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Olive Tree (*Olea europaea* L.) Diversity in Traditional Small Farms of Ficalho, Portugal

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Abstract: The genetic diversity of "Gama" and "Bico de Corvo", local cultivars of olive tree (*Olea europaea*) from seven traditional orchards of Ficalho (Alentejo region, Portugal), was studied to characterize the local diversity and assess the level of *on farm* diversity. Two different analytical systems were used: endocarp morphological characteristics and genetic analysis by microsatellite markers (Simple Sequence Repeats or SSR). The seven screened *loci* were polymorphic and allowed the identification of 23 distinct SSR profiles within the 27 trees analyzed. A total of 52 different alleles were scored, with an average of 7.43 alleles/SSR locus, and considerable genetic diversity was found. Neighbor-Joining algorithm cluster analysis and principal co-ordinate analysis (PCoA) allowed for the identification of the genetic relationships between several accessions. The 27 *Olea* accessions were clearly separated into three different groups. SSR analysis was more precise than endocarp characterization in the classification of genetic diversity among the olive tree cultivars. The study shows reasonable olive tree diversity in Ficalho, indicating that these traditional orchards are important reservoirs of old minor cultivars and incubators of new genotypes.

Keywords: olive tree local cultivars; endocarp characterization; SSR genotyping; *on farm* conservation

1. Introduction

The olive tree preceded the Romans in the Iberian Peninsula and olive oil has always been of great significance for the whole of southern Europe. The recognition of olive oil dietetic properties has lead to an increase in its consumption worldwide. Portugal is the eighth-largest worldwide olive oil producer and according to the Olive Oil Council is the fourth largest producer in the European Union [1]. This accounts for about 7% of the country's agro-food exports that have been increasing exponentially since 2000, particularly of high quality extra virgin oil. The activity of olive oil press industry has likewise been increasing [2].

The domestication of the olive (*Olea europaea* subsp. *europaea* var. *europaea*) was a continuous, and complex process [3] with many crossings occurring between cultivated trees and local oleasters (*O. europaea* L. subsp. *europaea* var. *sylvestris* (Miller) Lehr) across the entire Mediterranean region. Portugal has a large genetic patrimony of such germplasm represented by many "old" local cultivars, some of which have restricted distribution [4]. Olive trees are grown throughout the country, mainly further inland, and represent 9.2% of the Portuguese Utilized Agriculture Area (UAA). About 60% of the oil is produced in the Alentejo region using two different cultivation systems, traditional farming systems (85% of the area and 35% of total olive oil production) and semi-intensive and intensive systems (15% of area and 65% of total olive oil production) [5].

Traditional orchards still account for 80% of the cultivated area [6], where "Cordovil de Serpa" and "Verdeal Alentejana" are the dominant cultivars in these traditional systems in the Alentejo region [7]. This cropping system is an important repository of genetic diversity as other local cultivars are always present [8], although in small numbers [9,10]. Such genetic variability contributes to the distinctness of the oil and determines crop resilience. The ability to adapt to changes in biotic and abiotic factors, such as pests, diseases, and climatic stresses, is important, particularly in areas where climate change may become more severe. So, the sustainability of such agroecosystems could be endangered by the emergence of modern olive growing systems that have poor genetic diversity.

Identification of existing genetic diversity is essential for olive germplasm management and preservation. This process should start with morphological studies of the olive cultivars, such as the endocarp characteristics [11,12], which serve to discriminate among trees. Methodologies involving molecular markers should complement the morphological characterization. For instance, microsatellites (or Simple Sequence Repeats—"SSRs") have been shown to be very useful in the characterization of olive germplasm [13–16], even for small areas of cultivation [17–20].

In the light of increased risk of genetic erosion of minor olive cultivars, the present study was carried out in Ficalho which is an important area within the traditional Alentejo olive oil producing region. The study aims to (i) characterize the old olive trees using endocarp parameters and SSR markers, (ii) investigate the genetic relationships between these olive trees and the dominant cultivars "Cordovil de Serpa" and "Verdeal Alentejana", and (iii) assess the level of *on-farm* genetic diversity.

2. Materials and Methods

The study was performed at seven small traditional orchards in Ficalho within the Alentejo region, Portugal (Figure 1). Approximately 87% of the olive trees from these orchards are "Verdeal Alentejana" and "Cordovil de Serpa" [10] and so, our study focused on minor cultivars. A total of 27 olive trees including two oleasters and one unnamed tree ("Desco 05") were studied, following the labelling used by farmers. Two traditional cultivars from other regions were also studied, "Maçanilha Algarvia" (from Algarve region, Portugal) and "Cornezuelo" (from Andalusia region, Spain) as shown in Table 1.

Table 1. Olive tree accessions (cultivars and wild) characterized in the study.

Olive Tree	Designations	Area of Cultivation
Local Cultivars	Bico de Corvo Carrasquenha Cordovil de Serpa Galega Gama Maçanilha Verdeal Alentejana	Traditional orchards, Ficalho
From other Regions	Cornezuelo Maçanilha Algarvia	Germplasm collection, Herdade da Abóboda *
Unnamed Tree	Desconhecida	Traditional orchards, Ficalho
Wild	Zambujeiro	Traditional orchards, Ficalho

* Farm from Ministry of Agriculture.

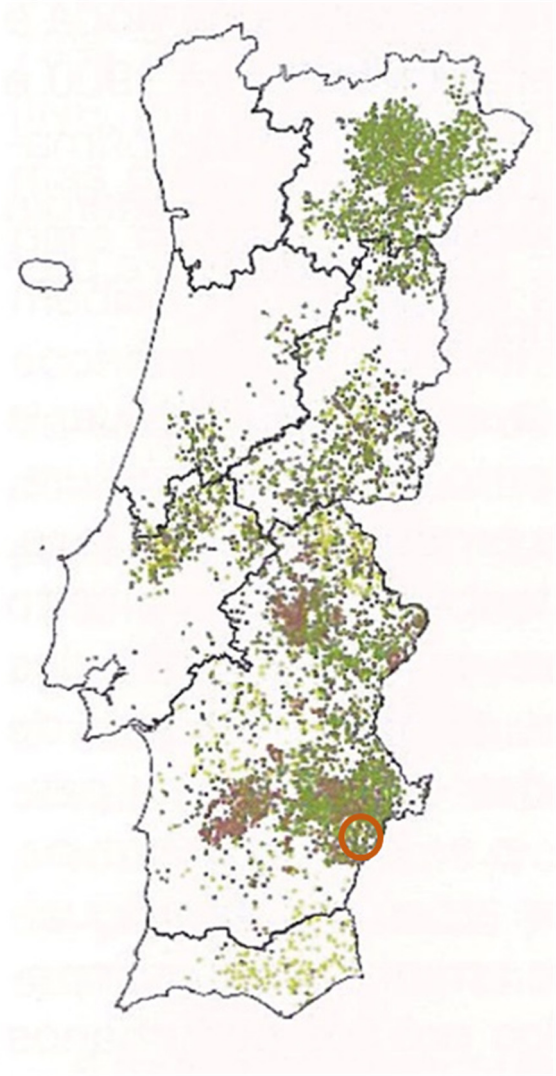


Figure 1. Olive tree distribution in Portugal, according to InstitutoNacional de Estatística (2011). The traditional orchards at Ficalho where the study was performed are delimited. 50 ha of olive tree with 60 trees/ha; 50 ha of olive tree with 61 to 100 trees/ha; 50 ha of olive tree with 101 to 300 trees/ha; 50 ha of olive tree with more than 300 trees/ha.

2.1. Morphological Study

Plant characterization is usually carried out using a large number of descriptors. In olives, descriptors based on endocarp characteristics can help determine the identity of a cultivar [12,21]. Forty fruits from each tree were collected during two seasons (2012 and 2013). Five endocarp characteristics were evaluated as follows: weight (g), length (mm), diameter (mm), and number and distribution of vascular bundles, using established methodologies [22]. Endocarp images were captured using a stereoscopic microscope Leica Wild MZ8 equipped with a Leica DC200 camera linked to a Leica IM50 image management software.

2.2. DNA Extraction, SSR Markers, PCR Amplification and Fragment Sizing

DNA was isolated from 100 mg of fresh young leaves ground in liquid nitrogen using the DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA quality was checked on 0.8% agarose gel, and the DNA concentration was estimated using a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Massachusetts, MA, USA).

For olive tree genotyping, nine nuclear SSRs were used, selected among those available in the literature [16] and previously proven to be suitable for the characterization and identification of olive varieties as follows: OeUA-DCA04, OeUA-DCA05, OeUA-DCA13, OeUA-DCA14, OeUA-DCA16, OeUA-DCA18 [13], GAPU-71B, GAPU103-A [14] and EMO90 [15]. PCR was conducted in a final volume of 25 µL containing 25 ng of DNA, 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM forward primer fluorescently labelled with WellRED dyes at the 5′-end and unlabelled reverse primers, and 0.05 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Capillary electrophoresis was performed to separate the PCR products using the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA). The sizes of the amplified products were determined based on an internal standard included with each sample. Data Analysis was performed using the CEQ 8000 Fragment Analysis software, version 9.0, according to the manufacture's recommendations (Beckman Coulter Inc., Brea, CA, USA). Sizes of SSR fragments were automatically calculated using the CEQ 8000 Genetic Analysis System. The information obtained was used to study the genetic diversity of the selected olive trees and to determine their relatedness.

2.3. Data Analysis

The endocarp morphological characteristics were analyzed for their means and standard errors. Principal Component Analysis (PCA) was performed to examine the morphological variation and to identify the most relevant characteristics in order to distinguish different accessions. The program NTSYS-pc, version 2.1 was used in all the statistical multivariate analysis [23]. The statistical analysis of SSR data was performed using Microchecker software v2.2.3 [24] for the detection of null alleles, stuttering and allele dropout, and FSTAT [25] for genetic diversity parameters (Polymorphism Information Content, allelic richness, private allele number, and heterozygosity). The genetic distance between each pair of individuals was calculated as one minus the proportion of shared alleles across all *loci* [26] using the MICROSAT program package [27].

To establish the genetic relationships existing between several accessions, two methods were used as follows: Neighbor-Joining Algorithm cluster analysis and the principal co-ordinate analysis (PCoA). The Neighbor-Joining algorithm, as implemented in the DARwin software package, version 6.0.12 [28], was based on a dissimilarity matrix, and the reliability of the tree topology was assessed via bootstrapping over 1000 replicates. Regarding the PCoA, the distance matrix was performed based on the proportion of shared alleles calculated from SSR markers following NTSYS—pc v.2.1 [23].

3. Results and Discussion

Two different analytical systems were used, endocarp morphological characteristization and genetic analysis with microsatellite markers, in order to identify the germplasm and to study the diversity of the olive trees in Ficalho.

The endocarp images and characteristics for the different accessions are shown in Figure 2 and Supplementary Table S1. We found that the surface roughness and the number of vascular bundles over the endocarp surface were characteristics with little variation in keeping with observations from the Spanish Extremadura region [19] as well as from the Italian Tuscany region [29]. However, our data revealed important differences and variability in other endocarp characteristics. The PCA analysis of the data allowed for the discrimination of the olive trees, with the first two principal components explaining 77.27% of the total variation (48.37% and 28.90% for the first (I) and the second (II) principal components, respectively). The I principal component is controlled by weight, length and diameter of the endocarps and the II principal component by the number and distribution of vascular bundles on the endocarp surface (Figure 3). According to the projections of the 27 accessions onto the plane defined by the I and the II principal components, the endocarps with higher diameter are on the right (e.g., "Bico 32" and "Maçanilha Algarvia–Maca Al"), and those with low diameter are on the left (e.g., "Zambujeiro 01–Zambu 01" and "Galega–Galeg 01"). On the other hand, on the upper side of the plane are endocarps with a lower number of vascular bundles (e.g., "Zambujeiro 17–Zambu

17") and on the lower side are endocarps with higher surface roughness (e.g., "Maçanilha 16–Maca 16" and "Maçanilha Algarvia–Maca Al") (Figure 3A and Supplementary Table S1). The oleasters ("Zambu 01" and "Zambu 17"), which have a typical wild aspect, are quite distant from all the other accessions, although quite distinct from each other. It was not possible to conclude if they are true oleasters or feral forms. The unnamed tree ("Desco 05") is close to the traditional cultivar "Galega–Galeg 01" (Figure 3) and could have resulted from hybridization with a wild olive tree (Figure 3A). The "Maçanilha Algarvia–Maca Al" which is not grown in Ficalho, is in Figure 3A far from the local varieties. Considering the remaining traditional local varieties, it is observed that the majority are in the center of the plane defined by the I and the II principal components. Two "Verdeal Alentejana" trees ("Verde 10" and "Verde 15") are distant from the central group due to the surface roughness (Supplementary Table S1). "Bico de Corvo 32" ("Bico 32") is also different from all the other "Bico de Corvo" trees due to its heavier weight and longer diameter.

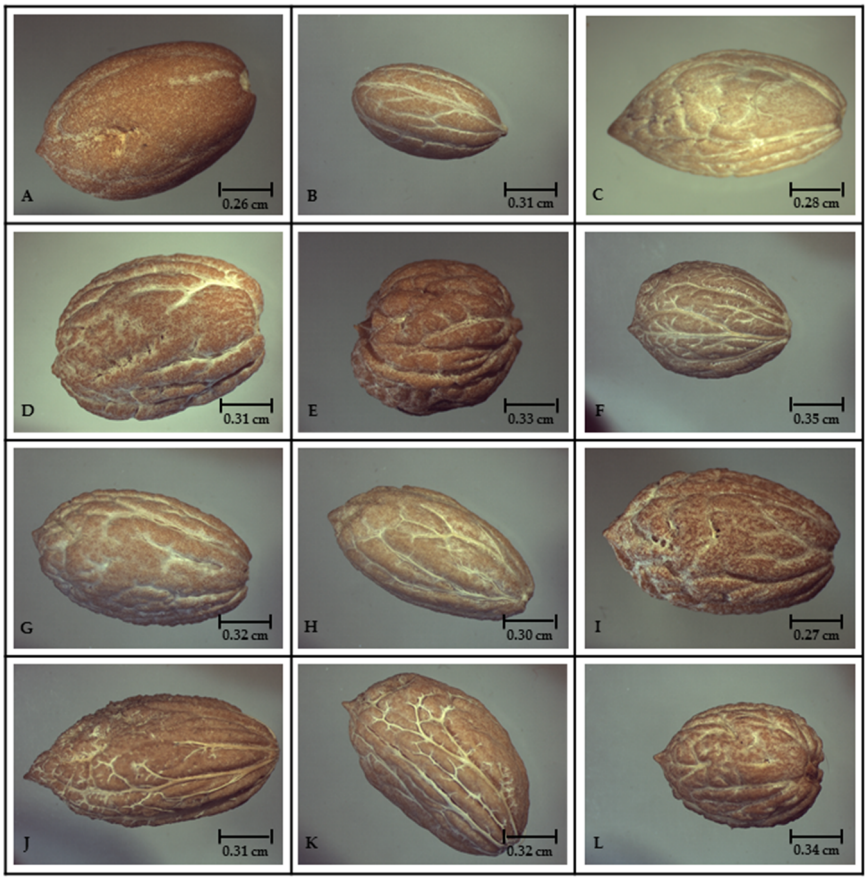
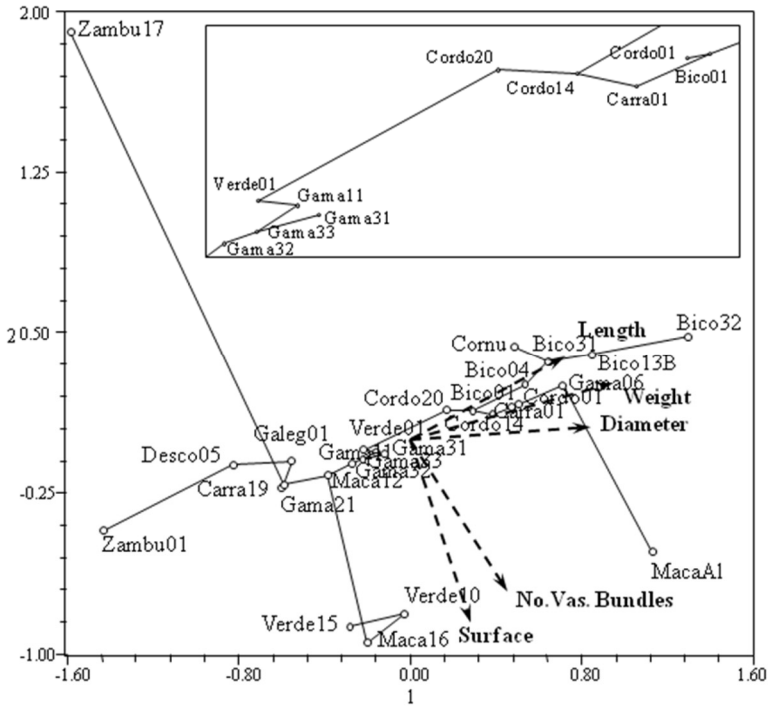


Figure 2. Endocarp images captured using a stereoscope Leica Wild. (**A**) "Zambu 17"; (**B**) "Zambu 01"; (**C**) "Galega 01"; (**D**) "Maca Al"; (**E**) "Maca 16"; (**F**) "Gama 32"; (**G**) "Cordo 01"; (**H**) "Verde 01"; (**I**) "Verde 15"; (**J**) "Bico 04"; (**K**) "Carra 01"; (**L**) "Maca 12".

The characterization of the accessions was further performed by SSR analysis. Using the microsatellite markers for the genetic analysis out of the nine SSR *loci* selected for the study, two (OeUA-DCA13 and EMO90) failed to amplify or yield monomorphic fragments and therefore, were not used. The seven remaining *loci* were polymorphic and allowed the identification of 23 distinct SSR profiles within the population of 27 olive trees (Supplementary Table S2). A total of 52 different alleles were scored, with an average of 7.43 alleles/SSR locus, ranging from 5 (for OeUA-DCA05 and GAPU71-B) to 14 alleles (for OeUA-DCA16), however, the average number of effective alleles was 3.352. Table 2 indicates that OeUA-DCA16 and OeUA-DCA18 *loci* had the highest number of unique alleles.

When evaluating the genetic diversity, it was found that the expected heterozygosity (He) was greater than 0.500 for the majority of the *loci*; the highest value (He 0.819) was observed with OeUA-DCA16 *locus*. The exception was the OeUA-DCA05 *locus*, with an He of 0.177. In regards to observed heterozygosity (Ho), the values were less than He except for those of OeUA-DCA05 (Ho 0.185) and GAPU71-B *loci* (Ho 1.000). The information obtained from the Polymorphism Information Content (PIC) value also indicated that the discriminatory power was quite good except for that of the OeUA-DCA05 *locus* (Table 2). Other studies of Portuguese cultivars [30,31] have also reported that OeUA-DCA05 and OeUa-DCA16 *loci* presented the lowest and the highest informative values, respectively. The number of alleles, the He and the PIC values per *locus* observed in our study are comparable to those reported by [16,19,20,32] for Mediterranean olive trees.



A

	PCA		
Characteristic	**1**	**2**	**3**
Weight	0.950	0.255	0.093
Length	0.724	0.381	-0.513
Diameter	0.841	0.059	0.497
Surface	0.287	-0.854	0.170
No. Vas. Bundles	0.454	-0.708	-0.406
Eigenvalue	2.418	1.445	0.712
Total Variance (%)	48.368	28.903	14.248
Total Variance Cumulative (%)		77.271	91.519

B

Figure 3. Principal Component Analysis (PCA) of the endocarp morphological characteristics. Projections of the olive tree endocarps onto the plane defined by the I (48.37%) and the II (28.90%) principal components with superimposition of the minimum spanning tree and eigenvectors. The variables correspond to weight, length, diameter and number and distribution of vascular bundles over the endocarps (**A**). Eigenvalues and total variance (%) describe the variation of the five endocarp characteristics analyzed (**B**).

Table 2. Genetic diversity of 27 *Olea europaea* accessions as assessed using seven SSRs *loci*. N—number of accessions; Na—number of alleles; Ne—effective number of alleles; Npa—number of unique alleles; Ho—observed heterozygosity; He—expected heterozygosity; PIC—polymorphism information content.

Locus	N	Na	Ne	Npa	Ho	He	PIC
OeUA-DCA04	27	6.000	2.666	1	0.222	0.578	0.538
OeUA-DCA05	27	5.000	1.211	3	0.185	0.177	0.170
OeUA-DCA14	27	7.000	2.285	1	0.667	0.671	0.618
OeUA-DCA16	27	14.000	5.080	6	0.667	0.819	0.782
OeUA-DCA18	27	9.000	3.636	4	0.630	0.739	0.684
GAPU71-B	27	5.000	4.599	-	1.000	0.797	0.747
GAPU103-A	27	6.000	3.984	1	0.704	0.753	0.693

Our data indicate that the olive tree is a highly polymorphic species, which confirms previous reports for Portuguese cultivars [30,31,33,34] and those for other Mediterranean olive trees [19,20,35–37].

To study the genetic relationships among the 23 different olive genotypes, an UPGMA dendrogram based on the proportion of shared alleles and a Neighbor-Joining Tree were constructed (Figure 4A,B). The UPGMA dendrogram showed a clear separation of cultivars from the wild olive trees. In the Neighbor-Joining Tree, the 27 *Olea* accessions were clearly separated into three different groups. The first group includes the wild trees ("Zambu 01" and "Zambu 17"), the "Galega–Galeg 01"cultivar, the unnamed tree ("Desco 05") and the cultivars not grown in Ficalho ("Maçanilha Algarvia–Maca Al" and "Cornezuelo–Cornu"). Concerning the two wild trees, it was not possible, at the molecular level, to disclose if they were truly wild or oleasters because they did not display close relationships with any of the studied varieties. The local cultivars are distributed in the second and third groups, the latter exclusively composed of "Gama". These two clusters are well supported by bootstrapping analysis, particularly in the case of "Gama" (Figure 4B). Two cases of identical plants were found: "Verde 01" and "Verde 15", on one side, and "Gama 11", "Gama 21" and "Gama 33", on the other side. The genetic uniformity of "Gama 11", "Gama 21" and "Gama 33" suggests that they may result from the clonal propagation of the same genotype. "Gama 32" differs in one *locus* (OeUA-DCA16) from the other "Gama" trees belonging to the same cluster (Figures 4 and 5). So, it probably resulted from a clonal mutation, although without phenotypic expression. Clonal variation in traditional olive varieties has been referred to in other studies [38]. Small genetic differences, probably due to somatic point mutations, were also reported in ancient olive trees [39] regardless of whether the phenotype was expressed [12]. The degree of correlation existing between somatic point mutations and phenotype variation remains to be clarified [39].

Three cases of mislabeling were also identified. One of them was "Gama 06" and "Bico 01", which are probably neither "Gama" nor "Bico de Corvo" trees. They have the same genotype and are in the first group due to the presence of the allele 141 (locus OeUA-DCA 04) that was found in the wild tree "Zambu 17". "Gama 31" has a different genotype than that of the "Gama" trees and has the allele 141 found in "Zambu 17" (Supplementary Table S2). "Bico 04", was also found to be different from the "Bico de Corvo" trees and very similar to "Cordovil 20", differing from it only by one different allele and is likely to be a "Cordovil" tree.

The unique allele 141 detected in the local cultivars "Bico 01", "Gama 06", "Gama 31" and in the oleaster "Zambu 17" (Supplementary Table S2) may suggest that these cultivars are the result of local tree domestication. This was similarly suggested for Moroccan trees [40,41] that some of the cultivated olive trees could result from local tree domestication. A similar conclusion can be made for the unnamed tree "Desco 05". Unique alleles at the OeUA-DCA05 and OeUA-DCA14 *loci* of wild material and alleles from local cultivars (GAPU71-B and GAPU103-A *loci*) suggest that "Desco 05" is autochthonous in origin. Earlier studies with Sardinian cultivated olive trees also suggested that local wild trees were involved in the domestication process [32].

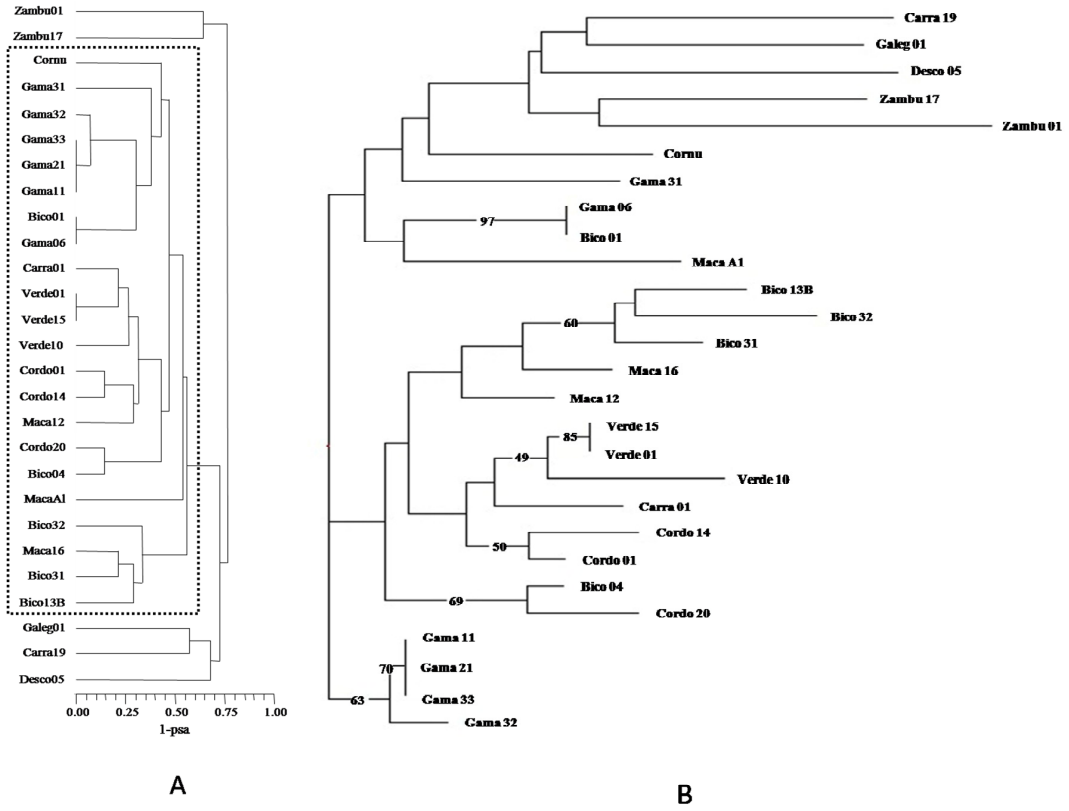


Figure 4. UPGMA (**A**) and Neighbor-Joining tree (**B**) of the 27 *Olea europaea* trees. All the cultivated olive trees are inside the dotted area (**A**). The tree construction was inferred from the bootstrapped dissimilarities, and a bootstrap value was given to each edge that indicates the occurrence frequency of this edge in the bootstrapped trees. Bootstrap values greater than 50 are indicated (**B**).

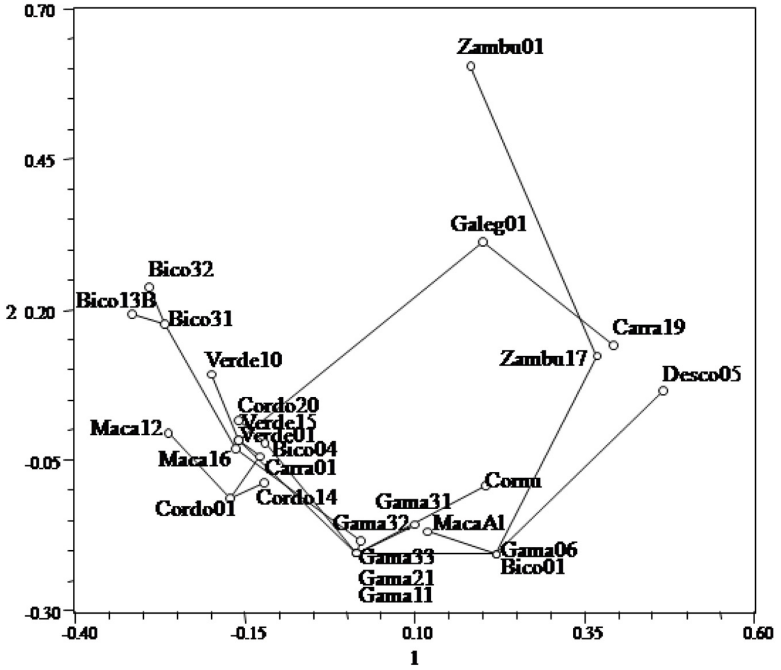


Figure 5. Principal co-ordinate analysis (PCoA) of the 27 *Olea europaea* trees based on the proportion-of-shared-alleles distance matrix. In all, 59.4% of the genetic variation was explained by the first two axes. The minimum length spanning tree was superimposed.

When performing the principal co-ordinate analysis (PCoA), it was found that considerable variation was explained by the first three axes (72.15%). The plane of the first two main PCoA axes accounted for 59.4% of the total genetic variation (Table 3).

Table 3. Percentage of variation explained by the first three axes as a result of a Principal co-ordinate analysis (PCoA) based on the proportion of shared alleles distance matrix for the 27 olive trees.

Axes	1	2	3
%	39.78	19.63	12.74
Cum %	39.78	59.41	72.15

The PCoA results are similar to those obtained by the UPGMA and the Neighbor-Joining algorithm. The wild trees ("Zambu 01" and "Zambu 17"), the unnamed tree ("Desco 05") and the cultivars not grown in Ficalho ("Maçanilha Algarvia–Maca Al" and "Cornezuelo–Cornu") are outliers. The local cultivars are distributed in three groups. "Bico de Corvo" ("Bico 13B", "Bico 31" and "Bico 32") and "Gama" ("Gama 11", "Gama 21", "Gama 32" and "Gama 33") constitute two separate groups. The remaining local varieties are clustered in the third group (Figure 5).

A comparison of the results obtained by endocarp characteristics and by means of SSR analysis showed that although both methods discriminate the 27 accessions in a similar way, some differences were observed as occurred for Italian cultivars from the Campanian region [42].The main differences relate to the "Verdeal" cultivar ("Verde 10" and "Verde 15") and the synonymy found between "Gama 21" and "Carra 19" using endocarp analysis which was not seen using SSR analysis (Figures 3–5). In summary, our data showed that the Ficalho area still has reasonable olive tree diversity. Such traditional orchards are important reservoirs of old minor cultivars and potential incubators of new genotypes. The genetic diversity observed in traditional farms is of great value and has been studied, for instance, in Morocco [40,41] and in Spain [39]. In Ficalho, there is a high probability that the observed genetic diversity of the traditional orchards is the result of mixed cultivars planting, of local trees originating from seed germination and of trees resulting from natural crosses between oleasters and domesticated trees. This was observed in the mountain region of Morocco, where apart from "Picholine marocaine", several other local varieties were found [40].

Keeping crop evolution on the farms contributes to the generation of a diversity of adaptive combinations of genes and traits in response to changing environmental conditions [43]. There is, presently, an increased risk of genetic erosion of olive germplasm due to the technological improvement in olive cultivation applied in the new commercial orchards [12,39].

It is recognized nowadays that *on farm* conservation is an important strategy of crop genetic resource conservation [38,44]. All over the Mediterranean region, traditional agroecosystems are of great importance as incubators of crop diversity [32,45,46] and as ecological infrastructures in general, providing essential ecological services. Portugal is very rich in traditional crop diversity, and a few studies [47–50] have demonstrated the existence of unexplored genetic resources. At Ficalho, the farmers have been developing an *on farm* conservation project by promoting the selection and utilization of local germplasm, and it is common to explore unnamed trees as a consequence of their agronomic characteristics. The *on farm* conservation is particularly important considering that, during the last several years, plant material from other provenances has been introduced, and some traditional orchards have been replaced by intensive systems in order to obtain higher yields. Some local producers are willing to adopt organic farming in these traditional orchards, thus producing higher quality products, in order to compensate for the financial loss associated to low yields and saving these orchards from being replaced. By promoting cultivar diversity, farmers benefit from the different fruiting times between cultivars, which allows staggering of field work such as harvest and olive oil mill work. They also benefit from the different tolerance/susceptibility to pests and diseases between cultivars [8,51,52], characteristics that may be used in breeding programs. Cultivar diversity also produces a diversity in flavors of the olives produced by a combination of cultivars.

4. Conclusions

Both methodologies used in this work showed that high genetic diversity still exists in the traditional orchards of Ficalho. Some cases of mislabeling were detected and it was found that several trees of "Gama" and "Verdeal Alentejana" are two distinct clonally propagated accessions. It was also found that the poorly represented cultivars "Gama" and "Bico de Corvo" are genetically distinct from all the other studied accessions, including the major local cultivars "Verdeal Alentejana" and "Cordovil de Serpa". "Gama", which is known to produce high quality oil, at a high yield, is specific to the Ficalho area and is a separate cluster from all the other accessions. Our work highlights the need to preserve minor cultivars in traditional orchards which can be reservoirs with great potential in the future.

Supplementary Materials: The following are available on line at www.mdpi.com/1424-2818/10/1/5/s1, Table S1: Endocarp characteristics of the 27 olive trees: Weight, Length, Diameter, Surface Roughness and Number of Vascular Bundles for the 27 olive trees. The values are mean values from 40 fruits from each tree, Table S2: SSR screening of 27 olive trees using 7 *loci.* The alleles sizes (in bp) are shown for each locus.

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Author Contributions: Maria Manuela Veloso designed the project. Maria Manuela Veloso and Cândido Pinto-Ricardo collected the plant material (fruits and leaves). Maria Manuela Veloso performed the endocarp characterization. Maria Cristina Simões-Costaperformed the DNA isolation and SSR analysis. Maria Manuela Veloso, Joana B. Guimarães and Luís C. Carneiro conceived and designed the statistical analysis. Maria Manuela Veloso, Célia Mateus and Cândido Pinto-Ricardo drafted the manuscript. Pedro Fevereiro critically revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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