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A Glimpse into the Genetic Heritage of the Olive Tree in Malta

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Abstract: The genetic diversity of the ancient autochthonous olive trees on the Maltese islands and the relationship with the wild forms growing in marginal areas of the island (57 samples), as well as with the most widespread cultivars in the Mediterranean region (150 references), were investigated by genetic analysis with 10 SSR markers. The analysis revealed a high genetic diversity of Maltese germplasm, totaling 84 alleles and a Shannon information index (I) of 1.08. All samples from the upper and the lower part of the crown of the Bidni trees belonged to the same genotype, suggesting that there was no secondary top-grafting of the branches. The Bidni trees showed close relationships with the local wild germplasm, suggesting that the oleaster population played a role in the selection of the Bidni variety. Genetic similarities were also found between Maltese cultivars and several Italian varieties including accessions putatively resistant to the bacterium *Xylella fastidiosa*, which has recently emerged in the Apulia region (Italy) and has caused severe epidemics on olive trees over the last decade.

Keywords: genotyping; biodiversity; genetic resources; *Xylella fastidiosa*; Bidnija olive grove

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1. Introduction

The olive tree (*Olea europaea* L.) is a typical Mediterranean oil plant that is of great socio-economic importance in many countries. In Malta, its cultivation, although older, flourished especially in the 15th century, when many olive trees were planted in the best areas, and towns were named after the olive groves surrounding them. At that time, large quantities of olive oil were exported every year, so much so that Malta was called the "*caricator d'olio*" or "oil exporter" [1]. Some Maltese villages still bear names that are clearly reminiscent of the olive cultivation that was traditionally practiced in this area. For instance, the name Żebbuġ, a town in the central part of the island, is derived from the Maltese word "*żebbuġa*", which means "olive". A very similar word is the Tunisian Arabic "*zabbūz*", which means "old olive tree that no longer bears olives and is mainly used for wood". However, the standard Arabic word for "olive" and "olive tree", namely "*zaytun*", has been largely preserved in the name of a town in southeast Malta, namely Żejtun, as the Maltese historian and archaeologist Giovanni Francesco Abela, Vice-Chancellor of the Order of the Knights of the Hospital of Saint John of Jerusalem, found out in the 17th-century book entitled *Della descrittione di Malta, isola nel Mare Siciliano con le sue antichità, ed altre notitie* [2]. Abela pointed out that olive cultivation and oil production were very common in the Żejtun area: "This area used to be very green and was cultivated with many olive trees. Therefore, it still deserves the name Zeytun, which means olives, and a large amount of olive oil was traditionally produced there".

Olive trees were not only cultivated but also grew wild or semi-wild in the valleys and vineyards of the Maltese islands. In the 19th century, however, the strong demand for cotton from Spain led to many olive groves being replaced by cotton fields. As a result, oil production ceased, and the remaining olive groves were only used to supply olives for pickling [1]. After this period of decline and the events of the war, interest in olive cultivation in Malta revived, leading to an intensification of research into the identification and restoration of the autochthonous Maltese olive germplasm [3,4]. Despite the strong introduction of foreign varieties, Malta still preserves a peculiar local germplasm. This includes the high-yielding Malti variety, which is tolerant to abiotic and biotic stress factors and was already characterized by quite large and robust trees at the beginning of the 20th century [1]. Another important local olive variety is the vigorous Bidni (or Bitni) variety from the nearby town of Bidnija, where some colossal trees are still preserved as remnants of a large old plantation from the Middle Ages [3,5,6]. The third variety typical of Malta is the white Bajda olive, which is prized as a table olive. It was probably introduced by the Knights of Malta and rediscovered in 2010 on a property owned by the Knights [7,8]. These varieties have been described for their biochemical and agronomic profiles [4,9], and a few studies have analyzed them at the genetic level [3,4,10]. Valeri et al. (2022) [3] discovered 46 unique Maltese genotypes, finding that almost 30% of trees were grafted and, in some cases, rootstocks presented unique profiles. Nevertheless, further genetic studies on Maltese olive germplasm are needed to study its genetic relationships to include the wild germplasm of the islands as well as germplasm of foreign origin. Although strategies based on single nucleotide polymorphism (SNP) are very efficient in crop genotyping and traceability [11–14], Simple Sequence Repeats (SSRs) are still a valuable and more economical tool for studying the highly heterozygous species olive, compared to SNPs [15–19].

The recent renewed interest in the Maltese olive oil sector contrasts with the few genetic studies that have been carried out on local varieties [5]. Knowing and protecting the Maltese olive germplasm and answering basic questions about its origin and whether this germplasm has an affinity with other germplasm would help to value this heritage. Using a consolidated set of highly polymorphic SSR markers, the genetic diversity of indigenous Maltese cultivars and their relationships with the wild germplasm still present in the peripheral areas of the islands were analyzed. The focus was on trees from the medieval orchard of Bidni on the island of Malta, where double sampling was carried out from the crown and base of the trees to determine whether they had been secondarily grafted. A comparison was made with the genetic profile of 150 other varieties from the Mediterranean region to determine their relationships with Maltese germplasm.

2. Materials and Methods

2.1. Plant Material

A total of 57 olive samples both cultivated and wild (*Olea europaea* L. subsp. *europaea* var. *europaea* and var. *sylvestris*) were selected from small populations or isolated trees on the Maltese archipelago islands of Malta, Gozo, and Comino (Table 1). As illustrated in Figure 1, the trees were found in the locations of Bidnija, Mellieħa, Żebbuġ, Rabat, San Martin, Lija, and Wied Qirda Valley, the latter being located between Żebbuġ, Qormi, Siġġiewi, and Luqa. An old olive grove that dates back to the Middle Ages [2], located in the Bidnija area on Malta Island was sampled by collecting randomly young leaves, taking them from both the upper part (samples named B1–B12 in Table 1) and the lower part of the crown (samples named G1–G12 in Table 1) three feet above the ground and analyzed separately. Seven samples (coded "K", Table 1) were collected from neglected rainfed trees growing on difficult soils in an abandoned private old garden in the Mellieħa area.

Table 1. List of the 57 Maltese genotypes analyzed in this study. For each sample, location, type of tree (cultivated (cv.) or wild (w.t.), sampling point, and suitability are indicated.

N.	Code	Sampling Location	Type	Sampling Point	Use
1	B1	Bidnija old olive grove	Cultivated	Lower Crown	Oil
2	B2	Bidnija old olive grove	Cultivated	Lower Crown	Oil
3	B3	Bidnija old olive grove	Cultivated	Lower Crown	Oil
4	B4	Bidnija old olive grove	Cultivated	Lower Crown	Oil
5	B5a	Bidnija old olive grove	Cultivated	Lower Crown	Oil
6	B5b	Bidnija old olive grove	Cultivated	Lower Crown	Oil
7	B6a	Bidnija old olive grove	Cultivated	Lower Crown	Oil
8	B6b	Bidnija old olive grove	Cultivated	Lower Crown	Oil
9	B7	Bidnija old olive grove	Cultivated	Lower Crown	Oil
10	B8	Bidnija old olive grove	Cultivated	Lower Crown	Oil
11	B9	Bidnija old olive grove	Cultivated	Lower Crown	Oil
12	B10	Bidnija old olive grove	Cultivated	Lower Crown	Oil
13	B11	Bidnija old olive grove	Cultivated	Lower Crown	Oil
14	B12	Bidnija old olive grove	Cultivated	Lower Crown	Oil
15	G1	Bidnija old olive grove	Cultivated	Upper Crown	Oil
16	G2	Bidnija old olive grove	Cultivated	Upper Crown	Oil
17	G3	Bidnija old olive grove	Cultivated	Upper Crown	Oil
18	G4	Bidnija old olive grove	Cultivated	Upper Crown	Oil
19	G5	Bidnija old olive grove	Cultivated	Upper Crown	Oil
20	G6	Bidnija old olive grove	Cultivated	Upper Crown	Oil
21	G7	Bidnija old olive grove	Cultivated	Upper Crown	Oil
22	G8	Bidnija old olive grove	Cultivated	Upper Crown	Oil
23	G9	Bidnija old olive grove	Cultivated	Upper Crown	Oil
24	G10	Bidnija old olive grove	Cultivated	Upper Crown	Oil
25	G11	Bidnija old olive grove	Cultivated	Upper Crown	Oil
26	G12	Bidnija old olive grove	Cultivated	Upper Crown	Oil
27	G14	Bidnija old olive grove	Cultivated	Upper Crown	Oil
28	G15	Bidnija old olive grove	Cultivated	Upper Crown	Oil
29	G16	Bidnija old olive grove	Cultivated	Upper Crown	Oil
30	G17	Bidnija old olive grove	Cultivated	Upper Crown	Oil
31	G19	Bidnija old olive grove	Cultivated	Upper Crown	Oil
32	G20	Bidnija old olive grove	Cultivated	Upper Crown	Oil
33	W.t. 13	Gozo (south-west coast)	Wild	Crown	-
34	Cv. 14	Mellieħa Xaghri	Cultivated	Crown	Oil
35	Cv. 15	Mellieħa Xaghri	Cultivated	Crown	Oil
36	W.t. 16	St. Martin	Wild	Crown	-
37	Cv. 17	St. Martin	Cultivated	Crown	Oil
38	Cv. 18	Comino	Cultivated	Crown	Oil
39	W.t. 19	Comino	Wild	Crown	-
40	W.t. 20	Comino	Wild	Crown	-
41	W.t. 21	Comino	Wild	Crown	-
42	W.t. 22	Comino	Wild	Crown	-
43	Malti 23	Wied Qirda	Cultivated	Crown	Oil
44	W.t. 24	Żebbuġ	Wild	Crown	-
45	Malti 25	Koronia-Rabat	Cultivated	Crown	Oil
46	Malti 26	Żebbuġ	Cultivated	Crown	Oil
47	Malti 27	Lija old tree	Cultivated	Crown	Oil
48	Malti 28	Unknown	Cultivated	Crown	Oil
49	Malti 29	Unknown	Cultivated	Crown	Oil
50	Malti 30	Unknown	Cultivated	Crown	Oil
51	K1	Old garden	Cultivated	Crown	Oil
52	K2	Old garden	Cultivated	Crown	Oil
53	K3	Old garden	Cultivated	Crown	Oil
54	K4	Old garden	Cultivated	Crown	Oil
55	K5	Old garden	Cultivated	Crown	Oil
56	K6	Old garden	Cultivated	Crown	Oil
57	K7	Old garden	Cultivated	Crown	Oil

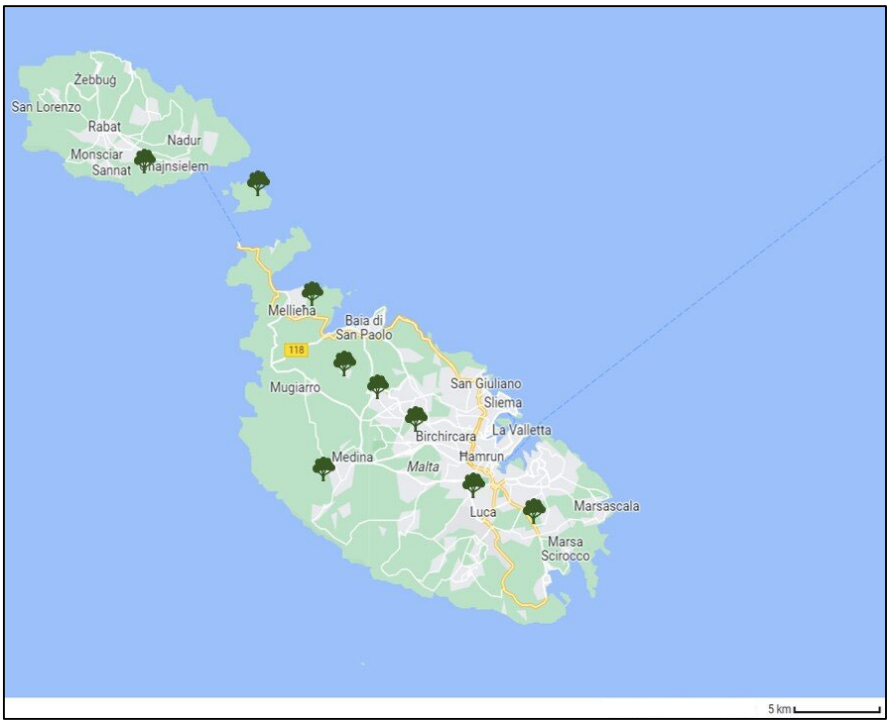

Figure 1. Map of the Maltese archipelago with the geographic locations of the olive samples considered in the study.

2.2. DNA Extraction

Young leaves were collected and immediately frozen. For each sample, genomic DNA was extracted from three lyophilized young leaves which were finely ground in a Tissue-lyser and following the protocol of Spadoni et al. (2019) [20]. DNA quality and concentration were assessed and checked by 0.8% agarose gel electrophoresis and a NanoDrop TM ND2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). The extracted DNA was transferred to a 96-well plate and was normalized to a standard concentration of 50 ng/µL using a 0.1 × TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA. DNA was stored at −20 °C until used.

2.3. Olive Genotyping

Genotyping of the 57 olive samples was performed using a set of 10 SSR markers highly informative for the study of genetic variability in olive and producing clear polymorphic amplicons in PCR reactions [21–23] (Table 2).

Table 2. List of the 10 SSRs used to genotype the Maltese olive trees under investigation.

SSR ID	Bibliographic Reference	Repeat Motif	Ta
DCA03	Sefc et al. (2000) [21]	$(GA)_{19}$	50 °C
DCA05	Sefc et al. (2000) [21]	$(GA)_{15}$	50 °C
DCA09	Sefc et al. (2000) [21]	$(GA)_{23}$	55 °C
DCA13	Sefc et al. (2000) [21]	$(CA)_{15}$	55 °C
DCA17	Sefc et al. (2000) [21]	$(GT)_9(AT)_7AGATA(GA)_{38}$	50 °C
DCA18	Sefc et al. (2000) [21]	$(CA)_4CT(CA)_3(GA)_{19}$	50 °C
GAPU71b	Carriero et al. (2002) [22]	$GA(AG)_6(AAG)_8$	59 °C
GAPU101	Carriero et al. (2002) [22]	$(GA)_8(G)_3(AG)_3$	59 °C
EMOL	De la Rosa et al. (2002) [23]	$(GA)_{12}$	50 °C
EMO90	De la Rosa et al. (2002) [23]	$(CA)_{10}$	50 °C

Amplifications were carried out in a final volume of 12.5 µL containing 1× Dream Taq buffer, 0.15 mM dNTP, 0.25 µM primer mix, 0.3 U Dream Taq, and 50 ng genomic

DNA following the protocol of Spadoni et al. (2019) [20]. In order to verify PCR efficiency, PCR products for each of the 10 SSR markers were randomly checked by electrophoresis on 2.5% SeaKem LE Agarose gel (Lonza, Visp, Switzerland). Amplicons were detected using the ABI PRISM 3100 Avant Genetic Analyzer automatic capillary sequencer (Applied Biosystems, Waltham, MA, USA) with the GeneScan Liz 600 dye as the internal molecular size standard (Applied Biosystems, USA). The allele size of each amplification product was estimated using GeneMapper v.5.0 software (Applied Biosystems, Foster City, CA, USA).

2.4. References for Comparison

To investigate the relationships of the 57 Maltese olive samples genotyped by SSRs with other Mediterranean germplasm, their genetic profiles were compared with the allelic profiles of 150 olive reference varieties representative of the Mediterranean cultivation area available at the databank of the DISSPA-UNIBA University (Bari, Italy). The collection included: Albania (ALB, 6), Algeria (ALG, 11), France (FR, 7), Greece (GR, 9), Italy (I, 63), Malta (4), Syria and Kurdistan (SY, 12), Spain/Portugal (SP, 12), Tunisia (TU, 13), and Lebanon (LE, 13). The Italian subset included 7 autochthonous Apulian genotypes selected as showing tolerance to the bacterium *X. fastidiosa* [24].

2.5. Data Analysis

2.5.1. Genetic Diversity

SSR markers with clear and distinct molecular patterns were used to estimate genetic indices using GenAlEx v.6.5 software [25]. The indices were the number of alleles (Na), the effective number of alleles (Ne), Shannon's information index (I), the observed (Ho) and expected (He) heterozygosity, and the fixation index (F). A Nei's unbiased genetic distance pairwise population matrix was obtained and used to determine the inter-individual relationship and verify if the molecular data supported the partitioning of the olive samples into specific groups, by performing a principal coordinates analysis (PCoA) [26]. The software GenAlEx v. 6.5 was also used to perform the Lynch and Ritland pairwise relatedness (LRM) analysis [27] which allows for the verification of the degree of allelic similarity between genotypes and the identification of synonyms.

2.5.2. Genetic Relationships and Population Structure

The genetic relationships of the Maltese varieties and 150 Mediterranean olive cultivars were estimated by a principal coordinate analysis (PCoA) performed with GenAlEx based on the Nei's pairwise unbiased genetic distance matrix. In addition, an unweighted neighbor-joining tree was constructed using the software DARWIN v. 6.0.010 (http://darwin.cirad.fr) [28]. The robustness of the branches was tested with 1000 replicates [29] and the tree was displayed with FigTree 2016-10-04-v1.4.3 available at http://tree.bio.ed.ac.uk/software/figtree/.

The genetic structure of the population was assessed using the Bayesian clustering method implemented in the software STRUCTURE v. 2.3.4 [30]. This assigned accessions to populations (K) based on the Markov Chain Monte Carlo (MCMC) algorithm, with ten independent runs for each K (from 1 to 10) and using 100,000 MCMC repetitions and 10,000 burn-in periods. The results obtained were analyzed using Structure Harvester software [31] based on an ad hoc statistic dK test [32]. Accessions with a membership coefficient [qi) higher than 0.7 were assigned to defined populations; otherwise, they were considered to be of admixed ancestry.

3. Results

3.1. Genetic Relationships and Genetic Diversity of Maltese Genotypes

SSR fingerprinting of the Maltese genotypes resulted in clear allele profiles for all samples which were used to study the genetic relationships among them. The neighbor-joining dendrogram divided the 57 Maltese varieties into two clusters, A and B (Figure 2). Cluster A comprised all the Bidni samples obtained from the upper (samples B1–B12) and

lower (G1–G19) parts of the crown, indicating that they were the same genotype. Cluster A also included several of the wildtype samples such as the w.t. 13 (from Gozo), w.t. 16 (from St Martin), w.t. 19, w.t. 20 (from Comino), and w.t. 24 (from Żebbuġ), as well as three cultivars, Malti 23 and Malti 26, and sample 14 from the locality of Mellieħa Xaghri. Cluster B included the samples K1/K4–K7 which were shown to be synonymous with sample Malti 27 (from Lija), and closely related to sample Malti 25 (from Koronja-Rabat). The same cluster included the wildtypes 21 and 22, the cultivars cv. 15 (Mellieħa) and 18 (Comino), and, at some distance, the samples Malti 28-29-30 with samples K3 and St Martin cultivar 17. Sample G20 was probably a misnomer with sample K2 in cluster A. According to these results, only one sample from each group of synonymies was retained, reducing the samples for further analyses to a total of 20.

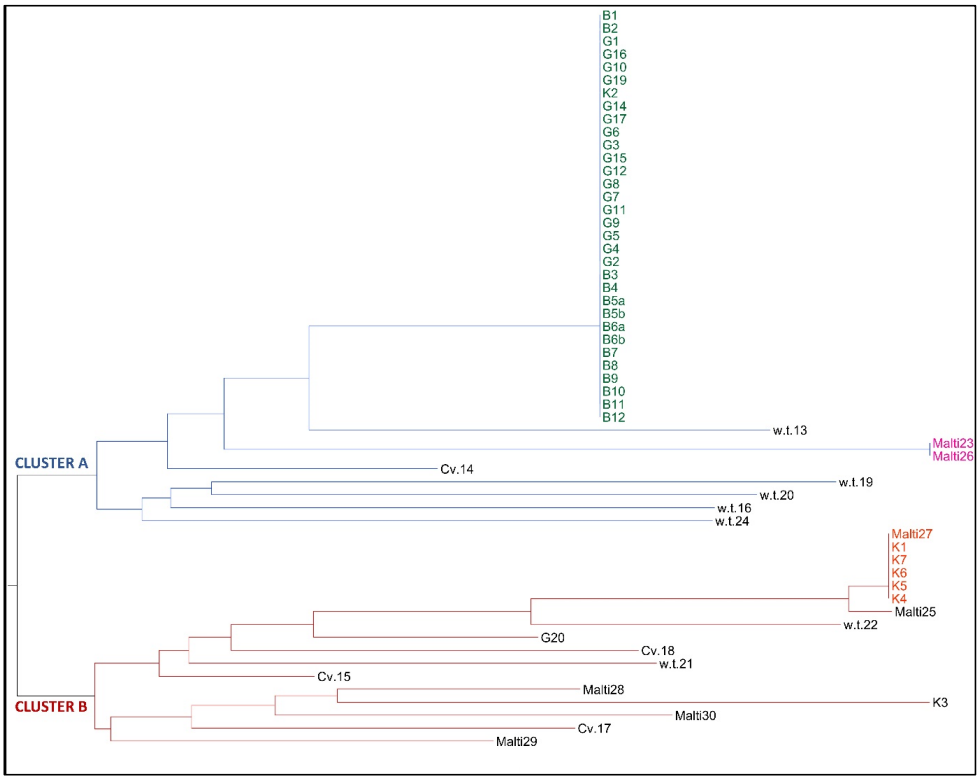

Figure 2. Neighbor-joining dendrogram illustrating the phylogenetic relationships among the 57 analyzed Maltese genotypes characterized by using 10 SSR markers.

3.2. Genetic Diversity of Maltese Genotypes

The genetic diversity indices of the Maltese samples expressed a total of 84 alleles (7.6 alleles/locus) with a 2.98 mean number of effective alleles (Ne) and a Shannon information index (I) of 1.08. The observed (Ho) heterozygosity averaged 0.77, the expected heterozygosity (He) averaged 0.65, and the mean inbreeding coefficient F was −0.167 (Table 3).

Table 3. Genetic diversity indices' (number of alleles (Na), effective number of alleles (Ne), Shannon's diversity index (I), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (F)) values revealed in the 57 olive Maltese accessions analyzed with 10 SSR markers.

	Na	Ne	I	Ho	He	uHe	F
Total	84.0	32.8					
Mean	7.6	2.9	1.323	0.765	0.651	0.657	−0.167
SE	0.8	0.191	0.065	0.065	0.022	0.022	0.088

3.3. Genetic Relationships of the Maltese Germplasm with the Mediterranean Germplasm

To analyze the relationships of Maltese olives with other Mediterranean germplasm, their molecular profiles were compared with the profiles of 150 olive varieties from 10 different Mediterranean countries. The pairwise relatedness analysis LRM made it possible to exclude an identity of the Maltese varieties with other germplasm but showed a high similarity between sample 1 from the Bidnija olive grove and cultivar 14 from Mellieħa-Xaghri (Table 4). Five synonymous pairs were also identified: Grossanne/Sigoise_2, Olivo_di_Berat/Konservolia, Androcarpos/Gordal_Sevillana, Aayrouni/Soury_2, and the misnomer, Lebanese_Taggiasca, which was instead Karidolia; only one sample of each pair was retained for further analyses.

Table 4. Results obtained by the pairwise relatedness analysis (LRM) for the identification of synonymies in Maltese and Mediterranean varieties. The value 0.50 indicates identity between genotypes.

Sample 1	Sample 2	LRM
ALG_Sigoise_2	FR_Grossanne	0.50
ALB_Olivo_di_Berat	GRE_Konservolia	0.50
GRE_Androcarpos	SP_Gordal_sevillana	0.50
ALG_Aayrouni	LIB_Soury_2	0.50
LI_Taggiasca	GRE_Karidolia	0.50
LIB_Baladi_8	LIB_Baladi_12	0.48
LIB_AbouChawkeh_1	LIB_AbouChawkeh_2	0.47
LIB_Baladi_1	LIB_Soury	0.45
LIB_Baladi_8	LIB_Aayrouni	0.44
LIB_Del_1	LIB_Del_2	0.43
GRE_Koroneiki_1	POR_Galega_Grada_de_Serpa	0.43
LIB_Aayrouni	LIB_Baladi_12	0.42
MAL_Bidnija_olive_grove_1	MAL_Mellieha_xaghri_cv.14	0.42
LA_Leccino_Lazio_PR	ALB_Kalinjot_Oval	0.41

The estimation of genetic indices highlighted a rich diversity in the whole collection, with a total of 637 alleles and 402 effective alleles, in particular in Italian and Maltese populations, which showed the highest allele numbers (11.9 and 10.4, respectively) and Shannon's information index (I) (1.881 and 1.922, respectively) (Table 5). The observed heterozygosity (Ho) fell between between 0.531 (Tunisia) and 0.843 (Greece), whereas the expected heterozygosity was highest in the Maltese population (0.801) which also showed a slight inbreeding.

Table 5. Genetic diversity indices obtained for the Maltese collection and olive populations of different Mediterranean countries, analyzed with 10 SSR markers. Number of alleles (Na), effective number of alleles (Ne), Shannon's diversity index (I), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), and fixation index (F) are listed.

Population	Na	Ne	I	Ho	He	uHe	F
Italy	11.9	5.4	1.881	0.681	0.786	0.792	0.140
Albania	4.9	3.7	1.378	0.783	0.689	0.752	−0.122
France	6.2	4.1	1.554	0.692	0.738	0.795	0.088
Greece	4.7	3.6	1.349	0.843	0.691	0.744	−0.232
Kurdistan	6.0	4.1	1.490	0.770	0.711	0.748	−0.065
Syria	5.4	4.0	1.465	0.709	0.725	0.762	0.015
Spain/Portugal	5.5	3.7	1.376	0.731	0.670	0.697	−0.073
Tunisia	4.6	3.0	1.157	0.531	0.571	0.594	0.107
Malta	10.4	5.8	1.922	0.735	0.801	0.819	0.085
Lebanon	4.1	2.6	1.065	0.774	0.577	0.599	−0.319
Grand Mean	6.3	4.0	1.453	0.728	0.692	0.73	−0.042
Total	637	402	-	-	-	-	-

The genetic structure of the entire Mediterranean collection, including the Maltese samples, was analyzed using the non-parametric alternative PCoA analysis performed on Nei's unbiased genetic distance matrix. The PCoA plot (Figure 3) based on the first two components (PC1 and PC2) clustered most of the Maltese samples with the Italian varieties and far away from the Spanish ones, while Tunisian, Syrian, and Algerian samples clustered together and fell in different quadrants.

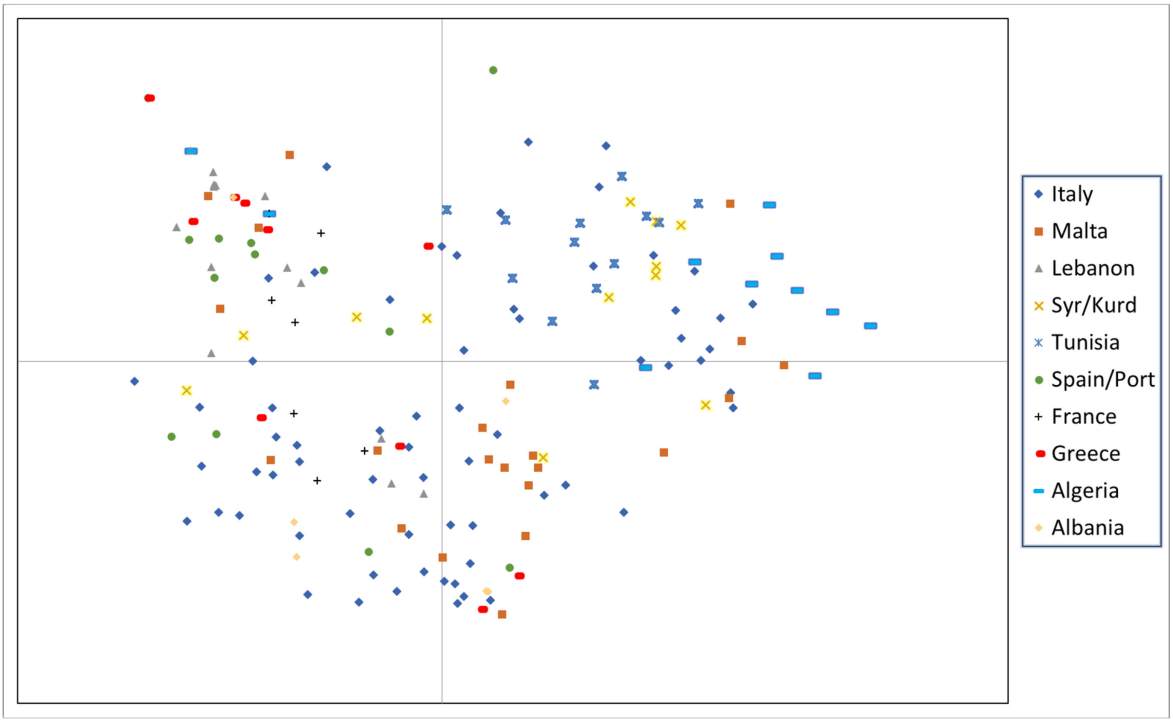

Figure 3. Scatter plot for genetic variation explained by the first two principal component PCs. Individual samples having different geographical origins are marked with different colors.

These results were confirmed by the hierarchical clustering of the entire collection (Figure 4). The dendrogram formed three clusters, with cluster C1 (yellow) including most Tunisian (TUN) and Apulian (PU) varieties, and cluster C2 (green) including most Algerian (ALG) and Syrian (SYR) as well as several Sicilian varieties such as Nerva, Calatina, Vaddarica, Erbano, Carbucia, Natisana, the Spanish Gordales, and three Maltese samples, Malti Lija, Santaton 1, Santaton 2, and Leucocarpa (Bajda).

Cluster C3 formed two subclusters, C3-A and C3-B. The large subcluster, C3-A (white), included the Maltese samples K3, cv. 17, and Malti 28, 29, and 30 together with varieties from different countries (Kurdistan, Spain, France, Greece, and Lebanon) characterized by medium-sized drupes. Most Maltese varieties were collected in subcluster C3-B (blue), together with most Italian varieties from the regions Apulia (PU), Tuscany (TO), Basilicata (BA), Calabria (CA), Abruzzo (AB), and Sardinia (SA). In particular, the sample Bidnja_old_Olive_grove 1, representative of the Bidnia variety, clustered with four wildtype samples (w.t. 13, 16, 19, and 20) and with the cultivated cv. 14 of Mellieħa Xaghri and Malti cv. 23. Interestingly, the subcluster C3-B also included the nine Italian genotypes currently under investigation in Italy (genotypes PR; light blue) as they are presumably resistant to the bacterium *Xylella fastidiosa* which is responsible for olive quick decline syndrome (OQDS) affecting olives in Apulia. Another group of Maltese cultivars, including the samples cv. 15 and cv. 25, as well as the wildtype w.t. 21 and w.t. 22 (from Comino), were clustered with the Italian varieties Calipa, Provenzale, and Spina, and the Albanian varieties White of Tirana and Mixan.

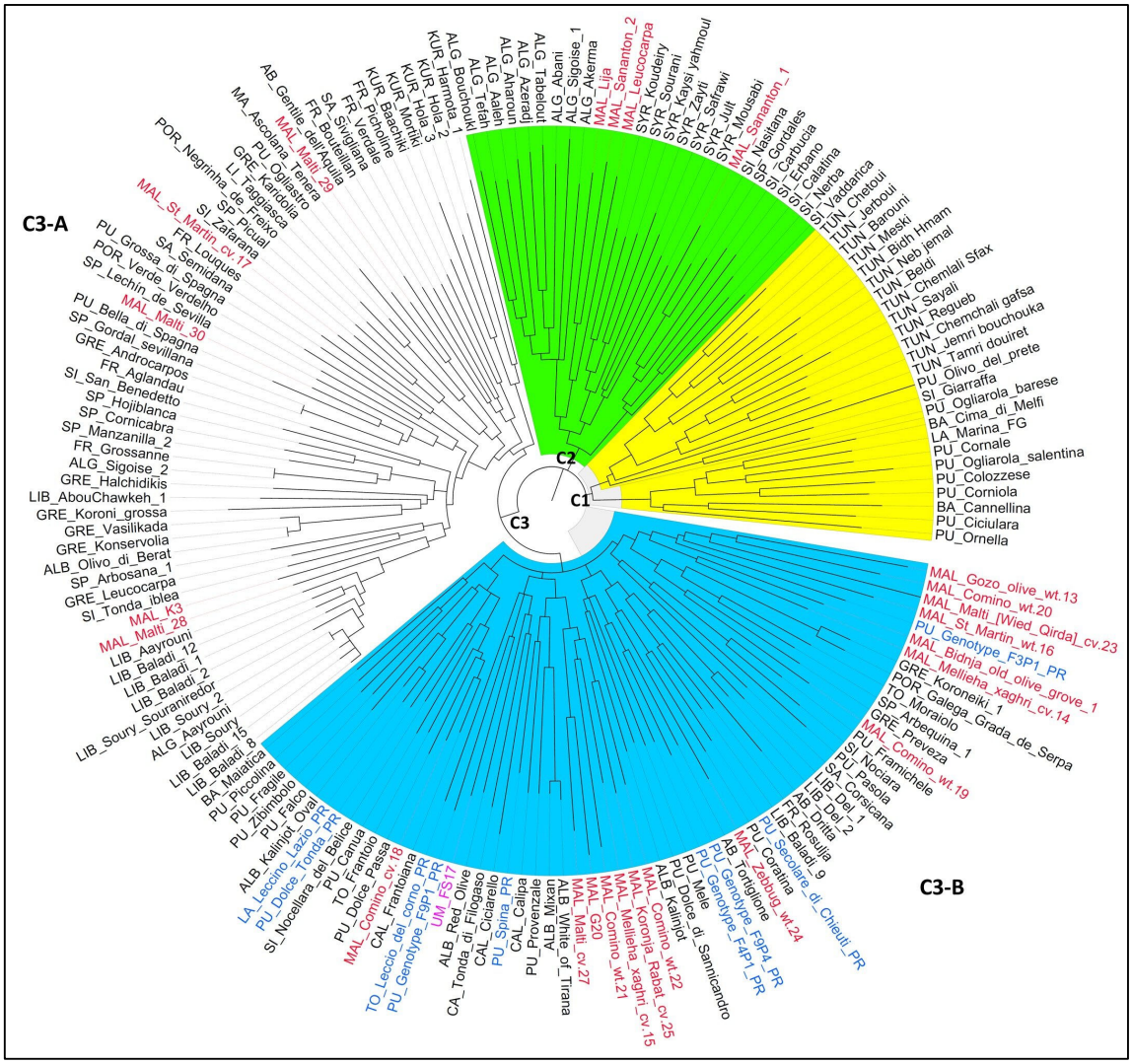

Figure 4. Dendrogram generated by the neighbor-joining clustering method using 10 SSR markers, illustrating the phylogenetic relationships among the Maltese genotypes and the Mediterranean ones. Clusters are indicated: C1 (yellow); C2 (green); C3-A (white); C3-B (blue). The Maltese samples are indicated in red; the Italian varieties include Putative Resistant (PR) genotypes (blue); Apulia (PU); Tuscany (TO); Basilicata (BA); Calabria (CA); Abruzzo (AB); Lazio (LA); Campania (CA); Sicilia (SI); Sardinia (SA). Maltese varieties are indicated in Purple.

3.4. Population Structure

The genetic structure of the olive germplasm under study was also investigated using the parametric model implemented by STRUCTURE [29]. According to Evanno's ΔK test, two ancestral subpopulations (K = 2) were assumed to best fit genetic data from the germplasm under study (Figure 5) and accessions with a membership coefficient (qi) higher than 0.7 were assigned to the two ancestries.

With few exceptions, the K1 ancestry was predominant in the germplasm from the eastern Mediterranean including Lebanon, Greece, and Italy, while the K2 ancestry was predominant in the Maltese germplasm, including the cultivated Bidni, Malti, and Bajda and the wildtype samples, together with several Italian varieties (Figure 6).

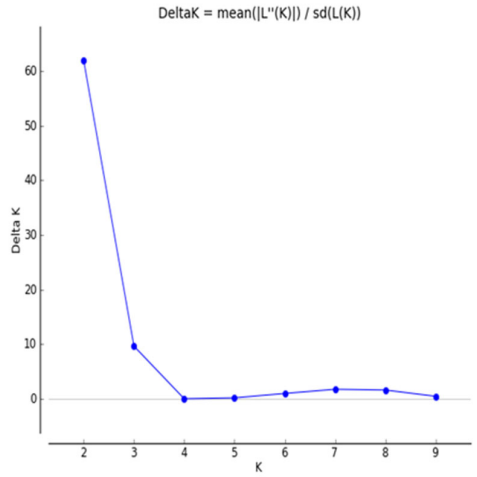

Figure 5. Evanno's ΔK plot associated with STRUCTURE genetic analysis.

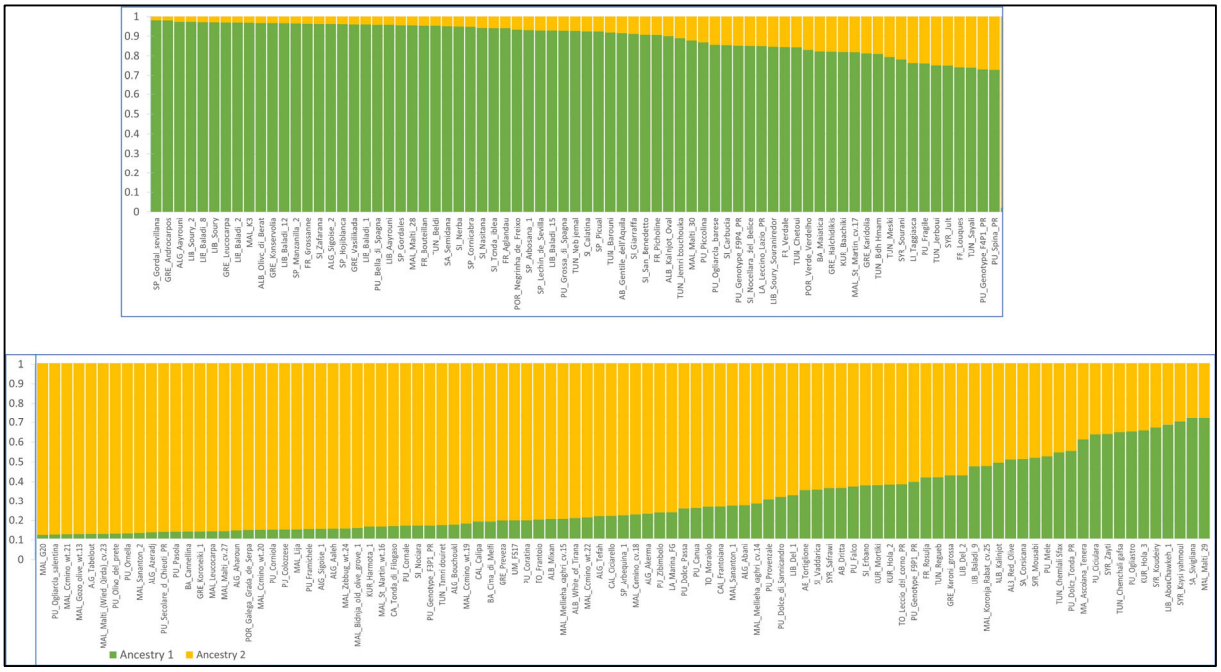

Figure 6. Genetic structure of Mediterranean olive genotypes. Bar plot showing clusters inferred by STRUCTURE. Each vertical line stands for a single accession, and it is divided into K-colored segments that represent the estimated membership coefficient (q).

4. Discussion

Archeological finds prove the presence of olive trees in Malta since Roman times, although cultivation was probably first introduced by the Phoenicians and intensified by the Romans [3]. Although the Maltese archipelago offers optimal geo-climatic conditions for olive cultivation, the olive oil sector in Malta is now secondary and has only recently experienced a resurgence that has led to programs to restore the ancient olive germplasm. Only a few autochthonous varieties such as "Bidni", "Malti", and the white-fruited "Bajda" (Leucocarpa variety), which are probably among the oldest varieties cultivated on the island, have survived in Malta. Malta also preserves wild genotypes that could be genetically related to the cultivated germplasm, due to the gene flow that is expected in areas where the two botanical varieties share a common environment.

Recently, Mazzitelli et al. [5] pointed out that indigenous Maltese varieties are characterized by a specific gene pool that distinguishes them from imported varieties, supporting the idea of local differentiation and limited genetic exchange with other germplasms. The

goal of this study was to verify this observation and to extend the investigation to the genetic relatedness of the Maltese germplasm with the wild local germplasm and with other Mediterranean germplasm, in order to shed light on the possible origin of this gene pool.

The genotyping carried out with 10 polymorphic SSRs confirmed that all samples from the medieval olive grove in the northwest of the island of Malta belonged to the Bidni variety. The only exception was sample G20 which was very similar to the Malti variety and was probably confused with sample K2, which instead belonged to the Bidni variety. The study also focused on verifying whether the old Bidni trees had been secondarily grafted. According to Valeri et al. [3], Bidni trees are basally grafted onto rootstocks, confirming the practice of grafting the better-performing genotypes onto wild rootstock, common in the whole Mediterranean region, and also on the island of Malta [33]. Our study showed that the upper and lower parts of the Bidni trees belong to a single genotype, suggesting that there was no secondary top-grafting of the branches. Moreover, our results confirmed a high similarity between the old Bidni variety and the local wild and cultivated genotypes included in the analysis, suggesting that the oleaster population played a role in the selection of the cultivar Bidni, as well as other local varieties. This is in accord with [2,3] which showed that both rootstock and scion genotypes belong to the genetic lineage E2, which is considered endemic to the central Mediterranean region and a remnant of a rich wild olive gene pool [34].

The genetic analysis of the Maltese germplasm with 150 olive varieties from 10 other Mediterranean countries resulted in high values of genetic indices for the Maltese germplasm, confirming the genetic richness and value of Maltese olives. This could be due to the wild and feral forms included in the analyzed collection, although the Maltese germplasm also showed a common genetic basis with the Italian varieties. Indeed, both the neighbor-joining dendrogram and the PCoA analyses pointed out that the Bidni cultivars share a genetic background with Leccino and other varieties closely related to this Italian germplasm. This is consistent with the results of Farrugia et al. [35], who found, by using Agglomerative Hierarchical Clustering analysis (AHC), the close relationship of cv. Bidni with the Italian varieties Pendolino and Leccino, and of cv. Malti with Frantoio and Ottobratica. The Bidni variety has also been found to be related to the main indigenous Albanian olive variety Kalinjot [36,37], and to the Lebanese variety Del, which raises an interesting question about the origin of the relationships among all these varieties. Human migrations and trade exchanges have played a key role in determining the current pattern of olive germplasm dispersal in the Mediterranean region. The olive is thought to have originated in the Syria/Turkey region about 5000 years ago, from where it spread westwards [38]. This is confirmed by the discovery of three main gene pools in the western (namely Q1), central (Q2), and eastern (Q3) Mediterranean [39,40]. Di Rienzo et al. [10] described the olive dispersal from Syria to Greece, followed by a secondary migration to Italy and Malta. Our results are consistent with these studies, but further studies on larger collections will be necessary to better clarify the origin of the Maltese germplasm.

The Maltese samples from the Mediterranean collection, including Malti Lija, Santaton 1, Santaton 2, and Bajda clustered with Algerian, Syrian, and Sicilian varieties, suggesting an influence of Arabic culture on the distribution of their common gene pool. Another group, which included the cultivated varieties K3, cv. 17, Malti 28, Malti 29, and Malti 30, clustered, instead, with the Spanish and Lebanese varieties, including the very popular polyclonal Baladi variety [41]. This cluster also appeared to be related to the medium/large size of the drupes, including varieties of different origins (Spain, Greece, Italy, etc.) grown for table olive production. Fruit size is a trait with high heritability [42,43], one of the most important domestication traits [44,45], and a target of modern olive breeding programs [46,47]. It will be interesting to further study the common genetic background of the cluster to identify genetic determinants associated with fruit size [48]. Finally, an interesting relationship was revealed between the cv. Bidni with the Italian variety FS17 and other Italian selections identified as resistant to the bacterium *X. fastidiosa* which has been destroying olive cultivation in Apulia since 2013 [49]. The Bidni variety is also of great interest for

other agronomical and economical/nutritional traits such as vigor, good oil yield, high polyphenol content [50], and resistance to the olive fly and *Verticillum* wilt disease [1]. The genetic relationship with the Italian genotypes resistant to *X. fastidiosa* makes the variety Bidni an interesting candidate whose profile of resistance should be further investigated in order to expand the range of varieties suitable for converting infected olive orchards into resilient areas and for the conservation of olive tree biodiversity.

5. Conclusions

The recently renewed interest, which is also economical, in the Maltese olive oil sector contrasts with the scarcity of genetic studies carried out on local cultivars (Mazzitelli et al., 2015) [5]. Basic questions relating to the migratory movements from which Maltese olive germplasm originate and whether this olive germplasm shows a closed gene pool or an affinity with other Mediterranean countries are still valid (Mousavi et al., 2017; Besnard et al., 2018) [17,45]. In this study, the genetic relationships of Maltese varieties with the wild local germplasm and other Mediterranean cultivars were investigated in order to contribute to the knowledge of the genetic differentiation of olive germplasm in the Mediterranean region. The study confirmed that Maltese germplasm preserves a great genetic diversity which could be due to the introgression of gene pools from wild local germplasm. Strong relationships were also found with the Italian germplasm, and of particular interest were the relationships of the Maltese germplasm with the Italian genotypes which are characterized by resistance to *X. fastidiosa.* This observation opens the possibility of studying the Maltese germplasm as a possible source of resistance to the bacterium in order to increase the number of available accessions, restore olive cultivation in the infected areas, and contain the spread of the pathogen.

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