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Research Article

GENETIC DIVERSITY ANALYSIS OF TUNISIAN OLIVE CULTIVAR BY SNP MARKERS

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ABSTRACT

To study the genetic diversity of 17 olive tree cultivars (*Olea Europaea* L.) sampled from different Mediterranean regions, we screened three SNP markers (*ACP-1, ANTHO3, SOD)* located in three different genes. The genotypes of the sampled cultivars were depicted via the genotyping analysis. The dendrogram based on cultivar genotypes generated by SNP markers revealed three clusters which were consistent with the established classification. In addition, we compared the results obtained with agro-morphological and chemical data using bioinformatic analyses. This work offers a more relevant classification of the genetic classification of the Tunisian olive cultivars.

Keywords: genetic diversity, olive, SNP markers.

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1. INTRODUCTION

Olive (*Olea europaea* L.) is one of the most known fruit crops in the Mediterranean region. The olive sector offers an important economic value for many Mediterranean countries including Tunisia [1]. Actually, Tunisia is estimated to have more than 65 million olive trees,

spread over 1 680 000 ha and to produce almost 340 000 tons of olive oil yearly, ranking second after the European Union in terms of olive oil production (http://www.tunisia-oliveoil.com/). Currently, the olive tree is being abandoned in this country, which promotes the genetic erosion and the loss of certain biotype data. It is however important to know and preserve the genetic heritage of this crop.

The genetic variation analyses are associated to chemical and morphological markers; however, these markers are insufficient to study the relationship between cultivars due to the environmental effects on the phenotype and chemical composition variation. Moreover, these markers require several expensive tests, which is the major constraint to their use [2,3]. Therefore, the use of molecular markers methods, such as random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFPLs), single sequence repeats (SSR) and single nucleotide polymorphism (SNP) allows the detection of DNA polymorphism without any environmental influence. Hence, the study of the genetic diversity is undertaken more efficiently.

In this study, we exploited three SNPs localized respectively in three different genes: *Cu-Zn superoxide dismutase* (SOD), *anthocyanidin synthase* (ANTHO3) and *steoryl_Acp desaturase* (ACP). *Superoxide dismutase* (SOD) plays an important role in the defense mechanism of the plant against oxidative stress. During biotic and abiotic stresses, an imbalance between the defense systems (antioxidants) and the reactive oxygen species (ROS) may occur and can damage the cellular components, such as DNA, lipids, proteins and sugars [4]. Indeed, Oxidative stress involves the appearance of events that are often irreversible for the cell [5,6].

ANTHO3, is involved in the flavonoid biosynthesis pathway. Flavonoids are produced in response to various stresses in higher plants. They are considered as antioxidants since they participate in ROS scavenging systems. Furthermore, they play a wide range of roles in mechanisms relating to UV protection, pathogen defense, symbiosis, variation of flower color, male fertility, pollination, allelopathy and auxin transport [7]. ACP, is implicated in fatty acids biosynthesis. The level of oleic/linoleic acid and oil accumulation throughout mesocarp development in olive oil is determined by the mutual function of *stearoyl-ACP* and *oleate desaturases* [8]. In plants, the transformation of *stearoyl-ACP* to *oleoyl-ACP* is catalyzed by

stearoyl-acyl desaturase (SAD). SAD's activity influences the rate of oleic acid content during mesocarp development. On the other hand, the activity of desaturase of fatty acids (FAD) is responsible for the linoleic acid content in olive oil [8,9]. These two genes influence the acidity of olive oil.

This work reports the use of the SNP markers to distinguish genetic diversity and relationship among 17 olive cultivars from different Tunisian regions among which 13 cultivars are originated from Tunisia and the others are from Morocco, Greece, Spain, and Algeria. It also checks the efficiency of the SNP markers to study the genetic diversity and the relationship among our studied olive cultivars.

2. MATERIALS AND METHODS

2.1. Plant material

A total of seventeen Tunisian olive tree cultivars were chosen and used. They were selected from different geographical regions from north to south of Tunisia. Four cultivars from four Mediterranean regions (Greece, Algeria, Morocco, and Spain) were included for comparison. For each cultivar, DNA was extracted from young leaves.

2.2. DNA extraction

DNA was extracted from young leaves using the CTAB methods described by Fabbri *et al.* [10]. The obtained genomic DNA was dissolved in TE buffer (1X) (50 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and stored at -20°C.

Table 1. Names and origins of the studied culivars

Cultivar	Country	Origin
koroneiki	Greece	Corone
Chemlali Zarzis	Tunisia	Zarzis
Sigoise	Algeria	Sig
Jarboui	Tunisia	El Kef, Béja, Siliana
Dhokar Ben Guerir	Morocco	Ben Guerir
Tounsi Gafsa	Tunisia	Gafsa
Chemlali Sfax	Tunisia	Sfax
Manzanille	Spain	Séville
Chehla	Tunisia	North
Zalmati	Tunisia	Zarzis
Zarrazi Zarzis	Tunisia	Zarzis
Rakhmi	Tunisia	Nabeul
Chemlali Tataouine	Tunisia	Tataouine
Besbessi	Tunisia	Besbessia
Chetoui	Tunisia	North
Jemri Benguerden	Tunisia	Benguerden
Bidh Hmam	Tunisia	All of Tunisia

2.3. Genotypic analyses

SNP markers depict most genetic variations in plants. They can discriminate very similar cultivars with a single nucleotide change. They are biallelic in most cases and transmitted co-dominantly [11,12]. The three studied SNPs were selected in the coding regions of genes involved in fruit characteristics. SNP SOD marker (insertion/deletion type), localized in the *Cu-Zn superoxide dismutase* gene, was genotyped by a simple polymerase chain reaction (PCR). The other two SNPs (ACP and ANTHO3) were genotyped by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (table 2). The PCR product (171 bp) of the SNP ANTO3 was digested with *MSPI* restriction enzyme at 37 °C

overnight. This restriction enzyme recognizes the sequence AA/GG. The G allele carrying the PCR product was cleaved once by the enzyme generating two fragments (64 bp and 107 bp). The PCR product (330 bp) of the SNP ACP was digested with the *TaqI* restriction enzyme at 65°C overnight. This restriction enzyme recognizes the sequence TT/CC. The T allele carrying the PCR product was cleaved once by the enzyme generating two fragments (263 bp and 67bp). Otherwise, the C allele carrying the PCR product was cleaved twice by the enzyme generating three fragments (158 bp, 105 bp, and 67 bp). All digestion products were separated by electrophoresis on 3% Nusieve ethidium bromide-stained agarose gel and visualized under UV light.

Table 2. Characteristics of the studied SNP markers used for DNA amplification.

Gene name	SNPs	SNP type	Tm[a]	Size of amplicon (pb)	Primer Sequence (5 3') → Enzyme
Anthocyanidine synthase	Antho3	G/A	57	171	5'-GTTGGTGGCATGGAAGAACT-3' *MSP I* 5'-GACCCATTTTCCCTCACAGA-3'
Cu–Zn-superoxide dismutase	SOD	I/D	57	176/128	5'-TTCCCACCGATATTGTTAAGGG-3' 5'-CATATAGCATAGCACCCGCAAC-3'
Stearoyl –ACP desaturase	ACP	T/C	57	330	5'-GCCAGCTCATCTGATGTACG-3' *Taq I* 5'-TGCACACGTAATCTTGTGCTT-3'

I: insertion, D: deletion, [a]: Annealing temperature for PCR amplification.

2.3. Statistical analyses

The analysis of the diversity of olive tree cultivars using SNP markers was performed at several steps using several statistical techniques. First, allele frequencies and heterozygositis (both observed and expected under Hardey-Weinberg equilibrium) were calculated using GDA program. The power of discrimination (PD) calculates the differences between the classes of qualitative trait in allele and genotype frequencies that were evaluated by the chi-square test, for each SNP locus according to Brenner and Morris.

$$PD = 1 - \sum_{i=1}^{g} Pi^2$$

Where *Pi* is the frequency of *i*th genotype for the locus and the sum of overall genotypes.

A dendrogramm was inferred using and arithmetic average (UPGMA) clustering algorithm (NTSysPc version 2.1).

3. RESULTS AND DISCUSSION

3.1. Genotyping Results and the Characteristics of the Studied SNP Markers

The expected fragments of each SNP are reported in the materials and methods section. The observed heterozygosis for each marker ranges from 0.266 (ACP) to 0.75 (ANTHO3) (0.475 average), while the expected heterozygosis varies between 0.391 and 0.5 with an average of 0.443 lower than the observed one (table 3).

Table 3. Characteristics of the studied SNP markers in 17 olive cultivars.

Locus	SNPs type	Tm[a]	Size of amplicon (pb)	Ho	He	PD	r
ANTHO3	A/G	57	171	0,75	0,5	0,406	-
SOD	Insertion/délétion	57	176/128	0,411	0.44	0,595	0.018
ACP	T/C	57	330	0,266	0,391	0,551	0,080
Average	-		-	0.475	0.443	0.517	-

For each locus, the type of polymorphism, the size of amplicon in base pairs, the frequency of heterozygotes found (Ho), the frequency of heterozygotes predicts (He) and the power of discrimination (PD) are reported.

$$r = (He - Ho)/(1 + He)$$

The probability of null alleles (r) varies from 0.018 to 0.080 with an incoherent value for the ANTHO3 marker, which indicates an excess of heterozygosity for this marker. The discrimination power (DP) varies from 0.406 for the ANTHO3 marker to 0.595 for the SOD

marker with an average value of 0.517. Although, the average value was lower than that shown by Ben Ayed et *al*. [13], with eight SSR markers (0.699), it is significantly higher than that reported by Muzzalupo et *al*. [14] in 39 Italian cultivars (0.38), using 11 SSR markers. Moreover, it is almost equal to that reported by Reale et *al*. [12] in 11 European and Australian cultivars (0.52), using 11 SNP markers. For the allele frequencies of each studied SNP marker, a dominance of one allele over another is noted, except for ANTHO3, in which both alleles are equal. Likewise, most of the studied cultivars are heterozygous genotypes (table 3), except for the ANTHO3 marker, in which the frequency of heterozygous genotype is 25% and that of homozygous genotype is 75%.

3.2. Dendrogram analysis

Genotypic data from SNP markers were used to construct the dendrogram shown in Figure 1 where four cultivar groups could be defined by cutting the dendrogram at a distance of 0.75. Group 1 consisted of seven cultivars (‘‘koroneiki’’, ‘‘Cheml_Tat’’, ‘’Sigoise’’, ‘’Tounsi_Gaf’’, ‘‘Bidh_Hmam’’,’’Zarrazi_Zar’’, ‘’Rakhmi’’). The second group comprised two cultivars (‘’Chemlali sfax’’ and ‘’Zalmati’’). The third group was made up of six cultivars (‘'Cheml_Zarz’’, ‘’Dhokar_BG’’, ‘’Chehla’’,’’Besbesi’’, ‘’Manzanille’’, ‘’Chetoui’’). The fourth group constituted two cultivars (’’ Jarboui ‘','‘Jemri_BG’’).

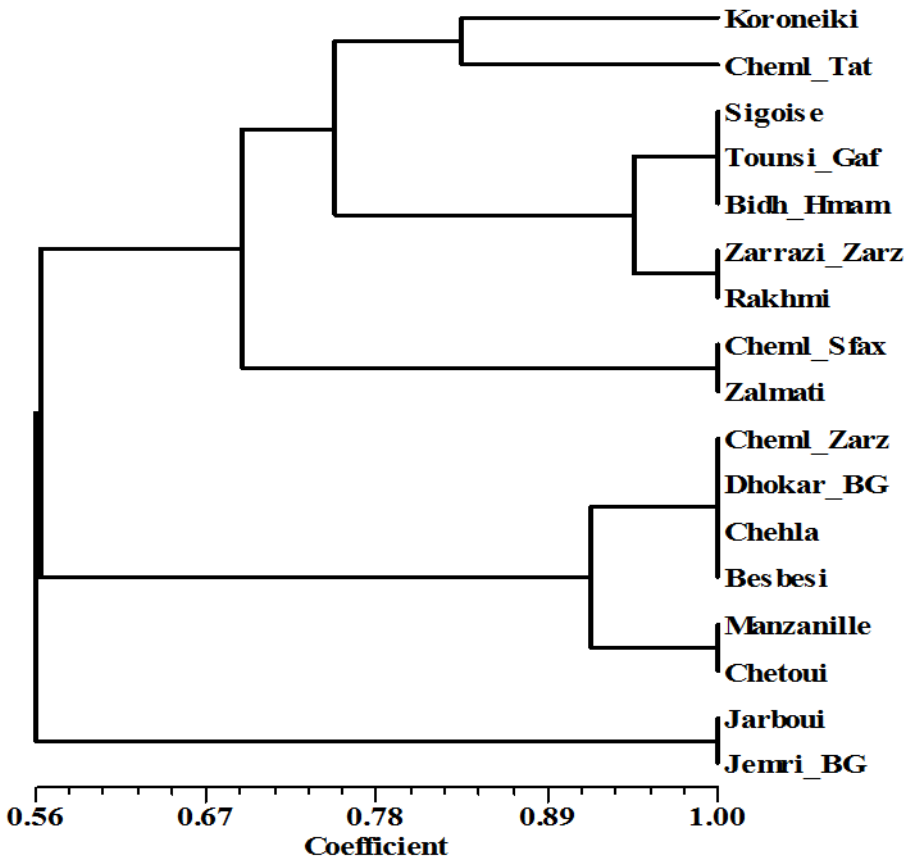

Fig.1. Dendrogram produced by UPGMA (Unweighted Pair Group Method using an Arithmetic average) clustering based on means of 3 SNPs markers of 17 olive tree cultivars.

Based on this graphical representation (Fig. 1), we firstly notice that group 1 contains seven different cultivars coming from different geographical origins. Moreover, the third group includes different origins and presents different susceptibilities to water scarcity. These observations can be explained by the fact that olive cultivars expand along agro-environments vegetatively [15]. In conclusion, this dendrogram reveals that there is no correlation between genetic variability and the geographic origin of cultivars, which was also reported by Ben Ayed et *al*. [13]. Our results indicate that SNP markers will be effective tools for the construction of the genetic map of the olive tree. Thus, to get a better study of genetic diversity, it would be necessary to increase both the number of olive cultivars and the number of SNP markers.

4. CONCLUSION

The main objective of this work was to study the genetic diversity of olive trees using SNP markers. In this study, we studied the polymorphism of these SNP markers located in three different genes (*ANTHO, SOD, ACP)* in a sample of olive cultivars and examined their genotype-phenotype association. Using the obtained data, we constructed a dendrogram that illustrates the groupings of genes and studies the family relationships that unite our cultivars. The results revealed that there are no existing correlation between genetic variability and the geographic origin of the Tunisian olive cultivars. In perspective, SNP markers could be used in the determination of olive oil characteristics, quality and to confirm its authenticity.

5. ACKNOWLEDGEMENT

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