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Genetic Relationships between Cultivated and Wild Olive Trees (*Olea europaea* L. var. *europaea* and var. *sylvestris*) Based on Nuclear and Chloroplast SSR Markers

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ABSTRACT

The olive is widely cropped in Tunisia where also oleaster trees thrive all around orchards and in natural sites. Little is known on the genetic relationships between the olive crop and oleaster trees in Tunisia. Fifty-two oleaster trees and fifteen cultivars were sampled from Tunisia. SSR genotyping was performed in polyacrylamide gels after fluorescent labeling. We used seven nuclear and two chloroplast SSR markers. AFC analyses showed close genetic relationships between cultivated and oleaster trees. Genetic relationships were also displayed in a dendrogram based on Unweighted Pair Group Method (UPGMA). Five clusters were defined mixing cultivar and oleaster trees suggesting close relationship between some cultivar and some oleaster trees. One oleaster is single in a cluster. The chlorotype SSR markers show probably three olive origins. Some cultivars have the CE chlorotype originates from the East of the Mediterranean basin, the CCK haplotype originates from Maghreb and the COM chlorotype originates from West Mediterranean. The cultivars were 1) introduced from the East; 2) selected in the West; 3) or selected in the North Africa region. The Tunisian oleaster trees carry eastern and western Mediterranean chlorotype CCK, COM and CE.

Keywords: *Cultivars, Oleaster, Genetic Relationship, SSR Markers, Haplotype, Origin*

1. Introduction

Two olive (*Olea europaea* subsp. *europaea* var. *europaea*) varieties are distinguished by botanists in the Mediterranean basin namely var. *europaea* which is the cultivated form and var. *sylvestris*, the wild olive tree or oleaster.

The olive is one of both of the oldest tree crops with the fig tree and it is cultivated for oil and table olives. The olive is the most important oil producing crop in the Mediterranean region. Olive oil has traditionally been used for pharmaceutical, industrial and consumer purposes. Tunisia is formerly a major producer of olive oil in North Africa. In Tunisia, about 60 million olive trees are cultivated in third of cultivated areas, most of them represented by two prevalent oil cultivars 'Chétoui' and 'Chemlali'. The rest is represented by several minor cultivars [1].

Little is known about the Tunisia oleaster trees and about their genetic relationships with cultivars. Genetic erosion and loss of biodiversity do not seem to be major issues for olive germplasms due to absence of turnover of new genotypes that do not occur as fast as in other woody crops. Moreover, old olive trees survive for a long time once abandoned [2,3].

Morphological traits in the olive do not enable differentiation between oleasters and cultivars. Although, several morphological descriptors show partial differentiation of them [4,5].

Recently, molecular markers have been developed in the olive [2,6-11] that enable cultivar differentiation and identification due to their high intra species variability.

The use of nuclear microsatellite markers for genetic analysis is well established in the olive [7,12-14]. The

principle has been extended to the chloroplast [15,16] and mitochondrial genomes [3,17,18]. The utility of molecular tools for evolutionary studies arises from the insensitivity of the genetic markers to environmental factors. Several markers based on DNA amplification technology have been used to look for genetic relationships between the cultivated olive (cultivars) and the oleaster trees as to structure its genetic diversity [3,8,15,16], including DNA from nucleus, chloroplast (cpDNA) and mitochondria (mtDNA). Simple Sequence Repeats (SSRs) lead to multiallelic fragments and are easily amenable to Polymerase Chain Reaction (PCR) based analysis. Microsatellite markers explore various independent portions of the olive genome and they have been identified in plants' nuclear and mitochondrial genomes [3,15,19] as well as in the chloroplast genome where they are mononucleotide [20]. With SSRs, Restriction Fragment Length Polymorphisms (RFLPs) are complementarily used to define chloroplast and mitochondrial RFLP data, using multiple pair wise combinations of probe and restriction enzyme to recognize distinct genetic patterns, called chlorotypes or mitotypes. Since the organelles are usually passed to offspring from the female parents, cytoplasm markers (mitochondria and chloroplast) trace maternal lineage only [21].

Many studies have shown the diversity of cultivars using morphological descriptors [1,22], but little attention has been given to the Tunisian oleasters [4,5]. In Tunisia a few studies have been made on cultivars using SSRs markers [23].

In the present study, an analysis of polymorphisms within and among the two olive taxa (cultivar and oleaster trees) was undertaken using nuclear and cytoplasm SSR markers. This will enable the determination of genetic groups or clusters to establish breeding programs that encompasses the genetic diversity of this species.

2. Materials and Methods

A total of 15 autochthonous Tunisian cultivars and 52 oleasters were sampled in the north of Tunisia (**Table 1**), all are presently cropped in wide area. They were subject to genotyping for chlorotypes previously described and developed. We used two markers ccmp5 and ccmp7 retained by authors [8,21,22] and seven nuclear microsatellite markers, three ssrOeUA-DCA04, 05, 09 [11]; one ssrOe-GAPU 101 [25] and three ssrOe-UD012, 017, 024 [9], chosen as used by Breton *et al.* [14], (**Table 2**).

2.1. DNA Amplifications

Total DNA preparation was performed using the method described by Besnard *et al.* [21]. PCR reaction was performed in 12.5 µl final volume, containing 40 ng genomic DNA, 0.75 mM $MgCl_2$, 2.5 mM dNTP, 1.25 U Taq polymerase and 0.19 mM M13-Fam. PCR amplifycation was conducted in a thermal cycler Gradient 96 Robocycler (Stratagene, Germany). The amplification program was 94℃C for 1 min, 52℃ for 1 min, 72℃ for 1 min, followed by 35 cycles at 94℃ for 30 s, at 50℃ for 45 s and at 72℃ for 1 min, with a final elongation cycle at 72℃ for 4 min.

Amplification products and ladders were labeled using the tailing method with the Fam fluorochrome. They were separated into 8% polyacrylamide gels enabling reading with a Hitachi scanner system associated with the FMBIO2 software [26].

The method used for chlorotype DNA-RFLP analysis was described by Besnard *et al.* [21] and Breton [13]. Two restrictions enzyme/probe combinations (HindIII/atp6 and *Xba*I/*atp*6) were used to identify the chlorotype CE, COM and CCK previously determined by Besnard *et al.* [21].

2.2. Statistical Analysis

Factorial Correspondence Analysis (FCA) was performed using GENETIX. Dendrogram was constructed with Unweighted Pair Group Method (UPGMA) algorithm based on Nei' genetic distances [27] and a neighbour-joining tree was constructed with the nuclear SSR data set using PHYLIP Version 3.5c [28].

3. Results

3.1. Factorial Correspondence Analysis

The plot of FCA coordinates for the first and the second axes, which explain 8.69% and 6.23% of variance, respectively and showed continuity in the distribution of oleaster and cultivar trees. However, most of cultivars clustered in the right of the cloud. Thus, we considered two clusters of genotypes (**Figure 1**). The cultivars cluster grouped three oleaster trees. In contrast, most oleaster trees clustered on the left with the cultivar C1 (Sayali).

3.2. Clustering

Olive cultivar and oleaster trees were clustered with the UPGMA method based on the Nei's similarity coefficient using SSR data (**Figure 2**). The clustering analysis showed five groups and one single oleaster (O11). Three clusters (CL1, CL2 and CL3) contain cultivars and oleaster trees and two other clusters (CL4 and CL5) contain only oleaster trees.

Cluster_1 (CL1) aggregated 11 out of 15 cultivars (C1 Sayali, C6 Marsaline, C10 Meski, C30 Rajou, C29 Limi, C26 Tounsi, C14 Gerboui, C20 Besbessi, C28 Zarras, C13 Neb Jmel and C22 Chaïbi) and 12 oleasters from several locations; six of them sampled from natural eco-

system (O6, O8, O9, O13, O22, O26) and six from agro-ecosystem (O3, O32, O34, O35, O40 and O42). Some cultivars were close to oleasters from natural ecosystem. The cultivars C1 'Sayali', C13 'Neb Jmel', C14 'Gerboui', C22 'Chaïbi', C28 'Zarras' and C29 'Limi', were close to oleasters from natural ecosystem O6, O22, O8, O13, O26 and O9, respectively. Whereas, others cultivars were related to oleasters from agro-ecosystem: the cultivars C6 'Marsaline', C10 'Meski', C20 'Besbessi', C26 'Tounsi', C30 'Rajou' related to oleasters O3, O32, O40, O34 and O42, respectively.

Cluster_2 (CL2) aggregated nine oleasters (O61, O57, O1, O23, O19, O20, O53, O30, and O21) and two cultivars (C8 Chemlali and C27 Roumi). Three oleasters (O61, O19, and O20) were from natural ecosystem and six from agro-ecosystem. The cultivars C8 'Chemlali' and C27 'Roumi' were close to two oleasters from agro-ecosystem O1 and O21, respectively.

Table 1. Origins of cultivated (cultivars) and wild (oleasters) olive trees used in the present study.

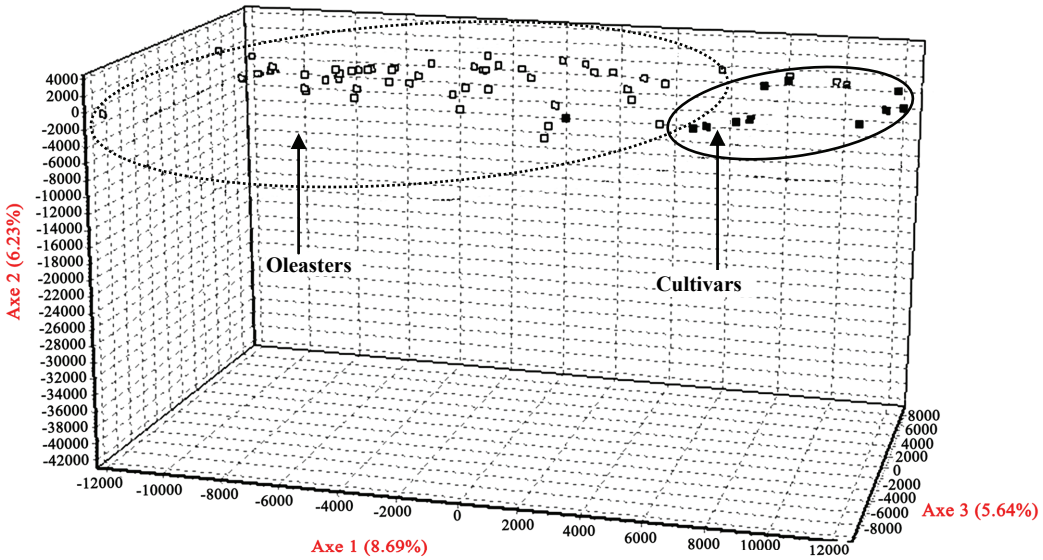
Code	Cultivar/oleaster	Location	Ecosystem	Governorate
C1	Sayali	Slouguia	Agro-ecosystem	Béja
C2	Chétoui	Slouguia	Agro-ecosystem	Béja
C6	Marsaline	Slouguia	Agro-ecosystem	Béja
C8	Chemlali	Slouguia	Agro-ecosystem	Béja
C10	Meski	Slouguia	Agro-ecosystem	Béja
C13	Neb jmel	Testour	Agro-ecosystem	Béja
C14	Gerboui	Slouguia	Agro-ecosystem	Béja
C20	Besbessi	Testour	Agro-ecosystem	Béja
C22	Chaïbi	Téboursouk	Agro-ecosystem	Béja
C26	Tounsi	Téboursouk	Agro-ecosystem	Béja
C27	Roumi	Téboursouk	Agro-ecosystem	Béja
C28	Zarras	Téboursouk	Agro-ecosystem	Béja
C29	Limi	Téboursouk	Agro-ecosystem	Béja
C30	Rajou	Ras jbel	Agro-ecosystem	Bizerte
C31	Nib	Ras jbel	Agro-ecosystem	Bizerte
O1	Oleaster	Slouguia	Agro-ecosystem	Béja
O3	Oleaster	Testour	Agro-ecosystem	Béja
O4	Oleaster	Testour	Agro-ecosystem	Béja
O5	Oleaster	Téboursouk	Agro-ecosystem	Béja
O6	Oleaster	Téboursouk	Natural ecosystem	Béja
O7	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O8	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O9	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O10	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O11	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O12	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O13	Oleaster	Ichkeul	Natural ecosystem	Bizerte
O15	Oleaster	Ras Jbel	Agro-ecosystem	Bizerte
O16	Oleaster	Ras Jbel	Agro-ecosystem	Bizerte
O17	Oleaster	Tunis	Natural ecosystem	Tunis
O18	Oleaster	Tunis	Natural ecosystem	Tunis
O19	Oleaster	Tunis	Natural ecosystem	Tunis
O20	Oleaster	Tunis	Natural ecosystem	Tunis
O21	Oleaster	Messaoudi	Agro-ecosystem	El Kef
O22	Oleaster	Midian	Natural ecosystem	El Kef
O23	Oleaster	Bahra	Agro-ecosystem	El Kef

Continued Table 1

Code	Cultivar/oleaster	Location	Ecosystem	Governorate
O25	Oleaster	Ettouiref	Natural ecosystem	El Kef
O26	Oleaster	Ettouiref	Natural ecosystem	El Kef
O27	Oleaster	Jendouba	Natural ecosystem	Jendouba
O28	Oleaster	Fernana	Agro-ecosystem	Jendouba
O29	Oleaster	Jendouba	Natural ecosystem	Jendouba
O30	Oleaster	Tbaba	Agro-ecosystem	Jendouba
O31	Oleaster	Zouaraa	Agro-ecosystem	Béja
O32	Oleaster	Zouaraa	Agro-ecosystem	Béja
O33	Oleaster	Tamra	Agro-ecosystem	Béja
O34	Oleaster	Sejnan	Agro-ecosystem	Bizerte
O35	Oleaster	Sejnan	Agro-ecosystem	Bizerte
O37	Oleaster	Aïn Ghlal	Agro-ecosystem	Bizerte
O38	Oleaster	Jbel Elwesr	Natural ecosystem	Zaghouan
O39	Oleaster	Zaghouan	Agro-ecosystem	Zaghouan
O40	Oleaster	Zriba	Agro-ecosystem	Zaghouan
O42	Oleaster	Jradou	Agro-ecosystem	Zaghouan
O43	Oleaster	Jradou	Agro-ecosystem	Zaghouan
O44	Oleaster	Oued Kenz	Natural ecosystem	Zaghouan
O45	Oleaster	Batria	Agro-ecosystem	Zaghouan
O46	Oleaster	Saouaf	Agro-ecosystem	Zaghouan
O47	Oleaster	Oued Touil	Agro-ecosystem	Zaghouan
O48	Oleaster	Saouaf	Agro-ecosystem	Zaghouan
O51	Oleaster	Mjez El Bab	Agro-ecosystem	Béja
O52	Oleaster	Kélibia	Agro-ecosystem	Nabeul
O53	Oleaster	Kélibia	Agro-ecosystem	Nabeul
O55	Oleaster	Kélibia	Agro-ecosystem	Nabeul
O56	Oleaster	Kélibia	Agro-ecosystem	Nabeul
O57	Oleaster	Kélibia	Agro-ecosystem	Nabeul
O59	Oleaster	Echraf	Agro-ecosystem	Nabeul
O61	Oleaster	Abderrahman	Natural ecosystem	Nabeul
O64	Oleaster	Abderrahman	Natural ecosystem	Nabeul

Table 2. Characteristics of microsatellites markers used for the genotyping of cultivated and wild olive trees in the present study.

Locus	Repeated motif	Directed sequence (5' – 3')	authors
ssrOeUA-DCA1	$(GA)_{22}$	CCTCTGAAAATCTACACTCACATCC	Sefc *et al.* [11];
ssrOeUA-DCA5	$(GA)_{15}$	AACAAAATCCCATACGAACTGCC	Sefc *et al.* [11]
ssrOeUA-DCA9	$(GA)_{23}$	AATCAAAGTCTTCCTTCTCATTTCG	Sefc *et al.* [11]
GapU101	$(GA)_8(G)_3(AG)_3$	CATGAAAGGAGGGGGACATA	Carriero *et al.* [25]
Udo012	$(GT)_{10}$	TCACCATTCTTAACTTCACACCA	Cipriani *et al.* [9],
Udo017	$(TG)_{11}$	TCACCATTCTTAACTTCACACCA	Cipriani *et al.* [9],
Udo024	$(CA)_{11}(TA)_2(CA)_4$	GGATTTATTAAAAGCAAAACATACAAA	Cipriani *et al.* [9],



■: olive cultivars; □: Oleaster olive trees

Figure 1. Factorial correspondence analysis on cultivar and oleaster olive trees based on SSR markers.

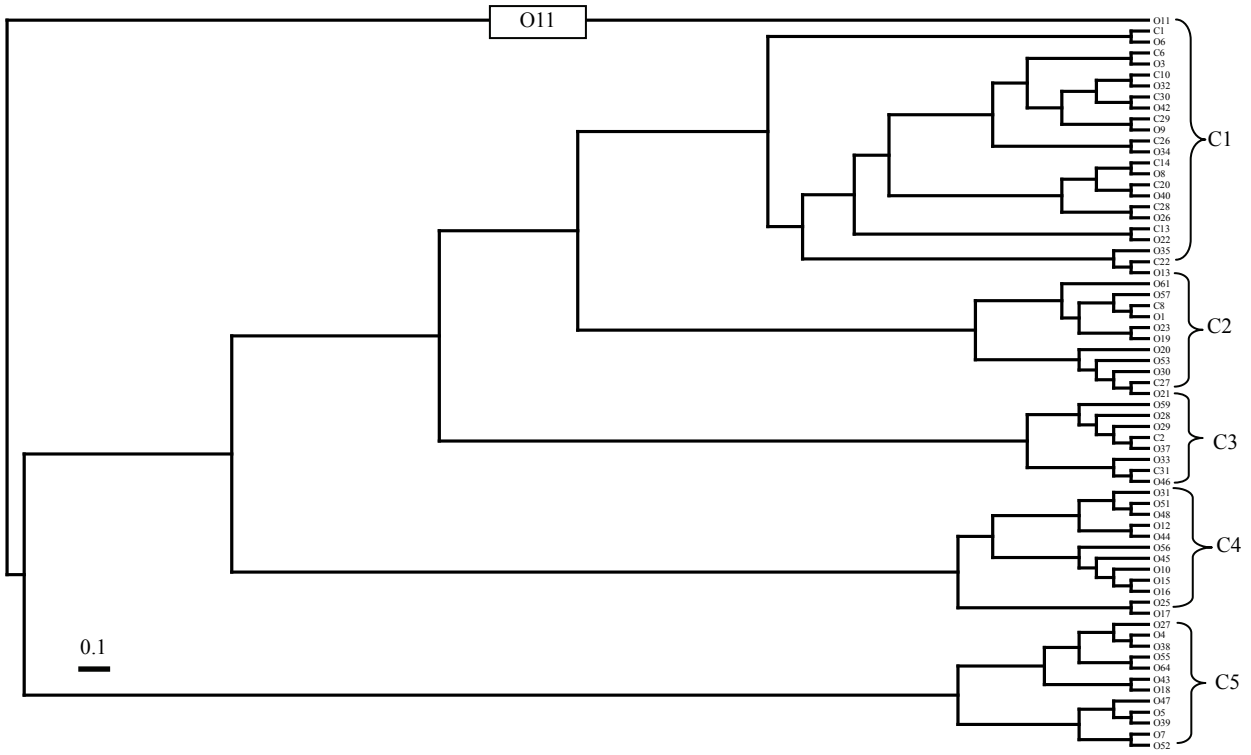


Figure 2. Dendogram based on the SSR data of 15 cultivars and 52 oleasters genotypes generated by UPGMA algorithm. C1 – C5 indicate five clusters, O11 is a single oleaster tree; C: olive cultivar and O: olive oleaster.

Cluster_3 (CL3) contains two cultivars (C2 Chétoui and C31 Nib) and six oleaster trees: O29 from natural ecosystem and the others (O59, O28, O37, O33, and O46) from agro-ecosystem. The two cultivars C2 'Chétoui' and C31 'Nib' were close to two oleaster trees from agro-ecosystem O37 and O46, respectively.

Cluster_4 (CL4) and cluster_5 (CL5) aggregated only oleaster trees. Cluster_4 contains five oleaster trees from

natural ecosystem (O12, O44, O10, O25, and O17) and seven from agro-ecosystem (O31, O51, O48, O56, O45, O15, and O16). Cluster_5 contains five oleaster trees from natural-ecosystem (O27, O38, O64, O18, and O7) and seven oleaster trees from agro-ecosystem (O4, O55, O43, O47, O5, O39, and O62). However, the oleaster O11 from natural ecosystem is single and represents a cluster by itself.

These results showed tight relationships between some oleaster trees and cultivars independently of locations and ecosystem.

3.3. Chloroplast SSR

In this study, four chlorotypes CE1, CE2, COM and CCK previously determined in olives were found in cultivars and defined the olive origins (**Table 3**). Oleaster trees from agro-ecosystem and natural sites carry CE1 (6/6), CE2 (0/0), CCK (9/5) and COM (10/7), respecttively, and the lattes do not reveal significant differences for chlorotype frequencies. Whereas, for olive cultivars six carry CE1, six CE2, one COM and six CCK chlorotype.

4. Discussion

We used seven nuclear and two cytoplasmic microsatellite markers over 15 cultivars and 52 oleasters that revealed several clusters of cultivars, oleaster trees and several chlorotypes. The morphological means showed a continuous variation between cultivated and wild olive trees [4]. In the present study, molecular markers show also continuous variation, but most cultivars clustered together. This genetic structure probably results from the origin of the cultivars and oleaster trees.

Besnard *et al.* [18] and Besnard and Bervillé [15] have shown that the CE1, COM, and CCK chlorotypes are prevalent in oleaster trees from the East (CE1) and the West (COM and CCK). In addition, Breton [13] and Breton *et al.* [14] have shown that CE2 and COM (COM1 and COM2 are variant of COM) originated in Cyprus and Tunisia where they are prevalent in oleaster trees. Consequently, the deep structure in chlorotypes infers that cultivars carrying CE1 or CE2 have ancestors in oleaster or in cultivars from the East. Whereas, cultivars carrying COM or CCK have ancestors in oleaster or

Table 3. Chlorotypes of cultivated (cultivars) and wild (oleasters) olive trees based on chloroplast SSR markers. (CE1, CE2: East Mediterranean chlorotype; COM, COM2: West Mediterranean chlorotype; CCK: Maghreb chlorotype; C and O: cultivars and oleasters codes, respectively, used in UPGMA analysis).

Chlorotype	Cultivars [a]	Oleasters[a]	Oleasters[b]
CE1	Sayali (C1) Chemlali (C8) Gerboui (C14) Roumi (C27) Zarras (C28) Nib (C31)	O32 O37 O45 O48 O56 O57	O6 O9 O18 O22 O44 O64
CE2	Besbessi (C20)		
COM	Neb jmel (C13)	O31 O43 O59	O7
COM2		O1 O3 O4 O23 O34 O35 O51	O8 O10 O11 O12 O61 O25
CCK	Chétoui (C2) Marsaline (C6) Meski (C10) Chaïbi (C22) Limi (C29) Rajou (C30)	O5 O15 O16 O20 O28 O40 O47 O52 O53	O19 O21 O26 O27 O29

a: agro-ecosystem; b: natural ecosystem

cultivars from the West. Tunisia offers a peculiar situation due to early colonization by Phoenicians, who have probably introduced cultivars from the East into Carthage colony and their further colonies in the West (Spain, Portugal). We can therefore deduce that oleaster trees carrying CE1 are feral trees (progenies of a cultivar by an oleaster or vice versa) either in the agro-ecosystem or natural sites (**Table 3**). Gene flow appears responsible for the diffusion of the CE1 chlorotypes, but also for the nuclear markers as shown by Breton *et al.* [14] using Bayesian methods [29].

From these results, we can deduce that 'Sayali' carrying CE1 that clustered with oleaster trees is probably of feral origin. 'Chemlali', carrying also CE1 but aggregated in the FCA intermediate between oleaster trees and cultivars, has probably similar origin. In the Dendrogram 'Chemlali' and 'Roumi' are in the same clusters with some oleaster trees suggesting that 'Roumi' could be a progeny of 'Chemlali' with local oleaster trees. 'Neb Jmel' characterized by the West chlorotype COM was probably selected in the Maghreb region. Oleasters carrying western Mediterranean chlorotypes (CCK, COM) may cluster with cultivars carrying the same chlorotypes in different clusters. Consequently, the chlorotypes are not correlated with the clusters (**Figure 2**).

Indeed, the UPGMA clustering revealed that each cluster is independent of the chlorotypes which means that kinship relationships by the chlorotypes have been hindered by gene flow between cultivars and local oleaster trees as well as between oleaster trees and cultivars. Obviously, we observed events that have occurred through the female side due to the chlorotypes which is maternally inherited in the olive [21]. The same events are likely existing through pollen flow, but too difficult to detect unless using Bayesian methods. However, in this study, the sampled trees are too limited to study per se gene flow events due to the absence of anchor references for COM and CCK chlorotypes. We suspect that the CCK chlorotype originated from Kabylia [7] where a refuge zone for the olive has been revealed [14], but we do not know whether it has been the only refuge for CCK or if other refuge zones in North Africa or Sicily may have preserved CCK. We also suspected that the COM chlorotypes (COM, COM1, COM2) originate from Tunisia where they are prevalent in natural sites and that correspond to a refuge zone [13,14], but we cannot exclude that, CCK chlorotypes, were kept in refuge zones from Sicily-Corsica. Unfortunately, oleaster trees from central and south Italy have not been genotyped for the chlorotypes [30].

Mixed stands of oleaster trees of natural sites in Tunisia display a huge diversity based on the chlorotypes and nuclear polymorphisms in comparison with other oleaster trees in Mediterranean forests. Oleaster trees transformed into new cultivars should be carefully examined since they are the result into crosses not usually done between genotypes from the East and West of the Mediterranean regions. Seed gene flow is locally detected when in a region where cultivars have been introduced and local oleasters do not carry the same cytoplasm [12,15].

Distinction of a crop from its wild relatives is based on several morphological traits and botanists have usually made distinct species of two taxa [31]. For the cultivated olive trees, it has been traditionally carried out by morphological, agronomic and chemical traits [1,32-35].

Based on the morphology and molecular markers, it is absolutely impossible to determine whether oleaster trees from natural sites are genuine oleasters or not. The phylogeography of the oleaster and cultivars trees is due to permanent and recurrent gene flow. Here, we clearly show that oleaster trees carrying the eastern CE1 chlorotype are present in natural sites of Tunisia. In the frame of the hypothesis that CE1 was absent from refuge zones in the west, it should have been introduced from the East into the West 2500 years ago. In this work, it appeared from the dendrogram (**Figure 2**) that the oleaster and cu-l tivar trees clustered by similarities whatever their chlorotypes showing tight genetic relationships. The same results were obtained by Bayesian methods [14].

In Tunisia, many studies have shown the diversity of the Tunisian cultivated olive trees [22,36] but little attention has been given to the Tunisian oleaster trees. Little is known about molecular identification of Tunisian olive.

A close genetic proximity between Tunisian oleasters and cultivars was showed by dendrogram based on nuclear SSR markers (**Figure 2**). This relationship has already been shown with isozymes [37,38], RAPD and RFLP [3,7,14].

Several molecular studies, including AFLPs, RAPDs, ISSRs, repetitive DNA sequence analysis, chloroplast and mitochondrial DNA polymorphism, have also contributed to elucidate the classification of the *Olea* complex and the origin of cultivated olive [2,3,12,15,16, 39-41].

It has been reported that oil composition for oleaster trees were in agreement with the olive oil norms [4]. Here, we show that those oleasters trees are unique to Tunisia. Crossing the oleasters suggest that breeding the olive and could be done in the country where cultivars may have non-equilibrated oil composition. Indeed, to improve the oil quality it should cutting olive oil with others to satisfy European norms. Screening oleaster trees from the agro-ecosystem and natural sites should lead to new genotypes that could be compared for oil

composition and yield as it has been done in Australia by Mekuria *et al.* [42] and Sedgley [43].

These trees are adapted to soil and climate found in Tunisia and therefore they should been screened for their behaviour in the agro-ecosystem to check the yield and quality of the product.

5. Conclusions

Cultivars found in Tunisia are of diverse origins based on their chlorotype and nuclear markers. Local genuine oleaster trees are difficult to differentiate from feral trees, and shown to share more or less kinship relationships with autochtonous and introduced cultivars. Those oleaster trees should offer opportunity to screen for new genotypes producing oil with more equilibrated composition than 'Chemlali' as an example and acceptable agronomic behavior to compete with local cultivars to ensure direct selling of the products to the Europe.

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