered in our study, only 160 ancient olive trees were prospected and analysed (Díez et al. 2011).

Estimating the age of these olive trees represents a very challenging task as the identification and interpretation of the annual tree rings is complicated, the inner part of the trunk is frequently absent due to wood rotting, and, with aging, a beam of many independent trunks replaces the original single tree trunk (Arnan et al. 2012; Cherubini et al. 2013; Pannelli et al. 2010). Furthermore, there is still lack of information on factors directly affecting plant growth and wood decay, such as olive wood physiology and wood development (Díez et al. 2011; Michelakis 2002). These factors may result in different growth speeds and distort interpretations of tree age. However, recent studies (Arnan et al. 2012; Díez et al. 2011; Michelakis 2002; Pannelli et al. 2010) on olive age have evidenced the utility of the algorithms, which are based on trunk size to estimate age of ancient olive trees. In this sense, it is worth mentioned that similar age estimations were obtained by using the algorithm described by Arnan et al. (2002) and

346 Pannelli et al. (2010), indicating the suitability of them. Consequently, based on these
347 calculations, it is conceivable that the age of most ancient trees found in the M-TdS area
348 ranges from 324 to 1,082 years. Similar age ranges (313-737 years) have been
349 previously estimated for olive trees in a neighbouring area (Montsià, Catalonia) of
350 North-East Spain (Arnan et al. 2012). Although there is no direct historical evidence for
351 the age of these olive trees, Cavallines (Cavanilles 1797) described very large
352 specimens of olive trees in the area in the late eighteenth century. According to these
353 authors, several olive trees included in the present study should be considered close to
354 the oldest recognised to date in the Mediterranean basin.
355 Despite the limited geographic area, a certain level of diversity among tree genotypes
356 was observed, similar to or even greater than that observed among other ancient olives
357 (Belaj et al. 2012; Charafi et al. 2008; Díez et al. 2011; Erre et al. 2010; Ipek et al. 2012;
358 Khadari et al. 2008; La Mantia et al. 2005; Lopes et al. 2004; Salimonti et al. 2013),
359 even though the presence of two varieties carrying the same rare chlorotype supports the
360 assumption that a local selection of varieties has occurred. The high H_o values
361 registered, at almost all loci, may be due to the male-sterility of 'Farga', which could
362 have favoured interbreeding among varieties.
363 'Farga' resulted as the main cultivar grown in the M-TdS area (Tous and Romero-Aroca
364 1993) and the predominant cultivar (97%) among the ancient trees studied, as the
365 preferred variety by past farmers, probably due to its high vigour and capacity to adapt
366 to poor soils. 'Farga' is characterized by the rare E3.1 chlorotype (Mariotti et al. 2010),
367 shared with a few other cultivars, such as the other local variety 'Canetera', the French
368 'Olivière' and the Sicilian 'Cerasuola', all male sterile cultivars. In fact, it has been
369 suggested that some polymorphisms in the chloroplast and mitochondrial genomes are
370 related to the male-sterility character (Besnard et al. 2000; Besnard et al. 2011), and

varieties carrying this chlorotype are all male-sterile. The E3.1 chlorotype, exclusively found in wild trees of the Western and Central Mediterranean Basin (Besnard and Bervillé 2002; Besnard et al. 2013), may have been introduced in the two local male-sterile varieties 'Farga' and 'Canetera' through breeding with wild plants or as their direct introduction into cultivation, as it has been reported for other olive cultivars (Belaj et al. 2010; Besnard et al. 2013; Breton et al. 2006; Breton et al. 2008).

Quite a high percentage of ancient trees (37.3%) shared the same endocarp profile to 'Farga' and had very similar SSR profiles, differing by only one or two alleles. These differences could be real or due to misinterpretations of alleles differing only by two base pairs (Baldoni et al. 2009; Charafi et al. 2008; Cipriani et al. 2002; Díez et al. 2011; Ipek et al. 2012; Khadari et al. 2008; Lopes et al. 2004; Muzzalupo et al. 2010). For this reason, samples showing this closeness were amplified and run twice to verify these small variations, and these subtle differences were confirmed. They are difficult to explain and numerous hypotheses may be conceived. Their origin as 'Farga' seedlings can be excluded because, due to its male sterility, they would have resulted from crossing, so resulting in a higher percentage of alien alleles. The small allelic differences could have originated from an accumulation of somatic mutations during the lifetime of ancient olive trees, as reported by some authors (Belaj et al. 2004b; Cipriani et al. 2002; Díez et al. 2011; El Bakkali et al. 2013; Sanz-Cortés et al. 2003). In fact, the probability of finding mutant loci may increase with tree age (Crespan 2004; Klekowski and Godfrey 1989; Petit and Hampe 2006). Olive plants older than four hundred years may have accumulated mutations, particularly in their microsatellite regions (Crespan 2004; Franks et al. 2002), without exhibiting any clear phenotypic difference. The numerous cases of intra-cultivar diversity previously reported in a wide range of olive genotypes, including 'Farga' (Sanz-Cortés et al. 2003) and revealed by different DNA

markers (Belaj et al. 2004b; Charafi et al. 2008; Cipriani et al. 2002; Díez et al. 2011; Gemas et al. 2000; Gomes et al. 2008; Khadari et al. 2008; Lopes et al. 2004; Muzzalupo et al. 2008; Ozkaya et al. 2008; Strikic et al. 2011), could be due to the use of previous generation markers or di-nucleotide SSRs, as in this case. But, the combined use of SSR markers and endocarp descriptors enabled us to identify ten previously unknown genotypes in 19 ancient olive trees with unique endocarp and SSR profiles. Two of these genotypes, with SSR patterns very similar to the cultivars 'Sevillenca' and 'Farga' (differences of only two or three alleles, respectively), but different endocarp profiles, are interesting cases of slight molecular variants combined with phenotypic differences. This finding supports the hypothesis that mutations may have also occurred in genes related to phenotypical traits, so producing visible changes. Similar results have been recently found, where few genotypes had little molecular differentiation but considerable variations in morphological traits (Trujillo et al. 2014). However, the possibility of changes due to phenotypic plasticity can not be ruled out.

Ancient trees, from the M-TdS, not related to known varieties could represent olive cultivars so far uncharacterized. Their classification as different cultivars is justified by the fact that, in some cases, different trees showed the same genotype, indicating they were vegetatively propagated, as any other cultivar. In these uncharacterized genotype, leaves were characterized by a longitudinal helical shape of their leaves, a quite rare trait among olive cultivars (Barranco et al. 2000). These trees could belong to an ancient local cultivar, possibly originating from a different gene pool than the other local varieties analysed.

5. Conclusions

Our results indicate that the ancient olive trees in the M-TdS area in the northeast Iberian Peninsula, preserved on-farm by local farmers, are a unique living and

exploitable heritage, represented by very ancient up to one thousand years old trees, holding profiles corresponding to well established varieties together with a number of closely-related genotypes showing in some cases also phenotypical differences. These trees carry a reservoir of genetic diversity that includes characteristics associated with resilience and adaptation to specific environmental conditions, and their longevity may be linked to their tolerance to unfavourable climatic conditions. New strategies for their conservation and exploitation should be defined. It is worth mentioning that most of the ancient olive trees analysed in the present study are currently used to produce extra virgin olive oil, marketed under the brand name "Millennium Oil", with a potential annual production capacity of 18,000-50,000 L, providing a potential supplementary source of income for local farmers.

Conflicts of interest

The authors state no conflicts of interest.

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722

Figure 1 Geographic area of prospecting in the north-east of the Iberian Peninsula (a) and distribution of the ancient monumental olive trees (b)

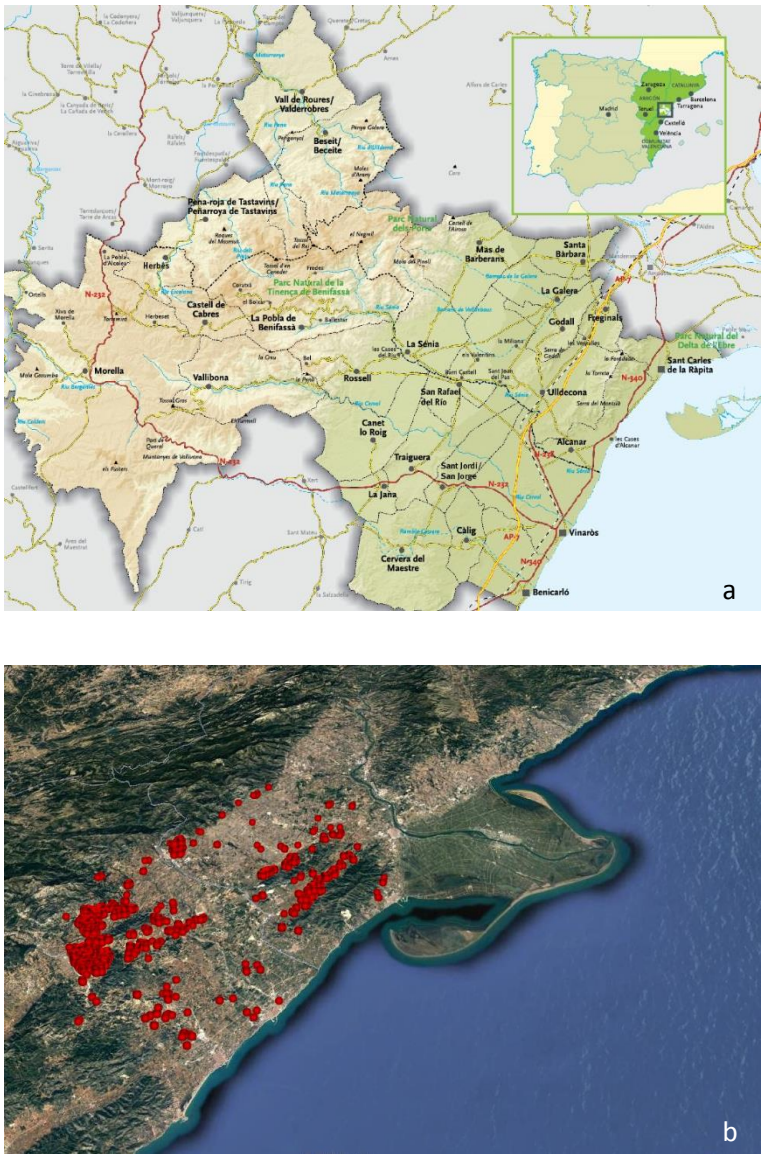

Figure 2 Ancient monumental olive trees inventoried in the M-TdS area. More than 4,500 trees with trunk circumferences of over 3.5 m (PBH) were included in the inventory. These included 41 olive trees with trunk circumferences of over 8 m. Specimen of PBH=9.00 m and age estimated of 694-955 years.

Figure 3. Global scheme of the study

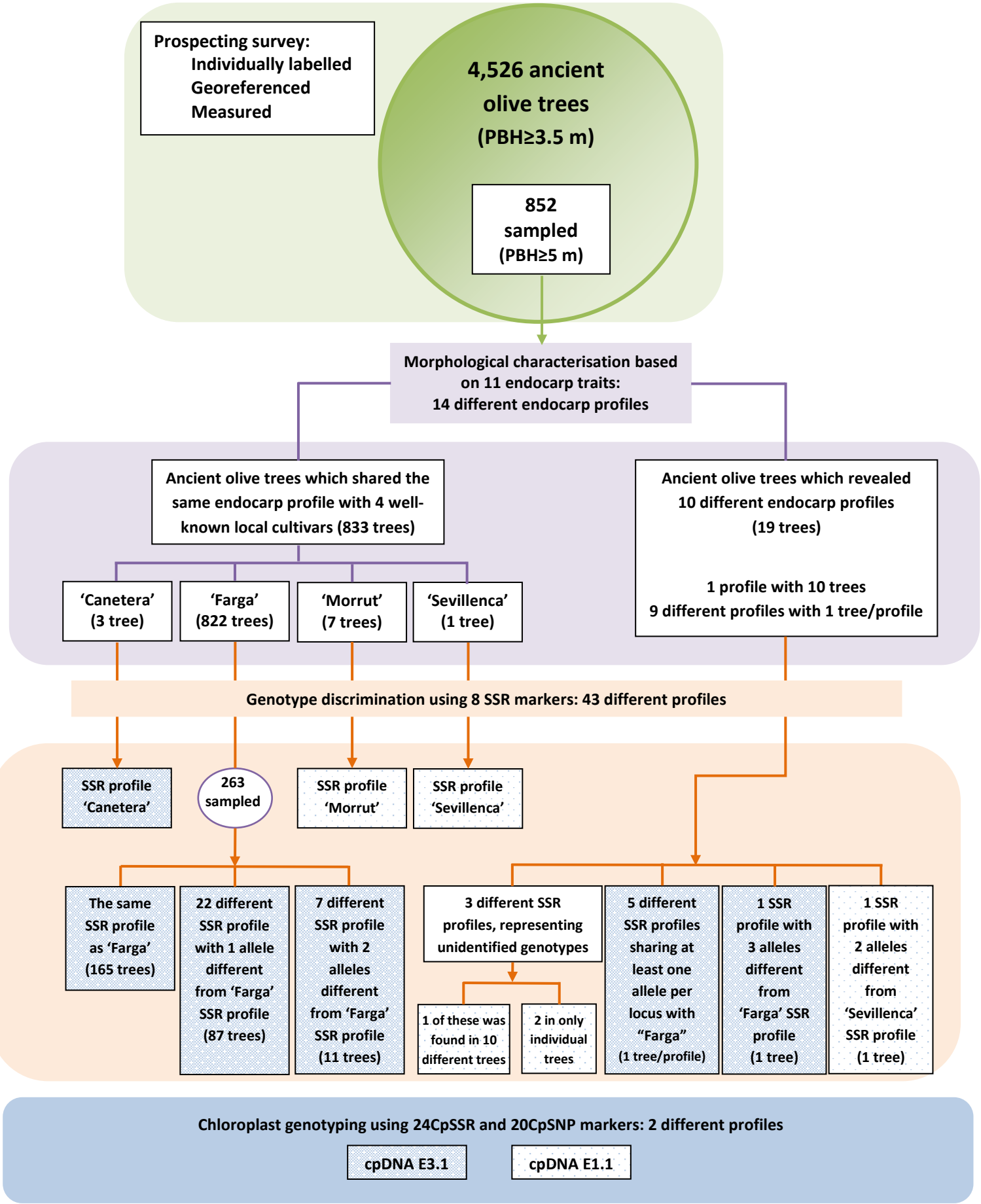

Figure 4 Phylogenetic analysis: NJ round tree by Darwin software, for the 293 ancient trees analysed (a) NJ dendrogram of the 43 genotypes of ancient olives identified by MEGA software (b); and endocarp profile (each colour represents a different profile) (c)

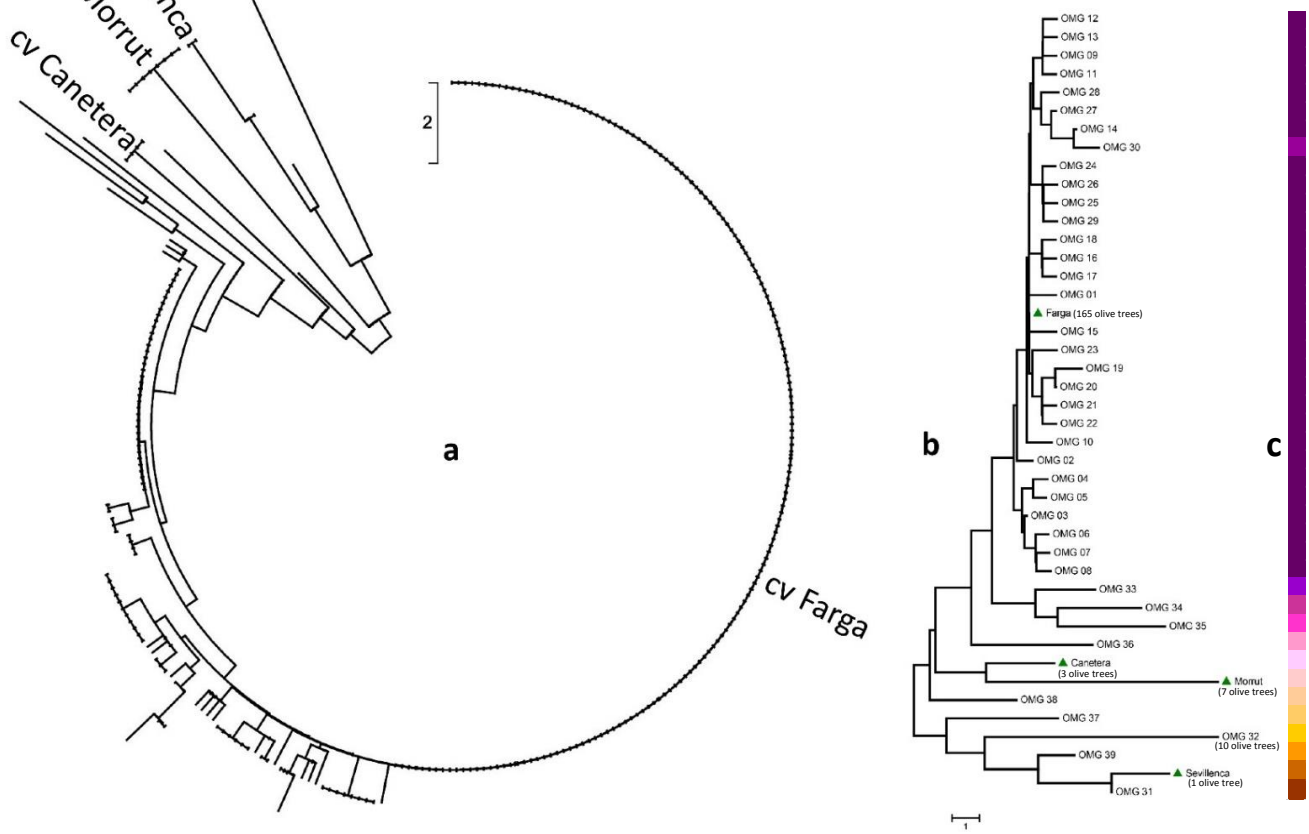

Table 1 Total ancient olive trees included in the inventory (4,526), the categories of monumentality (M1-M6) and their estimated ages (years) based on different algorithms

Algorithm	M1 (3.5 m-4.0 m)[A]	M2 (4.1 m-5.0 m)[A]	M3 (5.1 m-6.0 m)[A]	M4 (6.1 m-7.0 m)[A]	M5 (7.1 m-8.0 m)[A]	M6 (8.1 m-10.2 m)[A]	M3-M6 population
1 (Michelakis 2002)	371-424	435-531	541-637	647-743	753-849	859-1,082	1,103[B]
2 (Pannelli et al. 2010)	350-392	400-476	484-560	569-645	653-729	737-915	513[B]
3 (Arnan et al. 2012)	324-358	365-425	432-492	499-560	566-627	634-775	455[B]
Number of olive trees	1,409	1,889	857	241	89	41	1,228[B]

[A] Circumference in metres at 1.3 m above soil level.

[B] Weighted mean.

1 (Michelakis 2002): Radial growth rate: 1.5 mm/year.

2 (Pannelli et al. 2010): $y=5.2983x + 54.431$; y (years), x (radius at a height of 1.0 m, in cm).

3 (Arnan et al. 2012): $y=2.1125x + 88.925$; y (years), x (diameter at a height of 1.3 m, in cm).

Table 2 Morphological traits of the endocarps and the endocarp profiles found in 852 ancient olive trees

Number of trees	Endocarp profile[1]	Morphological characteristics of the endocarp										
		Weight[2]	Shape[3]	Symmetry (position A)[4]	Symmetry (position B)[5]	Position of maximum diameter (position B)[6]	Apex shape (position A)[7]	Base shape (position A)[8]	Surface[9]	Mucron[10]	Distribution of grooves[11]	Number of grooves[12]
822	1, Farga[A]	M	EL	A	SA	A	P	P	S-R	P	R	M
7	2, Morrut[B]	H	EP	SA	S	A	R	P	S	P	R	M
1	3, Sevillenca[C]	M	EL	SA	S	C	P	P	R	P	G	M
3	4, Canetera[D]	M	EL	SA	S	A	P	P	S-R	P	G	M
1	5	L	EL	SA	S-SA	C	P	P	S-R	P	R	M
1	6[E]	M	EL	SA	S	C	P	P	R	P	R	H
1	7	L	EL	SA-A	S-SA	B	P	P	S-R	P	R	M
1	8	M	EP	SA	SA	B	P	R	R	P	R	M
1	9	M	EL	A	SA	C	P	P	S-R	P	R	L
10	10[F]	M	EL	S-SA	S	B	P	P	R	P	R	M
1	11	H	EL	SA-A	S-SA	B	P	P	R	P	R	M
1	12	H	EL	S-SA	S	C	P	P	R	P	R	L
1	13	L	EP	SA	S	C	R	P	S-R	P	R	M

1	14	L	EL	SA	S-SA	A	P	P	S-R	P	R	M

[A]Endocarp profile identical to that of the 'Farga' cultivar; [B]Endocarp profile identical to that of the 'Morrut' cultivar; [C]Endocarp profile identical to that of the 'Sevillenca' cultivar; [D]Endocarp profile identical to that of the 'Canetera' cultivar; [E]Weeping growth habit; [F]Helical curvature of the leaf blade along the longitudinal axis.

[1]Olive endocarp profile: codes from 1 to 14 were assigned based on different morphological profiles.

[2]Weight: low=L (<0.3 g); medium=M (0.3-0.45 g); high=H (0.45-0.7 g); very high=VH (>0.7 g).

[3]Shape: EP= spherical or elliptical (length/width 1.8-2.2); EL= elongated (length/width >2.2).

[4]Symmetry (position A): S=symmetric; SA=slightly asymmetric; A=asymmetric.

[5]Symmetry (position B): S=symmetric; SA=slightly asymmetric.

[6]Position of maximum diameter (position B): B=towards base; C=central; A=towards apex.

[7]Apex shape (position A): P=pointed; R=rounded.

[8]Base shape (position A): P=pointed; T=truncated; R=rounded.

[9]Surface: S=smooth; R=rough; SC=scabrous.

[10]Mucron: P=present; A=absent.

[11]Distribution of grooves: R=regular; G=grouped around the suture.

[12]Number of grooves: L=low (<7); M=medium (7-10); H=high (>10).

Table 3 Summary of statistics for eight microsatellite markers on 293 ancient olive trees

Locus	N_a	Alleles (bp)	PIC	$F_{(Null)}$	H_o	H_e
DCA3	6	**243**,247,**249**,251,253,257	0.457	-0.294	0.990	0.557
DCA7	8	141,148,150,152,**154**,156,158,170	0.284	+0.106	0.268	0.297
DCA8	12	129,**135**,137,139,141,143,156,163,**167**,169,171,173	0.547	-0.247	0.962	0.621
DCA9	11	169,173,**179**,183,188,190,**192**,194,196,200,213	0.478	-0.288	0.997	0.572
DCA10	11	113,136,**145**,148,152,156,158,**160**,162,164,199	0.493	-0.279	0.993	0.582
DCA11	6	**128**,133,137,**143**,150,164	0.477	-0.283	0.972	0.571
DCA16	9	**122**,124,146,**150**,154,156,160,174,176	0.471	-0.293	1.000	0.566
DCA18	10	163,**165**,167,**169**,171,173,175,177,179,183	0.503	-0.268	0.961	0.589
Mean	9.13		0.464	-0.231	0.893	0.545

Alleles shown by the reference cv. 'Farga' are highlighted in bold.

Alleles with frequencies of less than 1% are underlined.

N_a: Number of alleles; PIC: Polymorphic Information Content; $F_{(Null)}$: Estimated frequency of null alleles; H_o: Observed heterozygosity; H_e: Expected heterozygosity.

Table 4 Number of trees as belonging to specific cultivar, SSR alleles, chlorotypes and endocarp profile of a representative sample (293) of the ancient olive trees conserved on-farm in the North-East of the Iberian Peninsula by the "Taula del Sénia" Association

Number of trees	SSR profile								Chlorotype	SSR identification	Endocarp profile	Observations
	DCA3	DCA7	DCA8	DCA9	DCA10	DCA11	DCA16	DCA18				
165	243-249	154-154	135-167	179-192	145-160	128-143	122-150	165-169	E3.1[3]	cv. 'Farga'	1	Local cultivars
7	247-249	158-170	143-156	192-213	152-199	150-150	150-176	171-183	E1.1	cv. 'Morrut'	2	
1	243-257	148-170	141-156	169-188	158-160	133-164	154-174	173-175	E1.1	cv. 'Sevillenca'	3	
3	249-249	154-156	143-171	192-200	152-160	128-150	122-124	165-169	E3.1	cv. 'Canetera'	4	
1	243-253	152-154	137-139	190-200	145-160	143-164	122-160	173-175	-	OM037	9	Unidentified genotypes, probably belonging to ancient unknown cultivars
10	243-253	150-170	141-141	179-200	113-158	137-164	124-146	179-179	-	OM038	10	
1	243-257	148-170	141-156	169-188	158-160	128-143	122-150	173-175	-	OM039	12	
1	249[1]-257	141-154	129-167	173-179	145-145	128-143	146-150	165-165	E3.1	OM031	5	Unidentified genotypes, probable 'Farga' seedlings
1	249-257	141-154	143-167	173-192	145-145	128-128	122-146	169-177	E3.1	OM032	6	
1	249-253	154-170	141-167	179-200	145-160	143-150	150-156	169-171	E3.1	OM033	8	
1	243-257	154-156	167-167	173-192	145-160	128-143	146-150	165-169	E3.1	OM034	11	
1	243-247	154-170	156-167	179-179	145-148	128-143	122-124	169-179	E3.1	OM035	14	
1	243-249	154-154	135-**169**[2]	179-**196**	145-160	128-143	122-150	**167**-169	E3.1	OM030	13	Probable somatic

												mutation of cv. ‘Farga’, with a different phenotype
87	Up to 22 SSR profiles with only one dissimilar allele (See Additional file 2)									E3.1	OM001-	1	Probable molecular variants of cv. ‘Farga’
11	Up to 7 SSR profiles with 2 dissimilar alleles (See Additional file 2)									E3.1	OM029	1	
1	243-257	148-**154**	141-156	169-188	**145**-160	133-164	154-174	173-175		-	OM036	7	Probable somatic mutation of cv. ‘Sevillenca’, with a different phenotype

[1]Underlined: possible maternal alleles shared with cv. 'Farga'.

[2]Bold: allelic differences with respect to the reference cultivar.

[3]E 3.1: chlorotype of cv. ‘Farga’ (Besnard et al. 2011; Mariotti et al. 2010).