sustainability

MDPI

Article

Exploring Olive Genetic Diversity in the Maltese Islands

Maria Cristina Valeri [1], David Mifsud [2], Clayton Sammut [2], Saverio Pandolfi [1], Emanuele Lilli [1], Marina Bufacchi [3], Vitale Stanzione [3], Valentina Passeri [3], Luciana Baldoni [1], Roberto Mariotti [1,*] and Soraya Mousavi [1]

[1] Institute of Biosciences and Bioresources, National Research Council, 06128 Perugia, Italy
[2] Institute of Earth Systems, Division of Rural Sciences and Food Systems, University of Malta, 2080 Msida, MSD, Malta
[3] Institute for Agricultural and Forest Systems in the Mediterranean, National Research Council, 06128 Perugia, Italy
* Correspondence: roberto.mariotti@ibbr.cnr.it; Tel.: +39-075-5014811

Abstract: A comprehensive effort was devoted to exploring, collecting and characterizing the local Maltese olive germplasm, often represented by ancient, monumental trees and by plants of uncertain origin. SSR and cp-SSR analysis of all samples enabled the identification of 46 genotypes and establishment of the correspondence between ancient trees, main local varieties and other Mediterranean cultivars. The application of plastid markers enabled identification of two lineages among Maltese genotypes, with more than 50% represented by lineage E2. Twenty-nine cases of grafting were identified among the various genotypes and lineages. In most cases, E1 canopies were grafted on E2 rootstocks, but reverse cases were also observed. The phylogenetic study of Maltese genotypes, together with hundreds of cultivars from the Mediterranean Basin and beyond, highlights the richness of Maltese olive diversity and drawing attention to the genetic similarity of some Maltese olive genotypes with neighboring Italian and Algerian varieties. These results underline the long-lasting presence of the olive in the country, contributing to the reconstruction of its phylogeny and demonstrating a possible autochthonous origin of many samples. Some still-living ancient trees are at serious risk of extinction due to abandonment, urban expansion and environmental threats. This study supports the preservation of the Maltese olive germplasm and highlights its importance as a rich genetic source to face new agronomical challenges and future climatic constraints.

Keywords: Malta; olive genotypes; ancient olives; gene flow; grafting; chloroplast lineage; local genotypes

Citation: Valeri, M.C.; Mifsud, D.; Sammut, C.; Pandolfi, S.; Lilli, E.; Bufacchi, M.; Stanzione, V.; Passeri, V.; Baldoni, L.; Mariotti, R.; et al. Exploring Olive Genetic Diversity in the Maltese Islands. *Sustainability* **2022**, *14*, 10684. https://doi.org/10.3390/su141710684

Academic Editor: Matteo Convertino

Received: 20 July 2022
Accepted: 24 August 2022
Published: 27 August 2022

1. Introduction

Olive (*Olea europaea* subsp. *europaea*), with a vast genetic heritage represented by cultivated (var. *europaea*) and wild (var. *sylvestris*) forms, has not suffered significant genetic loss, preserving its variability almost intact [1–3]. The domesticated olive comprises only a subset of the entire genetic variation in extant *O. europaea* [4]. The olive gene pool contains many autochthonous varieties, pollinizers, ecotypes and feral trees in various microenvironments and growing conditions [5–7]. Cultivated olives, with almost 1200 cultivars, coexists with the oleaster in areas around the Mediterranean basin [2,8]. Ancient olive trees have been reported throughout the entire Mediterranean area [9–14] and beyond [15]. These plants are a part of the cultural heritage of the region and are of invaluable agroecological interest due to their stress-tolerance and adaptability, constituting an underexploited pool of useful diversity [9,11,16].

Olive cultivation developed significantly in the eastern Mediterranean at least 6000 ya (years ago), spreading toward Anatolia and to Cyprus Island and from Crete Island toward Egypt [2,17], with subsequent expansion into the western Mediterranean, conveyed by Phoenicians, Greeks and Romans [8,18], who likely transported vegetative material from the center of origin toward the west, spreading cultivars throughout the Mediterranean [19].

The best cultivars were likely selected for their agronomic interest (e.g., high fruit set, large fruit size and high oil content), for their ability to grow in various environments and the ease of vegetative propagation through cuttings or grafting, techniques known since ancient Greece [20,21]. Grafting represents a major innovation in the history of fruit cultivation, and application of this technique has likely favored the spread of olive from the Middle East to western Europe [22]. The grafting of cultivated genotypes onto home-grown oleasters or overgrafting on ancient, poorly-performing trees is a tradition and a peculiarity of olive cultivation [9,11,23].

Traditional olive cultivars, mostly represented by monumental trees, constitute an important genetic patrimony that could represent a useful source of genetic diversity in the fight against climate change [24–26]. The genetic and phenotypic variability of these local varieties should be preserved and exploited, with a view toward variability protection and for their diffusion in eco-sustainable groves. Despite the richness of the olive varietal patrimony, few cultivars contribute a major portion of olive oil and table olives for commercial production, while the majority of them have only a limited local significance represented by few trees [24–27].

In recent years, the distribution and the genetic differentiation of olive has been the subject of numerous phylogeographic and phylogenetic studies involving the use of nuclear, chloroplast and mitochondrial markers and the application of archaeological, historical and ecological information [1,2,15,19,21]. The high level of polymorphism and transferability among olive species make SSRs the current markers of choice for identification and variability studies [10,15,28,29]. Cytoplasmic markers such as chloroplast may enable discrimination among cultivated olives, wild olives and other subspecies [30,31]. Three plastidial DNA lineages (E1, E2 and E3) have been characterized, but most olive cultivars possess the E1 chlorotype, and only a small percentage of them show the other two lineages typical of the Mediterranean wild olive populations [2]. SNP markers, as new application opportunities, could be useful to explore diversity within wild and cultivated olive genotypes [32].

The Phoenicians were the first to introduce olive in Malta; then, its distribution was maximized by Romans [33]. Olive oil production was extended through Roman domination, as evidenced by the presence of olive mills referred to that period (*trapetum*), such as that at San Pawl Milqi in the area of Burmarrad, where a remnant stand of indigenous olives has been reported, with trees considered to be as many as 1200 years old [34]. The Bidnija grove was established during the middle-late Medieval period, before 1450–1669 CE, rather than in considerably earlier Roman times, when the surrounding landscape could have been an important production area for olives [35]. In the Maltese Islands, three main local cultivars, namely Bidni, Malti and Bajda (or White Olive), were previously identified [36], in addition to rare wild olives. In Malta, the Italian cultivars Frantoio, Leccino, Carolea, Pendolino, Coratina, Ogliarola and Cipressino and French Picholine (Picholine Languedoc) are cultivated for olive oil, whereas Uovo di Piccione and Bella di Spagna are cultivated for table olives. The Maltese local varieties are still present in the islands, and recovery of their cultivation is being prompted despite the expansion of foreign varieties [36].

The issues addressed in our study refer to basic questions relating to the origin of the Maltese local olive germplasm and the role played by this small but ancient islands in the center of the Mediterranean in the development of Mediterranean olive varieties.

2. Materials and Methods

2.1. Plant Material and DNA Extraction

A total of 98 olive trees were sampled from the Maltese archipelago (Table S1), including the three Islands: Malta, Gozo and Comino (Figure 1). All trees were sampled from historical sites, such as Roman villas, ancient olive mills, ancient gardens or old farmland, represented also by ancient plants, based on trunk and stump size, with more than 1.0 m trunk diameter at 130 cm from the soil. Many ancient olive plants in several Mediterranean areas are grafted onto other varieties or wild olives [9,11]. In order to detect eventually

grafted trees, leaf samples were separately collected from canopy, rootstock and, in two cases, from trunk shoots, for a total of 190 samples. From eight trees, leaves were collected from the canopy only, as they were represented by bushes (Table S1). Total DNA was extracted using a GeneElute plant genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions.

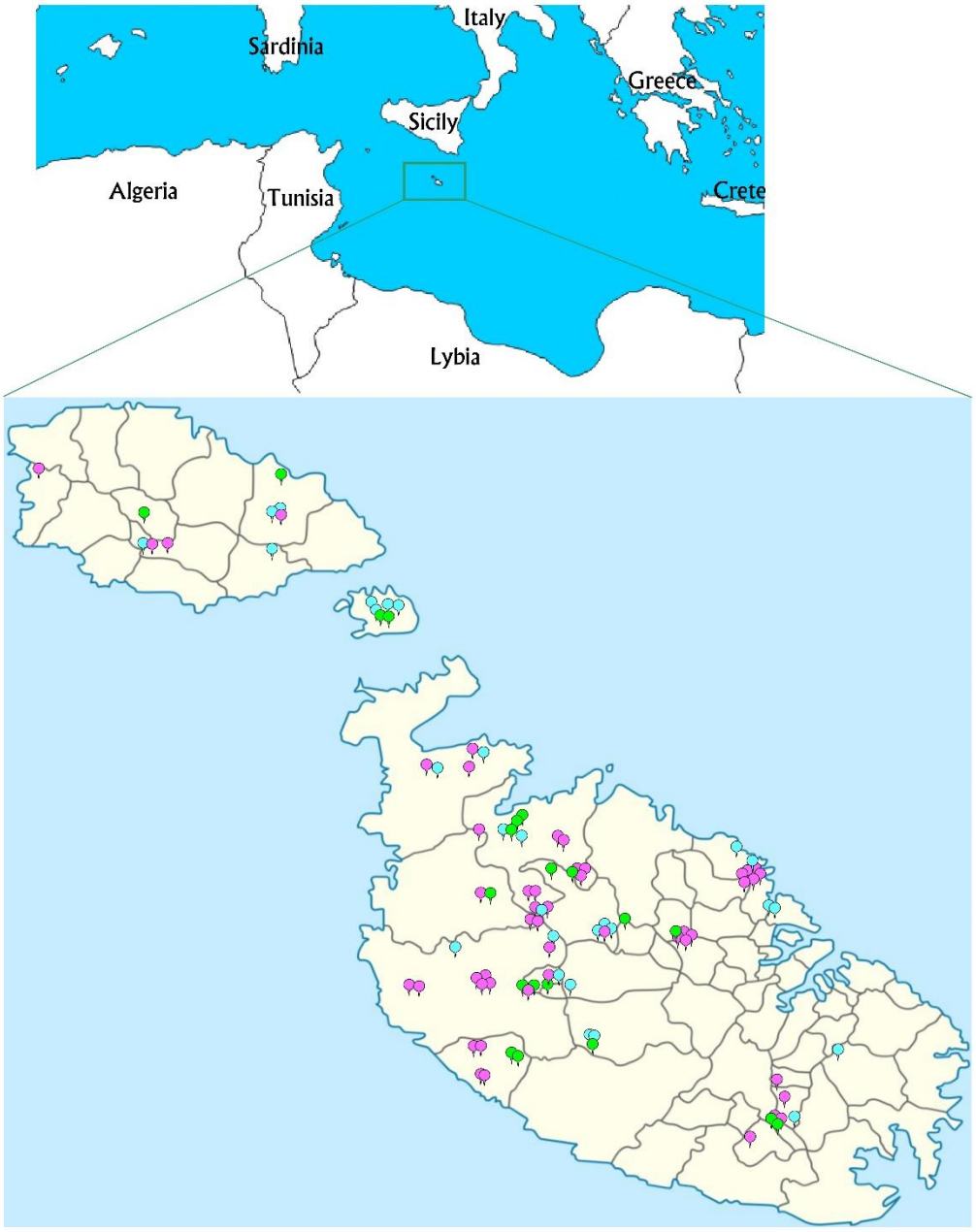

Figure 1. Geographic location of 98 olive accessions. Light blue indicates the E1 lineage, fuchsia represents the E2 lineage and light green represents trees with different lineages between canopy and rootstock.

2.2. Chloroplast and Nuclear Marker Applied to Maltese Olive Genotypes

To detect maternal inheritance of the Maltese samples, chloroplast genotyping was performed using cp-SSR p10–13 markers, including SSR, SNP and indel variation [30,31]. PCR amplifications were performed following a previously published method [15]. Output data were analyzed with GeneMapper 5 (Applied Biosystems-Hitachi, Applied Biosystems, Waltham, MA, USA).

In order to verify the identity of Maltese accessions, all samples were genotyped using standard dinucleotide SSR markers that are widely applied for cultivar characterization in most olive germplasm collections [10,28]. Ten highly polymorphic markers previously individuated as the best-performing loci [37] were selected, including DCA3, DCA5, DCA9, DCA16, DCA18 [38], EMO-90 [39], GAPU71B, GAPU101, GAPU103A [40] and UDO99-043 [41]. These markers were also applied in previous genotyping studies [7,14,15,27,42]. Forward primers carrying VIC, PET or NED labels at their 5′ end were used. The chromosome position of each SSR marker is reported in Table S2 based on a search of two olive genomes, *Olea europaea* subsp. *europaea* var. *sylvestris* [43] and var. *europaea* cv. Leccino (OLGENOME project, financed by MIPAAF DM 13938). The local blast analysis for each SSR in both genomes was performed by applying BLOSUM62 as a matrix [44] and 0.001 as the expectation value (E).

2.3. Frequency Analysis and Genetic Differentiation

A phylogenetic tree of Maltese genotypes was constructed with MEGA7 [45], with branch lengths in the same units as those of the evolutionary distances, to infer the phylogenetic tree. The evolutionary distances were computed using the neighbor-joining (NJ) method.

Genotypes with a unique profile were selected for frequency analysis. The following population-level genetic statistics were calculated using GenAlEx 6.5 [46]: number of alleles (Na), number of effective alleles (Ne) and observed (Ho) and expected (He) heterozygosity and fixation indices (F). Polymorphic information content (PIC) and the presence of possible null alleles (Fnull) were calculated for each microsatellite locus using CERVUS v.3.0.3 software (Field Genetics, London, UK) to determine the genetic uniqueness of each accession and to quantify redundancy [47].

The SSR data were further analyzed with STRUCTURE 2.3.4 software (Pritchard Lab, Stanford University, Stanford, CA, USA) [48], running 10,000 replicate Monte Carlo Markov chains (MCMCs) with a burn-in period of 10,000 for 100 iterations for each K. The range of possible number of clusters (K) was set to 1 to 20, considering independent alleles and an admixture of individuals. Bayesian analysis divided sampled individuals into a number of K clusters, and the most likely value of K was estimated using ΔK [49] with STRUCTURE Harvester [50].

GenAlEx 6.5 was also used to estimate pairwise population matrices of D_{est}, G_{st} statistics and F_{st} pairwise distance with 999 permutations in order to differentiate between the Maltese STRUCTURE groups.

SSR data of Maltese samples were compared with those of a representative sample of a wide range of cultivars from the Mediterranean and beyond [10,15,28], for a total of 665 genotypes (Table S1). The applied parameters were the same as those reported above. D_{est}, G_{st} statistics and F_{st} pairwise distance were calculated with GenAlex, considering Maltese unique genotypes as a separate population compared against the populations identified by STRUCTURE software (Pritchard Lab, Stanford University, Stanford, CA, USA) in the total set of cultivars.

3. Results

3.1. Genetic Diversity Characterized according to SSR and cp-SSR Markers

The neighbor-joining (NJ) tree based on the genetic distance matrix showed two distinct clusters (C1 and C2) among Maltese genotypes (Figure 2). The division of samples in these two clusters could be somehow related to their geographical position in the islands: C1 includes the samples collected from Dar il Bniet, Mellieha, Mgarr and Loretu, whereas C2 includes genotypes collected in Haz Zebbug and Kappara but mostly from Pembroke and Bidnija.

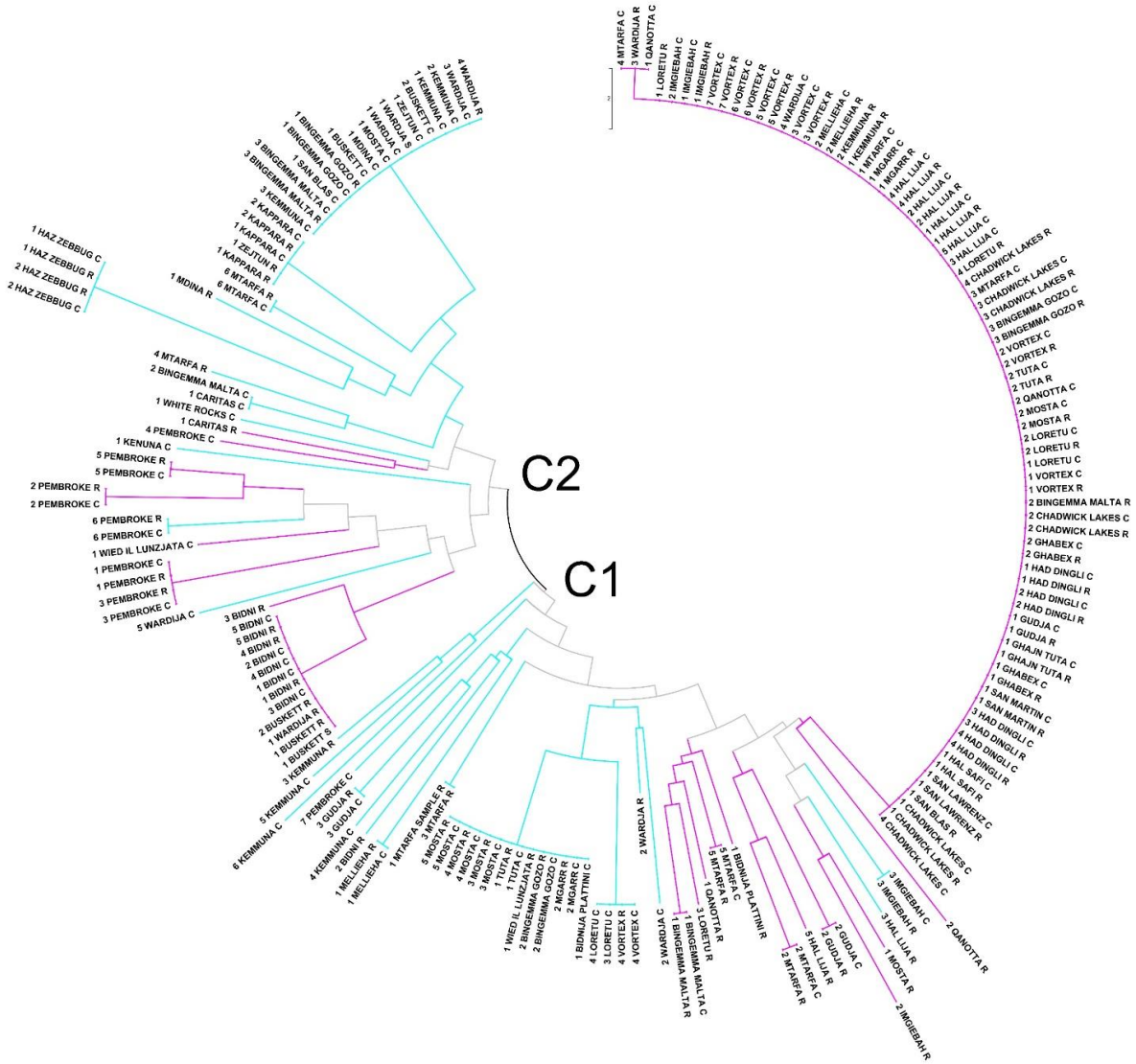

Figure 2. The evolutionary history of Maltese genotypes inferred using the neighbor-joining method. The branches in light blue belong to lineage E1, and the branches in fuchsia indicate lineage E2. C1 and C2 indicate the two main clusters.

From the 190 analyzed samples, 46 SSR profiles were identified, each corresponding to a unique genotype. These latter Maltese genotypes were enclosed in different groups (G) (Figure S1). Among all analyzed samples, 11 groups and 35 single genotypes, including trees with canopy and rootstock of the same genotype were clustered by NJ tree. The first group (G1) includes three identical samples, with only one allele difference with respect to the G2 group. This latter group includes a large number of samples (76), most of which were collected under different names and in different places throughout the islands (synonymous cases). The SSR profiling of canopies and rootstocks enable identification of 29 cases of grafting; in some cases the rootstock showed a unique profile, such as 2 Qanotta R, 3 Hal Lija R and 1 Mosta R. In other cases, the rootstock was genetically identical to the canopy of other trees, such as G1, G3, G4, G6, G10 and G11. Sixteen genotypes were detected only in rootstock, of which nine belong to the E1 lineage, with seven from the E2 lineage (Figure 2).

The ten loci used in this study showed a high degree of polymorphism among 46 unique Maltese genotypes, with a total of 116 alleles (Table 1). The number of alleles among all samples ranges from a minimum of 6 at the GAPU71B locus to a maximum of 20 alleles at DCA9 and DCA16. The number of effective alleles ranges from 2.50 to 5.75,

and the Shannon information index ranges from 1.35 to 2.13. Ho (0.76) was generally found to be higher than He (0.72), except in the cases of EMO90, GAPU101 and UDO43. Fixation values (F) were negative on average, excluding EMO90 and GAPU101, and a negligible or moderate number of null alleles was observed. The PIC values were higher than 0.5 at all loci, with an average value of 0.69 and the maximum discrimination power for DCA16 (0.81) and DCA9 (0.78).

Table 1. Indices of genetic diversity of 190 samples for each SSR locus: number of alleles (Na), number of effective alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He), fixation index (F), presence of null alleles (Fnull), polymorphism information content (PIC), private allele (PA) and Private allele frequency (PAf).

Locus	Na	Ne	I	Ho	He	F	Fnull	PIC	PA	PAf
DCA3	9.00	4.36	1.70	0.91	0.77	−0.18	−0.10	0.74	-	-
DCA5	8.00	3.28	1.46	0.78	0.69	−0.13	−0.07	0.65	-	-
DCA9	20.00	5.10	2.03	0.95	0.80	−0.19	−0.10	0.78	164, 170	0.022, 0.011
DCA16	20.00	5.75	2.13	0.94	0.83	−0.14	−0.08	0.81	216	0.011
DCA18	12.00	4.25	1.68	0.94	0.76	−0.23	−0.12	0.73	157	0.011
EMO90	7.00	2.50	1.14	0.41	0.60	0.32	0.22	0.53	-	-
GAPU71B	6.00	3.39	1.35	0.83	0.71	−0.18	−0.10	0.66	-	-
GAPU101	9.00	3.10	1.50	0.57	0.68	0.15	0.13	0.65	-	-
GAPU103A	11.00	3.29	1.47	0.78	0.70	−0.12	−0.07	0.65	-	-
UDO-043	14.00	3.09	1.65	0.48	0.68	0.29	0.21	0.66	184	0.011
Mean	11.60	3.81	1.61	0.76	0.72	−0.04	−0.01	0.69		

The cpSSR markers that were applied to discriminate the lineage of Maltese genotypes showed two distinct lineages, E1 and E2, among 190 analyzed samples (Figure 2). Seventy-one samples belong to lineage E1, whereas the majority of the samples (119) belong to lineage E2. Moreover, in 20 of 29 grafted trees, canopy and rootstock belong to two distinct lineages: in 13 trees, lineage-E1 canopy was grafted on rootstock of lineage E2, and in seven trees, lineage-E2 canopy was grafted on E1 rootstocks (Figures 2 and 3).

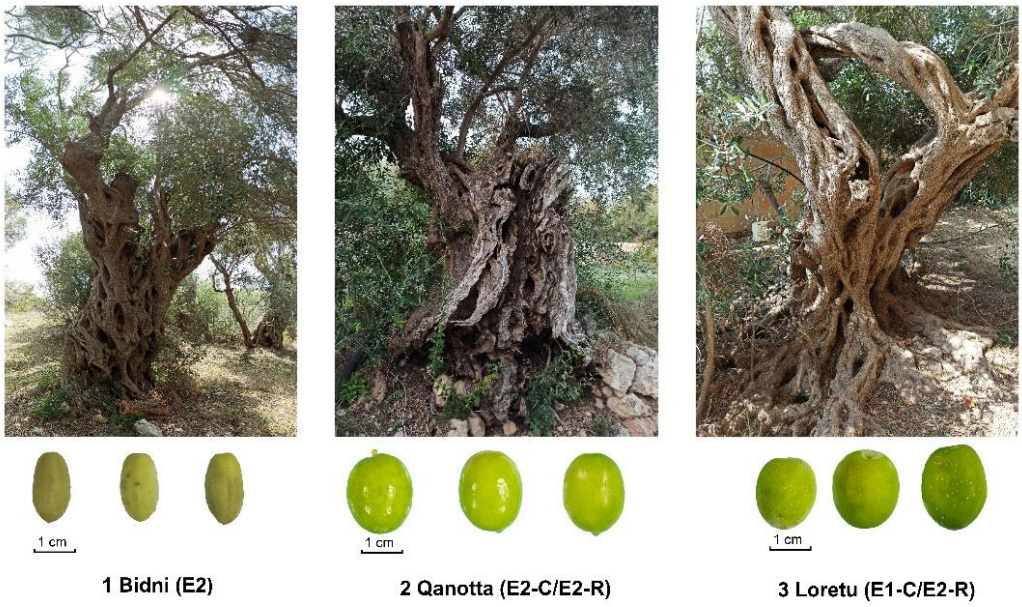

Figure 3. Ancient trees and their fruits from the analyzed genotype. The lineage of the tree (when not grafted) or the lineages of the canopy/rootstock in case of grafting among different genotypes is indicated in parentheses.

3.2. Genetic Differentiation of Maltese Genotypes

A population structure analysis of data on the 46 unique Maltese genotypes showed stabilization in terms of log-likelihood values of ΔK at K = 3, assigning individuals to a population for values above 60%, except in cases of intermixed genotypes (Figure 4). Eleven genotypes were placed in population 1 (POP1), 18 in POP2 and 17 in POP3. The first population is characterized by the presence of G1 and G2 groups. The second population (POP2) includes several samples from the Pembroke collecting site, whereas POP3 comprises almost all genotypes belonging to the E1 lineage. Among seven cases of grafting in which an E2-lineage canopy was grafted on an E1 lineage, five rootstocks were present in POP3, 3 Mtarfa R (G5), 1 Mtarfa R (G5), 2 Bidni R, 4 Mtarfa R and 4 Wardija R (G11), among which the Italian cultivar Nocellara del Belice (G4) was also present.

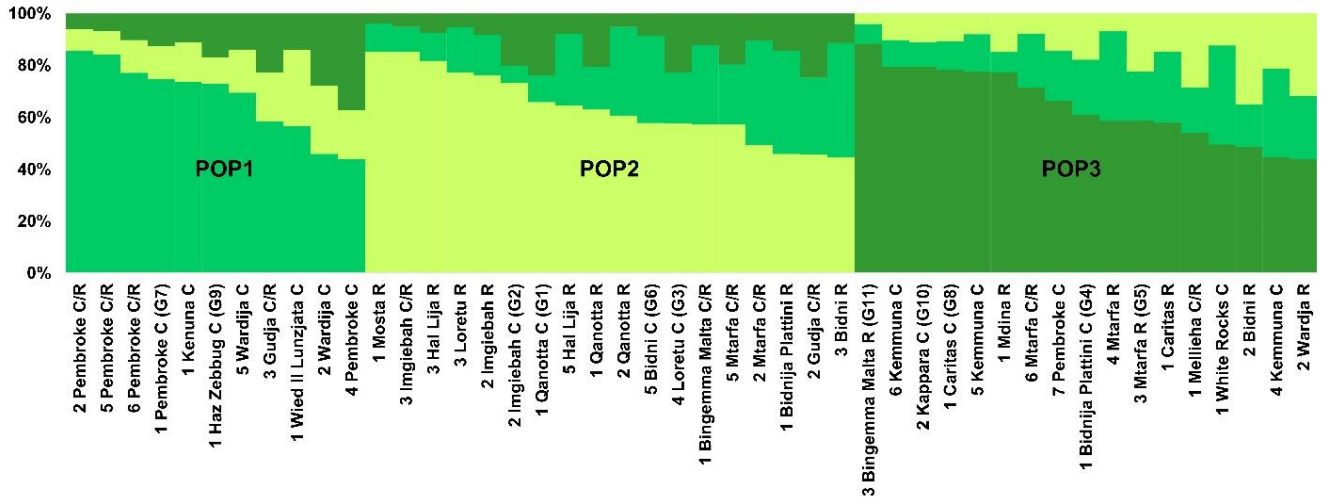

Figure 4. Genetic structure of the unique genotypes of the Malta Islands based on SSR markers. The group in which the genotype was clustered is indicated in parentheses. Each vertical bar represents single accession, and colors distinguish between three detected populations. Olive samples with more than one color indicate an admixture in their genetic composition.

Population differentiation values derived by D_{est}, G_{st} and F_{st} pairwise matrices (Table 2) calculated among the three identified populations, showing the highest values between Pop2 and Pop3 genotypes calculated by D_{est} and G_{st}, whereas for F_{st}, the highest value was among Pop1 and Pop2. The lowest values were between Pop1 and Pop3 samples for all three matrices.

Table 2. Pairwise population matrices of D_{est}, Hedrick's standardized G'_{st} (G'_{st} (*Hed*)) and F_{st} among unique Maltese genotypes.

		POP1	POP2	POP3
D $_{est}$	POP1	0.000	0.001	0.001
	POP2	0.333	0.000	0.001
	POP3	0.316	0.380	0.000
G′ $_{st}$ ***(Hed)***	POP1	0.000	0.002	0.002
	POP2	0.372	0.000	0.002
	POP3	0.348	0.413	0.000
Fst *	POP1	0.000	0.001	0.001
	POP2	0.077	0.000	0.001
	POP3	0.066	0.066	0.000

* F_{st} = inbreeding coefficient within subpopulations; relative to total = genetic differentiation among populations; F_{st} = (H_t-H_s)/H_t; G'_{st} (*Hed*) = Hedrick's standardized G'_{st} further corrected for bias when the population is small; D_{est} = Jost's estimate of differentiation. Values for D_{est}, G'_{st} (*Hed*) and F_{st} are below the diagonal. Probability (P) (rand ≥ data) based on 999 permutations is shown above the diagonal.

3.3. Genetic Diversity and Differentiation between Maltese and Worldwide Genotypes

The NJ tree based on SSR data of all Maltese samples (190), when analyzed with 475 worldwide genotypes, confirmed a high genetic diversity among Maltese samples and highlighted the cases of similarity or identity with cultivars from other countries. The 665 genotypes were divided in two clusters and five subclusters. The Maltese genotypes were group in three subclusters, mainly positioned near Italian, Algerian, Tunisian and Spanish cultivars (Table S1; Figure S2). The genotypes identical to known cultivars, positioned at G4 of the NJ tree (Figure S1), showed the same genetic profile as Sicilian cultivar Nocellara del Belice. The 3 Gudja samples were identical to the Ottobratica cultivar from the Calabria region of Italy (Figures S1 and 5).

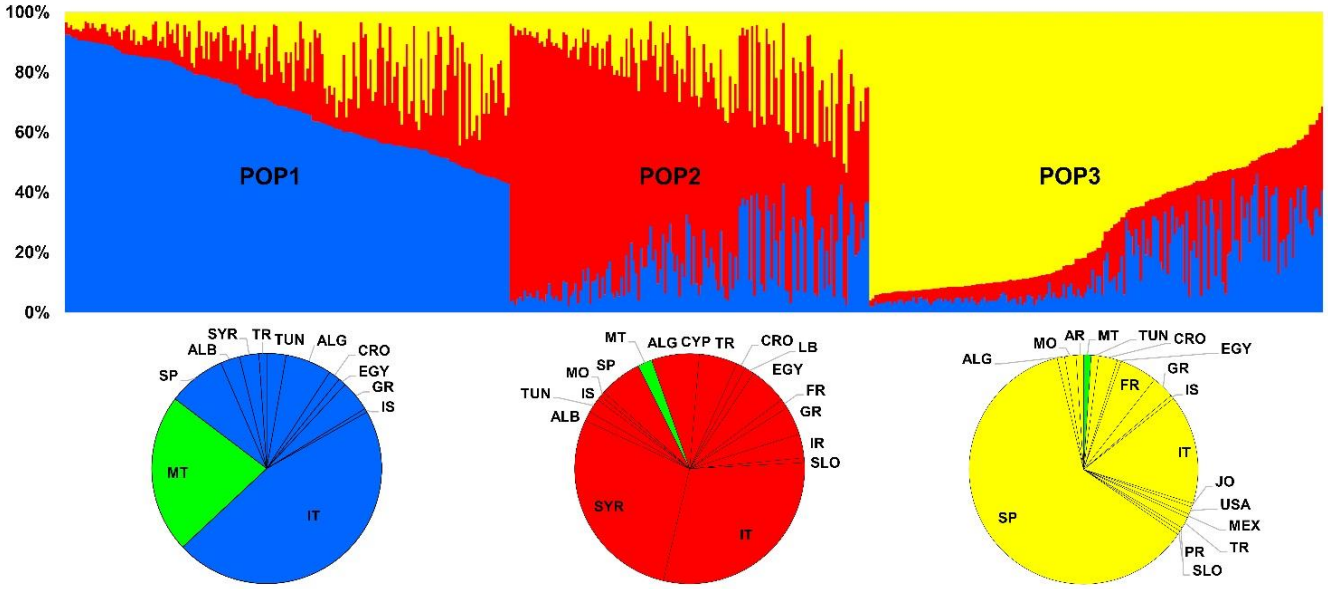

Figure 5. Genetic structure of the unique Maltese and international olive genotypes based on SSR markers. Each vertical bar represents single accession, and colors distinguish between three detected populations. Olive samples with more than one color indicate an admixture in their genetic composition.

Bayesian cluster analysis of the unique Maltese genotypes (46) and 475 international olive genotypes showed that the number of most likely subpopulations peaked at K = 3 (Figure 5). POP1 includes 184 genotypes, among which 41 out of 46 are Maltese genotypes, whereas the other olive cultivars are represented mostly by the Italian olive germplasm. There were a total of 22 intermixed genotypes with no exact assignment, including 1 Caritas C, 1 White Rocks C and 7 Pembroke C. The second population (POP2) comprises 149 genotypes, mostly from the eastern part of the Mediterranean Basin, such as Syria and beyond, with only three Maltese genotypes—1 Mdina R, 1 Kemmuna C and 3 Bingemma Malta R, in this population. It also includes 23 intermixed genotypes, none of which is from Malta. Finally, POP3 includes 188 genotypes, mainly of Spanish origin, with only two Maltese varieties, 1 Caritas R and 4 Pembroke C. POP3 contains a high percentage of intermixed genotypes, among which only 4 Pembroke C samples are from the Maltese genotype.

The genetic differentiation among populations, calculated with D_{est}, G_{st} and F_{st} pairwise matrices among the three POPs, showed the highest value between POP1 and POP3, with the lowest differentiation measured among POP1 and POP2 (Table S3A). To better explain the genetic affinity or differentiation among Maltese genotypes and the three others POPs identified by the worldwide genotype structure analysis, we performed a pairwise matrix analysis. The results confirm the highest differentiation among the Malta POP with POP3 and the lowest differentiation occurring among Malta POP and POP1 (Table S3B).

Using the same four populations we performed a frequency analysis in order to calculate genetic diversity between populations. The highest Na (18.80) corresponded to POP2,

with the fewest genotypes, whereas POP3, with 186 samples, had the lowest Na (14.40) and, as observed above (Table 1), the Malta POP, with just 46 unique genotypes, contained a considerable number of alleles (11.60) (Table 3). Moreover, in all three POPs, excluding the Maltese genotypes, the observed heterozygosity (Ho) was higher than expected (He), with a negative fixation index value, especially in POP3. The most private alleles (PAs) were found in POP2, whereas POP3 contained only seven PAs. The 46 unique Malta genotypes comprise five private alleles, with a considerably higher frequency than that of the three others POPs (Table 3).

Table 3. Genetic diversity among populations: number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He) and fixation index (F), number of private alleles (nPA) and average of private allele frequency (PAf).

POP		Na	Ho	He	F	nPA	PAf
Malta	Mean	11.60	0.80	0.81	0.01	5	0.013
	SE	1.59	0.04	0.02	0.03		
POP1	Mean	17.10	0.84	0.82	−0.02	15	0.006
	SE	2.10	0.02	0.02	0.03		
POP2	Mean	18.80	0.83	0.82	−0.01	33	0.007
	SE	1.87	0.03	0.03	0.01		
POP3	Mean	14.40	0.83	0.73	−0.13	7	0.004
	SE	1.63	0.07	0.06	0.04		

4. Discussion

A survey of Maltese olives and their genotyping through nuclear and chloroplast SSR markers on a wide set of local and ancient olive trees confirmed the rich diversity and uniqueness of this germplasm. According to archaeological and geoarchaeological evidence, olive trees were certainly present in Malta during the Roman period [34–36]. Palynological studies have shown that the intensification in Olea cultivation during the Roman period corresponded with that observed in Tripolitania, Libya, Spain and Levant [21,51,52]. The survival of ancient olives throughout the Mediterranean area and beyond has been reported in several studies [9,11–13,15], and molecular identification has confirmed the unknown genetic profile [9], recurrently with stable production and high quality and quantity of the oil [53]. Considering their long lifetime, a significant tolerance to biotic and abiotic stresses can be assumed. A carbon dating study on the ancient trees of Bidni (Bidnija grove) estimated an age of 400–600 years or slightly earlier [35]. The identity of characterized local Maltese cultivars Bidni, Malti and White olive or Bajda [36] could not be directly verified in the present study due to the nature of molecular markers that were applied in previous studies. Moreover, Mazzitelli et al. [36] reported that the sampling place of the Bidni cultivar that corresponds to the sampling place of the present research contained an "olive grove with 26 massive trees", confirming the uniqueness of the Bidni genotype not only as canopy genotype but also containing rootstock of other trees, which form group six in the NJ tree. The Malti cultivar, which was previously reported as the most diffused cultivar in Malta [36], could correspond to the group two of the NJ tree, which include the majority of analyzed samples from the Maltese Islands. It is possible to confirm the ancient cultivation of this genotype not only as grafted canopy but also as rootstock of other genotypes, such as the case of 1 Kemmuna, 2 San Blas, 2 Bingemma Malta and 4 Loretu that have this genotype as rootstock grafted with a different genotype. The White olive or Badja could correspond to the genotype including 1 Caritas and 2 Bingemma Malta canopies analyzed in the present study, which are white-colored drupes. Given that we found this genotype only in the canopy of two trees, it could be supposed that it is not a widespread cultivar in Malta but was probably introduced to the islands and grafted on the most diffused genotype (2 Bingemma Malta R, G2) and endemic oleaster, 1 Caritas R, both from the E2 lineage.

A separated genotyping of different tree parts, such as canopy and rootstock, showed the presence of at least two genotypes per plant, confirming the occurrence of grafting propagation in 29.6% of cases among total analyzed trees. Grafting practice was developed almost 4000 years ago and was the key element in the domestication process [54]. This observation confirms what has been previously shown in other countries, such as Spain, Italy, Palestine/Israel, Cyprus and Turkey [9,11,17,55,56]: that majority of ancient trees were grafted. In some cases, grafting occurred between two distinct lineages, confirming a different maternal origin and, presumably, different phases of olive cultivation that occurred in Malta during the last millennia. Studies based on chloroplast markers [2,3,15] have demonstrated that the majority of the cultivated varieties belong to the E1 lineage as a result of human-influenced dispersal from the east to the west of the Mediterranean. On the contrary, the E3 lineage, mostly diffused in the west Mediterranean [2], was not found in any of the Maltese samples. Thus, the high genetic variability of Malta rootstocks and, above all, the prevalence of the E2 lineage, which is endemic to the central Mediterranean, could be attributed to the survival of a rich wild olive gene pool [32,57].

The use of wild olive trees as rootstock to increase tree vigor has been reported in historical documents [58]. Some documents report the selection of wild olive as rootstock thanks to their high rooting ability and then their propagation [59]. Alternatively, sexual propagation has resulted in rootstock variation [60] of Maltese olive trees or from seed dispersal from neighboring countries by birds. Therefore, it is probable that scions were grafted on trees derived from seeds of cultivated trees or were spontaneous as wild types inside on the Malta Islands or beyond. From 1 Buskett tree, for which a shoot sample was also analyzed, the shoot lineage (grown on the main trunk) was the same as that of the rootstock (E2), whereas the canopy was found to belong to the E1 lineage. This evidence confirms that grafting was also applied to top of the trunk and not at its base, as was often the case. In the Levant area [60], to improve agronomical aspects, such abiotic stress tolerance, specific scion/rootstock genotype combinations were selected by farmers.

In the present study, different cases of canopy/rootstock combinations were found. In some cases, such as samples G1, G2 and G6, rootstock of a tree had the same molecular profile as the canopy of other trees and all of them were belonged to lineage E2. This evidence confirms (i) the clonal propagation of rootstock, probably from an autochthonous cultivar, followed by grafting with new or better performing-genotypes; (ii) the E2 lineage refers not only to wild genotypes with small fruit and bushy plants but also to the well-adapted ancient cultivation, in some cases with large fruits, as in the case of 2 Qanotta (G2), which has also been observed in other Mediterranean cultivars, such as Picholine Languedoc and Lechin de Sevilla, both from the E2 lineage [31,32], with large and medium fruit size, respectively. The other cases of canopy/rootstock combinations were observed in the G3, G5, G10 and G11 genotypes, and a unique genotype was clonally propagated to be used as rootstock or as canopy, confirming the use of a specific genotype with better performance in that place. In the latter case, all the genotypes were found to belong to the E1 lineage. A considerable number of unique genotypes were detected only in rootstock from both lineages. As reported by Besnard et al. [2], the E2 lineage is represented mostly by central Mediterranean oleasters, with only a few percent represented by cultivars. The presence of ancient trees (the case of Bidni trees) or canopies of the E2 lineage confirms the possible role of the oleaster population in the local selection of suitable genotypes with large fruit, which were clonally propagated through cuttings. Population structure analysis showed that genotype from the Pembroke site, included in POP1, showed a high level of relatedness within the group (excepting samples 6 and 7) carrying the E2 lineage, with a tree morphology characterized by bushes with few leaves and fruits, which may lead us to assume that they could represent a real, autochthonous remnant of a wild population in Malta.

For millennia, Malta has represented a site of strategic military importance, with no specialized management of crop systems, including olive. The relevant diversity of Malta ecosystems, with main vegetational assemblages represented by maquis, garigue and

steppe; limited infrastructure, such as terraces, water catchments and irrigation channels; and the presence of grazing animals [61], may have co-occurred with the establishment of a seminatural olive cultivation system and the development of an autochthonous olive germplasm, with a contribution of surrounding regions. Based on this evidence, the olive diversity developed in Malta Islands may have been almost solely contributed by local farmers by selecting the most promising wild trees based on fruit and tree size [62]. Similar olive domestication processes have been described in North Africa, where natural events and grazing could have favored the selection of vigorous oleasters, which were extensively used prior to the arrival of Punic, Greek and Roman influences [23,63]. In the southern and eastern Iberian Peninsula, olive domestication seems to have occurred two millennia before colonization by Phoenicians and Romans [3]. The introduction of cultivars from surrounding regions may have occurred long after, thanks to the trading activities carried out first by Phoenicians and later by Romans and Sicilian Arabs.

The phylogenetic study of Maltese samples together with 475 international genotypes also confirmed the genetic similarity among cultivars from Malta and Italy (particularly the nearby regions of Sicily and Calabria) and Algerian genotypes, such as in the case of 1 Mellieha, 5 Kemmuna C and 1 Bingemma Malta R (G11). The geographic position of these countries may have affected the local genetic diversity of the Maltese Islands. The most common genotype in Malta is clustered with an Algerian variety, Akenane, and others are grouped with Chemlal de Kabylie, with some samples corresponding to the Sicilian cultivar Nocellara del Belice and others were identical to the Ottobratica cultivar from a south Italian region, Calabria. Other Maltese genotypes, such as 4 Vortex, 3 Loretu C and 4 Loretu C are clustered near Dokkar, Canino, Blanqueta and Olivastra Seggianese, cultivars that are genetically close to wild olives [32] or directly belonging to the E2 lineage [3,31]. The assignment of almost all Maltese genotypes to the first population, together with 85 Italian cultivars, has again confirmed a strong gene flow between the neighboring countries and the Maltese Islands. Moreover, Maltese olives exhibited the highest genetic differentiation with the olive germplasms of western Mediterranean countries, such as Spain, France and Morocco, probably as a result of a founder effect followed by an isolation by distance and then low geneflow among these countries in ancient times.

5. Conclusions

Ancient and local olive genotypes may have developed in Malta Islands in traditional agricultural systems as a result of selection due to both natural factors and by farmers over long periods of time, followed by the direct introduction from neighboring regions and possible intercrossing with local olives. The Malta Islands represents a center of local domestication and selection of olive genotypes with a possible autochthonous origin. The availability of this large set of unique and highly differentiated genotypes is critical to ensuring the adaptability to new agro-environmental challenges and future climatic constraints.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141710684/s1, Figure S1: The evolutionary history of Maltese genotypes inferred using the neighbor-joining method. Samples with a different name but identical genotype are enclosed in eleven different groups; Figure S2: Phylogenetic analysis of Maltese genotypes and international olive germplasms inferred using the neighbor-joining method. The branches in light green indicate Maltese genotypes; Table S1: Names and GPS coordinates of Maltese olive samples collected from canopy, shoots and rootstock. International olive germplasms and their countries of diffusion; Table S2: Chromosome position of each of the ten SSR markers in *Olea europaea* subsp. *europaea* var. *sylvestris* [49] and var. *europaea* cv. Leccino (OLGENOME project); Table S3: Genetic differentiation among the three populations of structure (A) and between Maltese and worldwide genotypes (B) calculated through pairwise population matrices of D_{est}, Hedrick's standardized G'_{st} (G'_{st} (*Hed*)) and F_{st}.

Author Contributions: Conceptualization, S.M., R.M., L.B. and D.M.; methodology, M.C.V., C.S., S.P., S.M. and R.M.; software, S.M. and R.M.; validation, S.M. and R.M.; formal analysis, S.M. and R.M.; investigation, M.C.V., D.M., C.S., M.B., V.P., V.S., S.M. and R.M.; resources, D.M., C.S., S.M. and R.M.; data curation, C.S., S.P., E.L., S.M. and R.M.; writing—original draft preparation, M.C.V., S.M. and R.M.; writing—review and editing, M.C.V., C.S., S.P., M.B., V.P., L.B., S.M. and R.M.; visualization, S.M. and R.M.; supervision, S.M.; project administration, S.M., R.M. and M.B.; funding acquisition, S.M. and M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by GAL XLOKK of Malta as a part of Measure 19.3 of the EU LEADER Program 2014–2020.

Institutional Review Board Statement: The leaves of spontaneous olive trees used in this study for genetic identification were kindly provided by the GAL XLOKK Project of Malta as a part of Measure 19.3 of the EU LEADER Program 2014–2020. The collection sites (GPS coordinates) are reported in the Supplementary Materials of the present manuscript.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: Special thanks to Philip Von Brockdroff and Terrence Camilleri for their support in project administration. We would like to thank the OLGENOME Project, financed by MIPAAF DM 13938, for allowing access to the genome sequence data of cv. Leccino.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Díez, C.M.; Trujillo, I.; Martinez-Urdiroz, N.; Barranco, D.; Rallo, L.; Marfil, P.; Gaut, B.S. Olive domestication and diversification in the Mediterranean Basin. *New Phytol.* **2015**, *206*, 436–447. [CrossRef] [PubMed]
2. Besnard, G.; Khadari, B.; Navascués, M.; Fernández-Mazuecos, M.; El Bakkali, A.; Arrigo, N.; Baali-Cherif, D.; Brunini-Bronzini de Caraffa, V.; Santoni, S.; Vargas, P.; et al. The complex history of the olive tree: From Late Quaternary diversification of Mediterranean lineages to primary domestication in the northern Levant. *Proc. Natl. Acad. Sci. USA* **2013**, *280*, 20122833. [CrossRef] [PubMed]
3. Besnard, G.; Terral, J.F.; Cornille, A. On the origins and domestication of the olive: A review and perspectives. *Ann. Bot.* **2018**, *121*, 385–403. [CrossRef] [PubMed]
4. Kassa, A.; Konrad, H.; Geburek, T. Molecular diversity and gene flow within and among different subspecies of the wild olive (*Olea europaea* L.): A review. *Flora* **2019**, *250*, 18–26. [CrossRef]
5. Erre, P.; Chessa, I.; Muñoz-Diez, C.; Belaj, A.; Rallo, L.; Trujillo, I. Genetic diversity and relationships between wild and cultivated olives (*Olea europaea* L.) in Sardinia as assessed by SSR markers. *Genet. Resour. Crop Evol.* **2010**, *57*, 41–54. [CrossRef]
6. Marra, F.P.; Caruso, T.; Costa, F.; Di Vaio, C.; Mafrica, R.; Marchese, A. Genetic relationships, structure and parentage simulation among the olive tree (*Olea europaea* L. subsp. *europaea*) cultivated in Southern Italy revealed by SSR markers. *Tree Genet. Genomes* **2013**, *9*, 961–973. [CrossRef]
7. Mousavi, S.; Stanzione, V.; Mencuccini, M.; Baldoni, L.; Bufacchi, M.; Mariotti, R. Biochemical and molecular profiling of unknown olive genotypes from central Italy: Determination of major and minor components. *Eur. Food Res. Technol.* **2019**, *245*, 83–94. [CrossRef]
8. Baldoni, L.; Tosti, N.; Ricciolini, C.; Belaj, A.; Arcioni, S.; Pannelli, G.; Germana, M.A.; Mulas, M.; Porceddu, A. Genetic structure of wild and cultivated olives in the central Mediterranean basin. *Ann. Bot.* **2006**, *98*, 935–942. [CrossRef]
9. Díez, C.M.; Trujillo, I.; Barrio, E.; Belaj, A.; Barranco, D.; Rallo, L. Centennial olive trees as a reservoir of genetic diversity. *Ann. Bot.* **2011**, *108*, 797–807. [CrossRef]
10. El Bakkali, A.; Essalouh, L.; Tollon, C.; Rivallan, R.; Mournet, P.; Moukhli, A.; Hadidou, A.; Sikaoui, L.; Khadari, B. Characterization of Worldwide Olive Germplasm Banks of Marrakech (Morocco) and Córdoba (Spain): Towards management and use of olive germplasm in breeding programs. *PLoS ONE.* **2019**, *14*, e0223716. [CrossRef]
11. Barazani, O.; Westberg, E.; Hanin, N.; Dag, A.; Kerem, Z.; Tugendhaft, Y.; Hmidat, M.; Hijawi, T.; Kadereit, J.W. A comparative analysis of genetic variation in rootstocks and scions of old olive trees–a window into the history of olive cultivation practices and past genetic variation. *BMC Plant Biol.* **2014**, *14*, 146. [CrossRef] [PubMed]
12. Bernabei, M. The age of the olive trees in the Garden of Gethsemane. *J. Archaeol. Sci.* **2015**, *53*, 43–48. [CrossRef]
13. Beghè, D.; Piotti, A.; Satovic, Z.; De La Rosa, R.; Belaj, A. Pollen-mediated gene flow and fine-scale spatial genetic structure in Olea europaea subsp. europaea var. sylvestris. *Ann. Bot.* **2017**, *119*, 671–679.

14. Ninot, A.; Howad, W.; Aranzana, M.J.; Senar, R.; Romero, A.; Mariotti, R.; Baldoni, L.; Belaj, A. Survey of over 4, 500 monumental olive trees preserved on-farm in the northeast Iberian Peninsula, their genotyping and characterization. *Sci. Hortic.* **2018**, *231*, 253–264. [CrossRef]
15. Mousavi, S.; Mariotti, R.; Bagnoli, F.; Costantini, L.; Cultrera, N.G.; Arzani, K.; Pandolfi, S.; Torkzaban, B.; Hosseini-Mazinani, M.; Baldoni, L. The eastern part of the Fertile Crescent concealed an unexpected route of olive (*Olea europaea* L.) differentiation. *Ann. Bot.* **2017**, *119*, 1305–1318. [CrossRef] [PubMed]
16. Díaz-Rueda, P.; Franco-Navarro, J.D.; Messora, R.; Espartero, J.; Rivero-Núñez, C.M.; Aleza, P.; Capote, N.; Cantos, M.; García-Fernández, J.L.; de Cires, A.; et al. SILVOLIVE, a germplasm collection of wild subspecies with high genetic variability as a source of rootstocks and resistance genes for olive breeding. *Front. Plant Sci.* **2020**, *11*, 629. [CrossRef]
17. Yoruk, B.; Taskin, V. Genetic diversity and relationships of wild and cultivated olives in Turkey. *Plant Syst. Evol.* **2014**, *300*, 1247–1258. [CrossRef]
18. Breton, C.; Tersac, M.; Bervillé, A. Genetic diversity and gene flow between the wild olive (oleaster, *Olea europaea* L.) and the olive: Several Plio-Pleistocene refuge zones in the Mediterranean basin suggested by simple sequence repeats analysis. *J. Biogeogr.* **2006**, *33*, 1916–1928. [CrossRef]
19. Zohary, D.; Hopf, M.; Weiss, E. The origin and spread of domesticated plants in Southwest Asia, Europe, and the Mediterranean Basin. In *Domestication of Plants in the Old World*, 4th ed.; Oxford University Press on Demand: Oxford, UK, 2012.
20. Breton, C.; Terral, J.F.; Pinatel, C.; Médail, F.; Bonhomme, F.; Bervillé, A. The origins of the domestication of the olive tree. *C. R. Biol.* **2009**, *332*, 1059–1064. [CrossRef]
21. Kaniewski, D.; Van Campo, E.; Boiy, T.; Terral, J.F.; Khadari, B.; Besnard, G. Primary domestication and early uses of the emblematic olive tree: Palaeobotanical, historical and molecular evidence from the Middle East. *Biol. Rev.* **2012**, *87*, 885–899. [CrossRef]
22. Zohary, D.; Spiegel-Roy, P. Beginnings of Fruit Growing in the Old World: Olive, grape, date, and fig emerge as important Bronze Age additions to grain agriculture in the Near East. *Science* **1975**, *187*, 319–327. [CrossRef] [PubMed]
23. Aumeeruddy-Thomas, Y.; Moukhli, A.; Haouane, H.; Khadari, B. Ongoing domestication and diversification in grafted olive–oleaster agroecosystems in Northern Morocco. *Reg. Environ. Chang.* **2017**, *17*, 1315–1328. [CrossRef]
24. Abbott, A. Olive tree gridlock eases: Court ruling paves way for containment of deadly plant disease, but risk of spread remains. *Nature* **2016**, *533*, 299–301. [CrossRef] [PubMed]
25. Almeida, R.P. Can Apulia's olive trees be saved? *Science* **2016**, *353*, 346–348. [CrossRef] [PubMed]
26. Mousavi, S.; Mariotti, R.; Valeri, M.C.; Regni, L.; Lilli, E.; Albertini, E.; Proietti, P.; Businelli, D.; Baldoni, L. Characterization of differentially expressed genes under salt stress in olive. *Int. J. Mol. Sci.* **2021**, *23*, 154. [CrossRef] [PubMed]
27. Lazović, B.; Adakalić, M.; Pucci, C.; Perović, T.; Bandelj, D.; Belaj, A.; Mariotti, R.; Baldoni, L. Characterizing ancient and local olive germplasm from Montenegro. *Sci. Hortic.* **2016**, *209*, 117–123. [CrossRef]
28. Trujillo, I.; Ojeda, M.A.; Urdiroz, N.M.; Potter, D.; Barranco, D.; Rallo, L.; Diez, C.M. Identification of the Worldwide Olive Germplasm Bank of Córdoba (Spain) using SSR and morphological markers. *Tree Genet. Genomes* **2014**, *10*, 141–155. [CrossRef]
29. Fendri, M.; Trujillo, I.; Trigui, A.; Rodríguez-García, M.I.; Ramírez, J.D.D.A. Simple sequence repeats identification and endocarp characterization of olive tree accessions in a Tunisian germplasm collection. *Hortscience* **2010**, *45*, 1429–1436. [CrossRef]
30. Mariotti, R.; Cultrera, N.G.; Díez, C.M.; Baldoni, L.; Rubini, A. Identification of new polymorphic regions and differentiation of cultivated olives (*Olea europaea* L.) through plastome sequence comparison. *BMC Plant Biol.* **2010**, *10*, 1–13. [CrossRef]
31. Besnard, G.; Hernández, P.; Khadari, B.; Dorado, G.; Savolainen, V. Genomic profiling of plastid DNA variation in the Mediterranean olive tree. *BMC Plant Biol.* **2011**, *11*, 80. [CrossRef]
32. Mariotti, R.; Belaj, A.; De La Rosa, R.; Leòn, L.; Brizioli, F.; Baldoni, L.; Mousavi, S. EST–SNP study of Olea europaea L. uncovers functional polymorphisms between cultivated and wild olives. *Genes* **2020**, *11*, 916. [CrossRef] [PubMed]
33. Fenech, K. *Human-Induced Changes in the Environment and Landscape of the Maltese Islands from the Neolithic to the 15th Century AD*; BAR 1682; Archaeopress: Oxford, UK, 2007.
34. Gambin, B.; Andrieu-Ponel, V.; Médail, F.; Marriner, N.; Peyron, O.; Montade, V.; Gambin, T.; Morhange, C.; Belkacem, D.; Djamali, M. 7300 years of vegetation history and climate for NW Malta: A Holocene perspective. *Clim. Past.* **2016**, *12*, 273–297. [CrossRef]
35. Lageard, J.G.; Sultana, D.; Brearley, F.Q. Veteran trees in an historic landscape: The Bidnija olive grove, Malta. *J. Archaeol. Sci. Rep.* **2021**, *38*, 103094. [CrossRef]
36. Mazzitelli, O.; Calleja, A.; Sardella, D.; Farrugia, C.; Zammit-Mangion, M. Analysis of the molecular diversity of Olea europaea in the Mediterranean Island of Malta. *Genet. Resour. Crop Evol.* **2015**, *62*, 1021–1027. [CrossRef]
37. Baldoni, L.; Cultrera, N.G.; Mariotti, R.; Ricciolini, C.; Arcioni, S.; Vendramin, G.G.; Buonamici, A.; Porceddu, A.; Sarri, V.; Ojeda, M.A.; et al. A consensus list of microsatellite markers for olive genotyping. *Mol. Plant Breed.* **2009**, *24*, 213–231. [CrossRef]
38. Sefc, K.M.; Lopes, M.S.; Mendonca, D.; Dos Santos, M.R.; Laimer, M.; da Câmara Machado, A. Identification of microsatellite loci in olive (*Olea europaea*) and their characterization in Italian and Iberian olive trees. *Mol. Ecol.* **2000**, *9*, 1171–1173. [CrossRef]
39. De la Rosa, R.; James, C.M.; Tobutt, K.R. Isolation and characterization of polymorphic microsatellite in olive (*Olea europaea* L.) and their transferability to other genera in the Oleaceae. *Mol. Ecol.* **2002**, *2*, 265–267. [CrossRef]
40. Carriero, F.; Fontanazza, G.; Cellini, F.; Giorio, G. Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). *Theor. Appl. Genet.* **2002**, *104*, 301–307. [CrossRef]

41. Cipriani, G.; Marrazzo, M.T.; Marconi, R.; Cimato, A.; Testolin, R. Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. *Theor. Appl. Genet.* **2002**, *104*, 223–228. [CrossRef]
42. Rotondi, A.; Beghè, D.; Fabbri, A.; Ganino, T. Olive oil traceability by means of chemical and sensory analyses: A comparison with SSR biomolecular profiles. *Food. Chem.* **2011**, *129*, 1825–1831. [CrossRef]
43. Unver, T.; Wu, Z.; Sterck, L.; Turktas, M.; Lohaus, R.; Li, Z.; Yang, M.; He, L.; Deng, T.; Escalante, F.J.; et al. Genome of wild olive and the evolution of oil biosynthesis. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E9413–E9422. [CrossRef] [PubMed]
44. Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef] [PubMed]
45. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]
46. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research an update. *Bioinformatics* **2013**, *28*, 2537–2539. [CrossRef]
47. Marshall, T.C.; Slate, J.; Kruuk, L.E.B.; Pemberton, J.M. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* **1998**, *7*, 639–655. [CrossRef] [PubMed]
48. Pritchard, J.; Wen, X.; Falush, D. Documentation for Structure Software: Version 2.3. 2009. Available online: http://pritch.bsd.uchicago.edu/structure.html (accessed on 19 July 2022).
49. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef]
50. Earl, D.A. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
51. Barker, G.; Gilbertson, D.; Jones, B.; Mattingly, D. *Farming the Desert: The UNESCO Libyan Valleys Archaeological Survey*; UNESCO: London, UK, 1996.
52. Pantaléon-Cano, J.; Yll, E.I.; Pérez-Obiol, R.; Roure, J.M. Palynological evidence for vegetational history in semi-arid areas of the western Mediterranean (Almería, Spain). *Holocene* **2003**, *13*, 109–119. [CrossRef]
53. Cicatelli, A.; Fortunati, T.; De Feis, I.; Castiglione, S. Oil composition and genetic biodiversity of ancient and new olive (*Olea europaea* L.) varieties and accessions of southern Italy. *Plant Sci.* **2013**, *210*, 82–92. [CrossRef]
54. Harris, R.J. Root coverage with connective tissue grafts: An evaluation of short-and long-term results. *J. Periodontol.* **2002**, *73*, 1054–1059. [CrossRef]
55. Anestiadou, K.; Nikoloudakis, N.; Hagidimitriou, M.; Katsiotis, A. Monumental olive trees of Cyprus contributed to the establishment of the contemporary olive germplasm. *PLoS ONE.* **2017**, *12*, e0187697. [CrossRef] [PubMed]
56. Schicchi, R.; Speciale, C.; Amato, F.; Bazan, G.; Di Noto, G.; Marino, P.; Ricciardo, P.; Geraci, A. The monumental olive trees as biocultural heritage of Mediterranean landscapes: The case study of Sicily. *Sustainability* **2021**, *13*, 6767. [CrossRef]
57. Gros-Balthazard, M.; Besnard, G.; Sarah, G.; Holtz, Y.; Leclercq, J.; Santoni, S.; Wegmann, D.; Glémin, S.; Khadari, B. Evolutionary transcriptomics reveals the origins of olives and the genomic changes associated with their domestication. *Plant J.* **2019**, *100*, 143–157. [CrossRef] [PubMed]
58. *Foxhall, L: Olive Cultivation in Ancient Greece: Seeking the Ancient Economy*; Oxford University Press: Oxford, UK, 2007.
59. Connell, J.H.; Catlin, P.B. Root physiology and rootstock characteristics. In *Olive Production Manual*, 2nd ed.; Sibbett, G.S., Ferguson, L., Coviello, J.L., Lindstrand, M., Eds.; University of California, Agricultural and Natural Resources: Los Angeles, CA, USA, 2005; pp. 39–48.
60. Barazani, O.; Waitz, Y.; Tugendhaft, Y.; Dorman, M.; Dag, A.; Hamidat, M.; Hijawi, T.; Kerem, Z.; Westberg, E.; Kadereit, J.W. Testing the potential significance of different scion/rootstock genotype combinations on the ecology of old cultivated olive trees in the southeast Mediterranean area. *BMC Ecol.* **2017**, *17*, 3. [CrossRef] [PubMed]
61. Schembri, P.J. The Maltese Islands: Climate, vegetation and landscape. *GeoJournal* **1997**, *41*, 1–11. [CrossRef]
62. di Rienzo, V.; Sion, S.; Taranto, F.; D'Agostino, N.; Montemurro, C.; Fanelli, V.; Sabetta, W.; Boucheffa, S.; Tamendjari, A.; Pasqualone, A.; et al. Genetic flow among olive populations within the Mediterranean basin. *PeerJ* **2018**, *6*, e5260. [CrossRef]
63. Camps, G. *Massinissa, ou les Débuts de L'histoire*; Imprimerie Officielle: Alger, Algeria, 1961.