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Polymorphism Of Microsatellite (SSR) Markers In Tunisian Olive (Olea Europaea L.) Cultivars

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***Abstract*—Olive (Olea europaea L. subsp. europaea var. europaea) is one of the oldest fruit tree in the Mediterranean basin, and is cultivated for oil and canned fruit. Part of this interest is driven by the economic importance of olive oil which is increasing throughout the world due to its beneficial effect to human health. In Tunisia, olive has great socio-economic importance, with more than 60 millions olive trees cultivated for olive oil production including a wide range of cultivars which are widely extended from the north to the south regions of the country for its high economic value. Here, we applied microsatellites (SSRs) molecular markers to assess the genetic variability of the most important Tunisian olive variety cultivated in Southeastern Tunisia. In total, the 7 simple sequence repeats (SSR) loci revealed 54 alleles with a mean number of 7.714 alleles per locus were detected. The observed heterosigosity ranged from 0 at DCA5 to 1 at DCA3, while the expected heterosigosity varied between 0.408 and 0.905 respectively at GAPU71A and UDO39.**
The UPGMA cluster analyses using Jaccard's index permitted a segregation of the sixteen olive cultivars in six main groups and revealed that no clear clustering trends were observed according to their geographic origin.

Keywords— Olea europaea, genetic diversity, microsatellite, genetic relationships

I. INTRODUCTION

Olive (*Olea europaea L.*) is one of the subtropical fruit tree species with remarkable cultural and economic importance. *Olea europaea L.* is considered as one of the most widely grown fruit crop in the countries of the Mediterranean basin. Many varieties with a significant phenotypic and genotypic diversity result from centuries of natural selection [37]. The olive oil sector plays a very important role in the economy of Tunisia and constitutes one of the main factors of economic and social stability in the country. In Tunisia, the olive trees spread almost over the whole of the territory. According to recent estimates, there are 78 million olive trees in Tunisian covering an area of 1.77 million hectares (Ministère de l'Agriculture, DGPA). This puts Tunisia second after Spain with nearly 19% of the worldwide olive-growing area. Tunisian orchards are rich in many varieties which have been the subject of a catalogue listing fifty-six different varieties [46]. A study by [24], found more than seventy olive tree varieties in the country. Nevertheless, the Tunisian olive grove is dominated by the two major varieties Chetoui in the North and Chemlali in the Centre and the South. These two varieties account for 95 % of the total olive tree orchards and contribute more than 90 % of the national production of olive oil. Chemlali alone covers 60 % of the olive-growing surface. The distribution of Olea varieties in the Mediterranean basin gave rise to a very complex and highly articulated structure of olive culture which was marked by the existence of a considerable number of different olive cultivars [8]. The great number of existing varieties led to the need of a powerful method of genetic analysis for the development of conservation management strategies for the genetic resources and for the protection of the commercial varieties quality label. In addition, there is the problem arising from the existence of homonyms and synonyms [38]. This makes cultivar identification very difficult and complex. The study of genetic diversity of olive is of interest for the conservation of genetic resources, broadening of the genetic base and practical applications in breeding programs. Several characteristics such as morphological [49, 15], biochemical characteristics [22] have already been used to study the genetic variation of olive germplasm. This is noticeable that morphological characteristics usually vary with environment [29]. Now, with the emergence of molecular markers such as simple sequence repeat markers (SSR), this is

possible to evaluate genetic divergence of plant germplasm in greater detail. Molecular markers are stable and detectable in all tissues, regardless of growth, differentiation and development or stage of the cell. They are not subject to environmental, pleiotropic or epistatic effects [3, 33]. In this sense, several studies were developed SSR markers [15, 35, 31] to reveal genetic diversity of olive. Microsatellite are still considered major tool in cultivar fingerprinting, simple sequence repeat (SSR) analysis is becoming the preferred choice for its high discriminatory power and simpler interpretation [11]. Microsatellite markers have proved successful for germplasm fingerprinting of woody plants. These markers exhibit a high level f polymorphism. In diversity studies, because of their codominant character, they are more effective than others in estimating heterozygosity. The capacity of microsatellite primers for evaluating genetic diversity between different genotypes is the first prerequisite for genetic characterization of germplasm collections. Microsatellites were used to fingerprint olive genotypes and study the genetic structure of olive cultivars [42, 47, 30, 6]. In order to exploit genetic diversity for targeted olive cultivar improvements, several Tunisian research teams have used PCR-based markers for basic and applied research to assess the genetic diversity of Tunisian olive cultivars. These markers types include RAPD [50], AFLP [23, 44]), SSR [39, 1, 32] and SNP [40].
The aim of this work is to assess the overall degree of polymorphism of microsatellites or single sequence repeats (SSR) markers useful for distinguishing olive genotypes of the different olive varieties cultivated in the South East of Tunisia and to determine the genetic diversity and relationship among local olive cultivars.

II. MATERIALS AND METHODS

A. Plant material

Molecular analysis was performed on 16 Tunisian cultivars collected from the various region of the South East of Tunisia. The cultivars name, geographic origin and the end use are shown in table 1.

Table1. List of the different olive cultivars analyzed, growing region, and use of fruits.

Code	Cultivar name	Growing region	End use
1	Zalmati Zarzis	Zarzis	Oil
2	Zalmati Djerba	Djerba	Oil
3	Chemlali Dakhla	Boughrara-Medenine	Oil
4	Jemri Ben Guardène	Ben Guardène	Both
5	Jemri Matmata	Matmata	Both
6	Chemlali Zarzis	Zarzis	Oil
7	Chemlali ontha	Douiret-Tataouine	Oil
8	Neb jmel	Boughrara-Medenine	Both
9	Zarrazi Zarzis	Zarzis	Both
10	Fakhari	ElGorthab-Tataouine	Both
11	Fougi	Matmata	Both
12	Dokhar el Gorthab	ElGorthab-Tataouine	Both
13	Zarrazi Tataouine	Tataouine	Both
14	Dokhar Drina	Drina -Tataouine	Both
15	Zarrazi injassi	Mareth-Gabès	Both
16	Chemlali Douiret	Douiret-Tataouine	Oil

B. DNA extraction

Total genomic DNA was isolated from young leaves lyophilized and powdered using the CTAB method according to the protocol of [39] with some modifications. The quality of the DNA was checked by agarose gel electrophoresis and the quantity by spectrophotometry using a Nanodrop 2000 system.

C. SSR analysis

A total of seven microsatellite primer pairs were used to test the polymorphism in the sixteen olive cultivars. The primers were selected from previous literature: DCA3, DCA4, DCA5, DCA9 [43], GAPU71A [13], UDO 28 and UDO39 [14] and were chosen for their high discriminative power.
Amplification reactions were performed in a final volume of 20µl in the presence of 50 ng template DNA, 2µl of buffer (10x Colorless GoTaq Flexi Buffer) PCR,25 mM MgCl2(2 µl), and 500U of Taq polymerase(0,5 µl),2 mM DNTP(0,5 µl), 10 µM of forward and reverse primers.
PCR amplification was performed in thermal cycler (DNA-Amp Master cycler gradient eppendorf) with an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1min, annealing temperature 60°C for 1min and 72°C for1min, and a final extension at 72°C for 10 min.
The amplified products were tested on 0.8 % agarose gel to check for the amplification of the PCR products. An aliquot of 2µl of PCR product was mixed with a 10 µL of deionised formamide and 2 µL Gene Scan 500 (ROX) size standard markers. The resulting mixture was denatured at 95°C for 5 min and then quickly cooled on ice. The detection of amplification products was carried out using Genetic Sequencer (Applied Biosystems, USA).

D. Data analysis

SSR data were scored and verified using GeneMapper Software 3.7v (Applied Biosystems). The DNA bands were scored as (1) for the bands present and (0) for the bands absent. Based on the banding pattern scored, a similarity matrix among olive tree accessions was calculated using the Jaccard coefficient [26]. Genetic relationships between olive genotypes were studied on the estimated similarities, using the unweighted pair group method with arithmetic average (UPGMA) algorithm and the resulting clusters were expressed as a dendrogram using PAST software v.2.12. The number of alleles (Na) and their frequency observed (Ho) and expected (He) heterozygosity and fixation index (Fis) were determined using POPGENE32 Software [48]. The probability of null alleles was estimated according to the formula of [12]:

$$r = (He - Ho)\ (1+He) \quad (1)$$

Discriminating power (PD) [27] was calculated for each primer pair according to the formula of [45]:

$$PD=1-\sum Pi^2 \quad (2)$$

where pi is the frequency of the ith genotype for the locus.

III. Results and Discussion

The use of molecular markers and especially SSRs are becoming the preferred choice in olive identification because of their high discriminatory power and usually straight forward interpretation. The research results reported here constitute the first genetic study on olive varieties cultivated in Southeastern Tunisia.

The 7 SSR markers used in this study were chosen among three sets of primer pairs developed on olive [43, 13, 14]. The used primers were selected on basis of their high degree of polymorphism, their high discriminative power allowing to distinguish between cultivars and their amplification of a single locus [7, 18, 34, 35].

Our results of SSR analysis rely on efficiency of SSR marker in evaluation of olive germplasm and existence of suitable genetic variability in the studied olive cultivars. These findings are accommodated with findings of [36, 32, 6].

A total of 54 alleles were amplified ranging from 4 for GAPU71A to 11for UDO28 with an average of 7.714 alleles per locus (Table 1). The number of distinguished cultivars (NDC) varied from 3 (locus DCA9) to 11 (locus DCA4).The allelic size ranged from 108 bp for UDO39 to 253 bp for DCA3. Allele frequencies varied from 0.029 (all loci except loci GAPU71A and DCA5) to 0.764 (locus GAPU71A). The most frequent allele was allele 212 of locus GAPU71A ,followed by allele 204 of locus DCA5, with frequency 0.47 0, and by allele 232 of locus DCA3, with frequency 0.352. The observed heterozygosity ranged from 0.000 at DCA5 to 1.000 at DCA3with an average of 0.428.

The average expected He was 0.775, ranging from 0.408 (GAPU71A) to 0.905 (UDO39).

To evaluate the ability of each locus to discriminate among accessions the Power of discrimination (PD) was measured; PD varied from 0.633 for locus GAPU71A to 0.996 for locus UDO39 with a mean value of 0.922 (Table1).

The results of the SSR profiles showed that the number of the unique profiles amplified by the 7 SSRs varied from zero, at locus DCA3 to 9 at locus UDO39. All olive cultivars analyzed showed only one amplified allele at DCA5 locus were assumed to be homozygotic at this locus.

Table1. SSR locus, allelic number (Na), number of distinguished cultivars (NDC), allele frequency (f), observed(Ho) and expected (He) heterozygosity, fixation index (Fis), Power of discrimination (PD), probability of null alleles(r) and product size range.

Locus	Na	NDC	F	Ho	He	Fis	PD	r	Range size
GAPU71A	4	5	0.058-0.764	0.235	0.408	0.4061	0.633	0.122	210-228
DCA3	6	4	0.029-0.352	1.000	0.775	-0.328	0.929	-0.290	232-253
UDO39	10	5	0.029-0.176	0.352	0.905	0.598	0.996	0.290	108-232
UDO28	11	9	0.029-0.235	0.705	0.885	0.179	0.989	0.095	150-250
DCA4	9	11	0.029-0.235	0.529	0.868	0.371	0.970	0.181	145-220
DCA5	5	-	0.117-0.470	0.000	0.727	1.000	0.950	0.420	198-208
DCA9	9	3	0.029-0.264	0.176	0.859	0.788	0.992	0.367	172-210
Total	54								
Mean	7.714	5.285		0.428	0.775		0.922	0.169	

The high level of polymorphism observed is consistent with results obtained by several authors that investigated olive germplasm with SSR markers [28, 4] and reflects the great diversity within olive cultivars [21]. The co-dominant nature of SSR markers permitted to detect a large number of alleles per locus and a high level of heterozygosity. The number of average polymorphic alleles per primers (7.714) is consistent with results obtained by [9] whose analyses included 35 Spanish and Italian olive varieties assayed with nine SSR markers, which gave an average number of alleles per locus of 7.5. On the contrary, this is lower compared to those found by [28, 2, 41].Variations reported in the number of alleles in olive cultivars by different scientists may be related to variation in the loci studied as well as the number of genotypes and their localities [28].

The number of alleles, He and PD values indicate clearly that these markers were valid tools to discriminate among and between the studied cultivars. According to statistical analysis, all 7 loci PD values (except GAPU71A locus) were superior to 0.75, which was similar to the PD values reported in olive cultivars by [2]. Moreover, this is greater than those found by [39] in Tunisian olive cultivars (0.71) and by [42] in Italian cultivars (0.5).The averages of observed and expected heterozygosity (Ho=0.428and He=0.775) are lower than those described by [1] in Tunisian

olive, by [30] and by [16] and the presented levels can be similar or higher than the results of other research [21, 39, 35, 47]. Indeed, [4] found that a higher PD value does not always correspond to a high NDC value. Our results showed the same tendency. We found high expected heterozygosity than observed one. [35, 41]reported higher expected heterozygosity than observed one respectively among 489 Italian olive cultivars by using 11 nuclear SSR markers and among 76 Turkish olive using 14 microsatellite markers.

[2] were described a higher values of the Ho compared to He, which is reflected by the negative Fis values. Furthermore, these negatives values would suggest an excess of heterozygotes due to random matting. [25] stated that clonal reproduction enables the two alleles at each locus to independently accumulate mutations and therefore diverge within individuals and thus leads to a negative Fis values. [7] reported similar finding when establishing a consensus list of microsatellite markers for the olive genotyping using 21 cultivars. As well, [4] reported accordance between Ho and He values, while [17] described higher values of Ho compared to He. These authors stated that the SSRs applied to olive and in general to the majority of out-cross species, which are clonally propagated; seem to be characterized by medium levels of heterozygosity.

Present study indicates high genetic diversity among olive cultivars grown in different region of the South East of Tunisia and the used SSR markers revealed high discriminating capacity for olive cultivars. Previously, most scientists conclude that SSR markers are a powerful tool for cultivar identification and analysis of genetic structure in olive [5, 19, 35, 2].

The results obtained by microsatellite DNA analysis revealed a clear separation of most Tunisian olive cultivars and showed a significant degree of inter-varietal genetic diversity. A dendrogram was made using the genetic similarity between cultivars based on SSR markers (Fig. 1). A high range of similarity was found among analyzed samples ranging from 0 to 0.7 (with average of 3.5). The dendrogram in Figure 1 depicts the pattern of relationships between the studied cultivars. There is no clear clustering of cultivars in relation with their growing area or end use. Nevertheless, five major clusters can be defined by cutting the dendrogram at a GS value of 0.28. The first cluster included the two cultivars of Dokhar el Gorthab (12) and Dokhar Drina (14). The second group formed by the cultivars Zalmati Zarzis (1), Zalmati Djerba (2) and Fougi (11). The third cluster grouped all cultivars of chemlali variety (Chemlali Dakhla (3), Chemlali Douiret (16), Chemlali Ontha (7), Chemlali Zarzis (6)). The fourth group formed by the five cultivars Zarrazi injassi (15), Zarrazi Zarzis (9), Zarrazi Tataouine (13), Fakhari (10) and Neb Jmal (8). The last group included the two cultivars of Jemri Ben Guardène (4) and Jemri Matmata (5).

There is no clear structure with their geographical origin that has been observed in this work. These results were in accordance with previous researches [21, 30] that showed no clear correlation between olive genotypes and their geographical origin and a wide genetic variability was described independently from the geographical origin. Also, [41] were indicated that grouping genotypes based on the geographic origin is not useful in olive. [10] found that olive genotypes from different countries clustered together within a group and they did not find any grouping based on their geographical origins.

But there is in contrast with those described by [20, 49] who were indicated a relationship between the geographical origin and the genetic relationships in Italian and Moroccan olive cultivars respectively. The same result was found [15] that reported olive cultivars were grouped according to their geographical origin. Additionally, [6] were noted that classification of olive genotypes based on SSR markers is somewhat coincidence with the geographical distribution of genotypes.

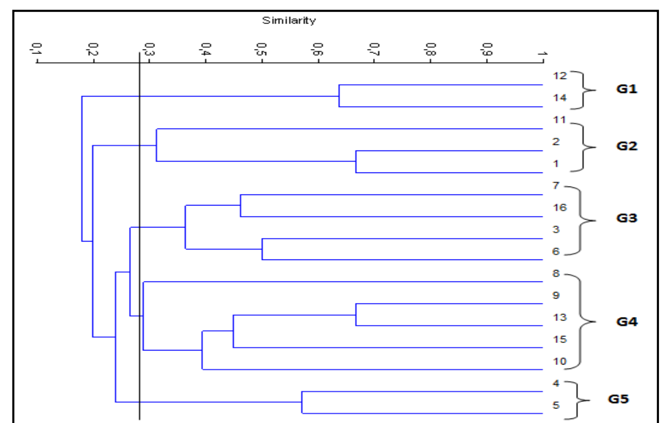

Fig.1. *Dendrogram of different olive cultivars studied based on SSR data using Jaccard's GS matrix and the UPGMA clustering method.*

IV. CONCLUSION

It was concluded that the genetic diversity in olive was much higher at the molecular level. Evaluation of molecular genetic diversity is useful for the detection of genetic differences among the olive accessions, for conservation of genetic resources, identification of cultivars. The genetic relationships among olive cultivars may facilitate the selection of parents in breeding programs. Microsatellite markers used in this study may be used for establishing a molecular database for Tunisian olive identification and to construct a molecular catalogue that can compare the molecular pattern of cultivars as well as to avoid redundant genetic entities to make a reference collection. These are preliminary results, further study is needed to corroborate these results, and it can be increase the number of samples for each cultivar, use more SSR markers.

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