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Genetic Characterization of Tunisian Olive Table Cultivars (*Olea europaea* L.): Inventory Based on Microsatellite Analysis

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ABSTRACT

Olive (*Olea europaea* L.) is an ancient crop which spread from the Middle East towards the western Mediterranean region. In addition to its agronomical and economical importance, the olive industry has a great social and cultural interest as part of our heritage. In order to characterize and to study the genetic relationship among cultivars, five polymorphic microsatellite loci were used in this study and were generated a total of 56 polymorphic alleles with an average of 11 alleles by locus. For all genotypes, the highest number of polymorphic alleles was obtained by the locus UDO043 and GAPU 103. The polymorphism index content ranged from 0.836 for the locus ssrOeUADCA-04 and 0.919 for the locus GAPU-103. The analysis of the dendrogram and the principal coordinates analysis produced by SSR markers grouped studied cultivars into four distinct clusters by cutting the dendrogram at genetic similarity value of 0.1. Accessions of 'Marsaline', 'Fouji' and 'Fokhari' are grouped in one clear cluster. 'Zarrazi' and 'Meski' varieties are grouped in separated clear class. The last group was formed by 'Picholine' and 'Chemcheli' varieties.

Keywords: genetic diversity, genetic relationships, polymorphism, molecular markers, simple sequence repeats

INTRODUCTION

Since the Romans and through all the civilizations that have marked the history of Tunisia, the olive tree (*Olea europaea* L.) occupies a large place in the Tunisian landscape and plays a vital role in its social and economic life. This species is extended throughout the country, under various biotopes and climates showing its spatial and temporal geographical distribution. Tunisia accounts approximately 24,500 ha of olive table which was widespread in the north (70-80%) (GICA 2008).

According to statistical data (IOOC 2003), Tunisian table olive annual production is about 23,000 tons, this production is steadily increasing due to the appreciation of olive fruit (good taste), as well its nutritional properties. Olive table is gaining the favor of consumers due to the beneficial effect that is attributed to a favorable fatty acid profile and to the presence of some minor components (Sakouhi *et al.* 2008; Yamada *et al.* 2008).

A total of 20 native olive table varieties are known in Tunisia and 4 indigenous varieties, 'Lucques' and 'Picholine' (French), 'Ascolana' (Italy) and 'Manzanille' (Spain). Currently, the most used cultivar for processing is 'Meski'. This cultivar is one of the most important and widely planted covering more than two thirds of the area for table olives (15,043 ha) and especially in the irrigated system.

'Meski' is known and appreciated for its taste and its technological interest in the local market and for export. It is characterized by a spherical fruit with an excellent external appearance, very fleshy and tastes good. The mean weight of the fruit is around 4 g. The pulp/stone ratio is very important (4: 6) and the core is easily removable (Mehri and Hellali 1995). Diagnosis of the sector's current situation shows that production levels are still modest and have not achieved the large genetic potential of existing varieties, which can be explained by the heterogeneity and the alternation of production (Mehri *et al.* 1990; Msallem and Hillali 2000; Mehri *et al.* 2003). However, some genetic improvement programs that were established 20 years ago have led to new descendants that take into account the productivity and quality of fruits (Trigui 1996; Ben Amar *et al.* 2006).

Despite the wide genetic patrimony and the large number of synonyms and homonyms in olive table require powerful and precise methods of discrimination for cultivar identification and classification (Bracci *et al.* 2009).

Morphological characterization of Tunisian olive cultivars is carried out based on methodology adapted by the International Olive Oil Council (IOOC) (Mehri and Hellali 1995; Trigui and Msallem 2002). Morphological characters which are considered very heritable were used for describing olive cultivars held in the germplasm collection and in the field. The morpho-pomological characterization is based on a set of distinctive descriptors for qualitative and quantitative characters related to the tree (vigour, growth habit, canopy density and length of internodes), to the leaf (length, shape, width and longitudinal curvature of the blade), to the inflorescence (length, number of flowers/ inflorescence), to the fruit (weight, shape, symmetry, colour presence of lenticels, etc.) and to the characters of the endocarp (stone) (weight, shape, symmetry, position of maximum transverse diameter, surface, number of grooves, distribution of the grooves, etc.) (Hannachi *et al.* 2006). Moreover, this approach yielded a considerably redundant number of different olive table cultivars. However, because of most discriminating characters related to fruit and flower, morphological identification has to be completed by a molecular approach.

Some authors applied isozymes techniques to identify and to study the genetic variability of olive (Trujillo *et al.* 1995). The use of isozyme markers to characterize and to study the genetic variation between and within cultivars, depend on several factors (environmental, limited in number) that limit the wider adoption of this technique (Grati-Kamoun *et al.* 2006).

Recently, molecular markers were used to identify olive

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Table 1 Studied accessions of olive table genotypes and their origin.

Genotype	End use	Collection	Geographic origin	Mean weight of fruit (g)
Zarrazi	Oil and olive table	-	Zarzis	3.9
Picholine1	Oil and olive table	Ksar Ghriss	Sidi BouZid	5.0
Picholine2	Oil and olive table	Ksar Ghriss	Sidi BouZid	5.0
Marsaline1	Oil and olive table	Ksar Ghriss	Sidi BouZid	7.0
Marsaline2	Oil and olive table	Ksar Ghriss	Sidi BouZid	7.0
Chemcheli	Oil and olive table	Boughrara	Sfax	2.3
Fouji	Oil and olive table	Boughrara	Sfax	1.3
Fakhari	Oil and olive table	Boughrara	Sfax	2.0 -2.5
Meski1	Olive table	Boughrara	Sfax	>6.0
Meski2	Olive table	Bouighrara	Sfax	>6.0

Table 2 Sequence of primers, temperature of hybridization (°C), product size range, allelic number, He, Ho, and PIC of the 5 SSR loci studied.

Locus	Repeat motif	Sequence of primers (5′- 3′)	Hybridization temperature (°C)	Size range (bp)	Number of alleles	Ho	He	PIC
UDO099-043	(GT)12	TCGGCTTTACAACCCATTTC TGCCAATTATGGGGCTAACT	52	157-223	15	0.985	0.968	0.914
GAPU-103	(TC)26	TGAATTTAACTTTAAACCCACACA GCATCGCTCGATTTTATCC	57	127-199	15	0.996	0.973	0.919
ssrOeUA DCA-04	(GA)16	CTTAACTTTGTGCTTCTCCATATCC AGTGACAAAAGCAAAAGACTAAAGC	55	128-184	10	0.600	0.894	0.836
ssrOeUA DCA-03	(GA)19	CCCAAGCGGAGGTGTATATTGTTAC TGCTTTTGTCGTGTTTGAGATGTTG	50	122-252	11	0.900	0.921	0.863
EMO-90	(CA)10	CATCCGGATTTCTTGCTTTT AGCGAATGTAGCTTTGCATGT	55	123-200	14	0.987	0.952	0.897

cultivated and wild clones and cultivars (reviewed by Muzzalupo and Perri 2008). DNA molecular markers techniques based on *in-vitro* enzymatic amplification of specific fragments of DNA via PCR (polymerase chain reaction) are of greater suitability in genetic diversity estimations and identification of olive genotypes (Doveri *et al.* 2008). Previous studies have assessed the genetic variability of olive using random amplified polymorphic DNA (RAPDs) (Belaj *et al.* 2003), amplified fragment length polymorphism (AFLPs) (Sanz-Cortés *et al.* 2003), inter simple sequence repeats (ISSRs) (Terzopoulos *et al*. 2005) and simple sequence repeats (SSRs or microsatellites) (Charafi *et al*. 2008; Besnard *et al*. 2009).

The objectives of this study were i) to identify and to verify by SSR markers DNA fingerprints of studied accessions of olive trees and ii) to test the above-mentioned markers for their ability to discriminate between and among cultivars.

MATERIALS AND METHODS

Plant material

In the present study, plant material was collected from morphologically different varieties of different collections in Tunisia. The origin of studied accessions of Tunisian varieties and are listed in **Table 1**.

DNA extraction

Total DNA was extracted from young leaves using hexadecyltrimethyl ammonium bromide (CTAB) according to the method described by Doyle and Doyle (1990). High salt concentrations (100 mM Tris-HCl [pH 8.0], 20 mM EDTA, 1.4 M NaCl, 1% CTAB) and polyvinylpolypyrrolidone (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) were added separately to remove polysaccharides and polyphenol compounds. Extraction was performed by an extended RNase A digest (Promega) treatment and a phenol-chloroform method (Rekik *et al.* 2008). DNA was resuspended in TE (pH 8) solution and stored at –20°C.

DNA amplification by SSRs

The five selected microsatellite loci were used in other work on the characterization of varieties of cultivated and wild olive trees are considered as reliable molecular markers and reproducible. Amplification was performed among three sets of SSR primers pairs: DCA (Sefc *et al.* 2000), GAPU (Carriero *et al.* 2002) and UDO primer pairs (Cipriani *et al.* 2002) (**Table 2**).

Amplification reactions were carried out in final volumes of 10 µl. The reaction contained 1X PCR buffer, 0.75 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of each primer, 0.5 U/µl *Taq* polymerase (Gotaq, Promega) and 50 ng/µl template DNA. PCR reactions were performed at the following conditions: 3 min at 94°C for initial denaturation, 35 cycles of 1 mn at 93°C (denaturation), 1 mn at optimal temperature ranging from 50 to 57°C (annealing) and 90 sec at 72°C (extension). A final extension step at 72°C for 10 min followed. Polymerase chain reaction (PCR) was carried out using a thermocycler (PE Applied Biosystems). The forward primer was 5′ labeled with one of two fluorophores (6FAM or HEX) (Sigma).

0.5 µl of PCR product was mixed with a 12 µl of deionised formamide and 0.5 µl Gene Scan 500 (LIZ) size standard marker. The resulting mixture was heated for 2 min at 95°C and then quickly cooled on ice. Each sample was loaded and run on an ABI-310 automated DNA sequencer (capillary electrophoresis). GeneMapper v.4 software (PE Applied Biosystems) was used to score the SSR profiles.

Data scoring and analysis

For each individual and for each primer that yielded a clear pattern, polymorphic DNA pics for SSR markers were scored as present (1) or absent (0). For the data set, dendrograms was constructed by UPGMA (Unweighted Pair-Group Method with Arithmetic Averages) cluster analysis according to Jaccard's coefficient (Sneath and Sokal 1973) using NTSYS software analysis and grouping.

Polymorphism Index Content (PIC), observed and expected heterozygosy were calculated by the software CERVUS v.2 (Marshall *et al.* 1998).

Principal Coordinates Analysis (PCoA) was performed by XLSTAT software.

Average fruit weight was determined according to the method indicated by IOOC (1997).

RESULTS AND DISCUSSION

Molecular markers

The results obtained by microsatellite DNA analysis revealed a clear separation of most Tunisian olive table cultivars and showed a significant degree of inter-varietal

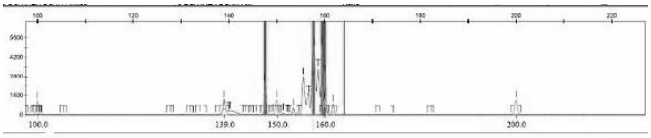

Fig. 1 Example of peakks revealed on automatic sequencer for ssrOeUADCA-04 locus of 'Meski1' individual.

genetic diversity. The use of microsatellite techniques and their application in genetic diversity studies has allowed the discrimination between and among cultivated olive cultivars and with wild olive forms and the estimation of their genetic inter-relationships (Pollastri 2008).

The microsatellite method consists of short stretches of DNA tandemly repeated is becoming a powerful tool for genetic analysis and has a greater information content that can be helpful for organizing germplasm, for conservation of genetic resources, for the identification of cultivars and for selection of parents for breeding programs (Brito *et al.* 2008). The five microsatellite loci used in this study generated a total of 56 polymorphic alleles with an average of 11 alleles by locus (**Table 2**). For all genotypes, the highest number of polymorphic alleles was obtained by the locus UDO043 and GAPU 103.

The size of the alleles found for the five loci varies between 57 and 167 bp for ssrOeUA DCA-04 and UDO-43 respectively.

The PIC values were in a range from 0.836 for the locus ssrOeUADCA-04 and 0.919 for the locus GAPU-103 and the five used loci as suitable for mapping (PIC > 0.7) (**Table 2**), indicating a high discriminating power of the analysed microsatellite markers (Poljuha *et al.* 2008).

Genetic diversity

Analysis of the dendrogram in **Fig. 1** and PCoA in **Fig. 2** depict the pattern of relationships between and among cultivars produced by SSR markers and grouped cultivars into four distinct clusters by cutting the dendrogram at a Genetic Similarity (GS) value of 0.1. There is no clear structure of the variability relative to the geographic origin and to the end use of olive table cultivars. Accessions of 'Marsaline', 'Fouji' and 'Fokhari' are grouped in one clear cluster. 'Zarrazi' and 'Meski' varieties are grouped in two clear clusters. The latest group was formed by 'Picholine' and 'Chemcheli' varieties.

In fact, 'Meski' (cultivated specially in the north of the country) and 'Zarrazi' cultivars (cultivated specially in the south: Zarzis) were grouped separately. The first variety is among the most important Tunisian table cultivars which used for canning and have a high average fruit weight (6.0 g), whereas the letter is used for both oil and canning and have a high average fruit weight (3.9 g). Genetic differentiation based on fruit size and use has been observed in previous study (Grati-Kamoun *et al.* 2006). Taamalli *et al.* (2006), in their AFLP-SSR analysis of Tunisian olive varieties show that olive accessions inherent small fruit and medium fruit were clustered in separated groups. This result is consistent with previous findings indicating that Greek cultivars were clustered according to fruit size (Nikoloudakis *et al.* 2003).

Cluster 2 is composed of autochthon cultivars native to Tunisia: 'Marsaline' (Siliana and Zaghouan), 'Fouji' (Gafsa) and 'Fokhari' (Tataouine), whereas cluster 4 is composed by one foreign variety 'Picholine' from French and 'Chemcheli' from Gafsa. As indicated in **Table 1**, the geographic distribution of studied varieties was from the south to the north of the country and there is no clear clustering of the variability relative to the geographic origin of growing area or relative to the weight of fruit. This result may be explained by the fact that Tunisian olive germplam is a rich assortment of polyclonal varieties, which are the results of a clonal selection process, generally done by farmers in terms of their needs or in terms of their aspiration to superior quality of olive fruit for oil or for canning (Rekik *et al.* 2008).

Lack of a clear clustering of olive cultivars according to their geographic origin was observed previously in several studies based on allozymes (Ouazzani *et al.* 1995) or based on molecular markers (Owen *et al.* 2005; Rekik *et al.* 2008). The agro-ecological adaptation area of cultivars (not wide geographical distribution), a possible exchange of plant material and seed among the different regions of Tunisia and the old cultural traditions may explain the clustering obtained by SSR's data among cultivars (Bartolini and Petruccelli 2002).

The presence of two varieties 'Picholine' and 'Chemcheli' in the same cluster could be explained by the common origin of olive trees. As reported by some previous studies (Hannachi *et al.* 2008), shown that Mediterranean olive was widely diffused in Mediterranean countries by human activities.

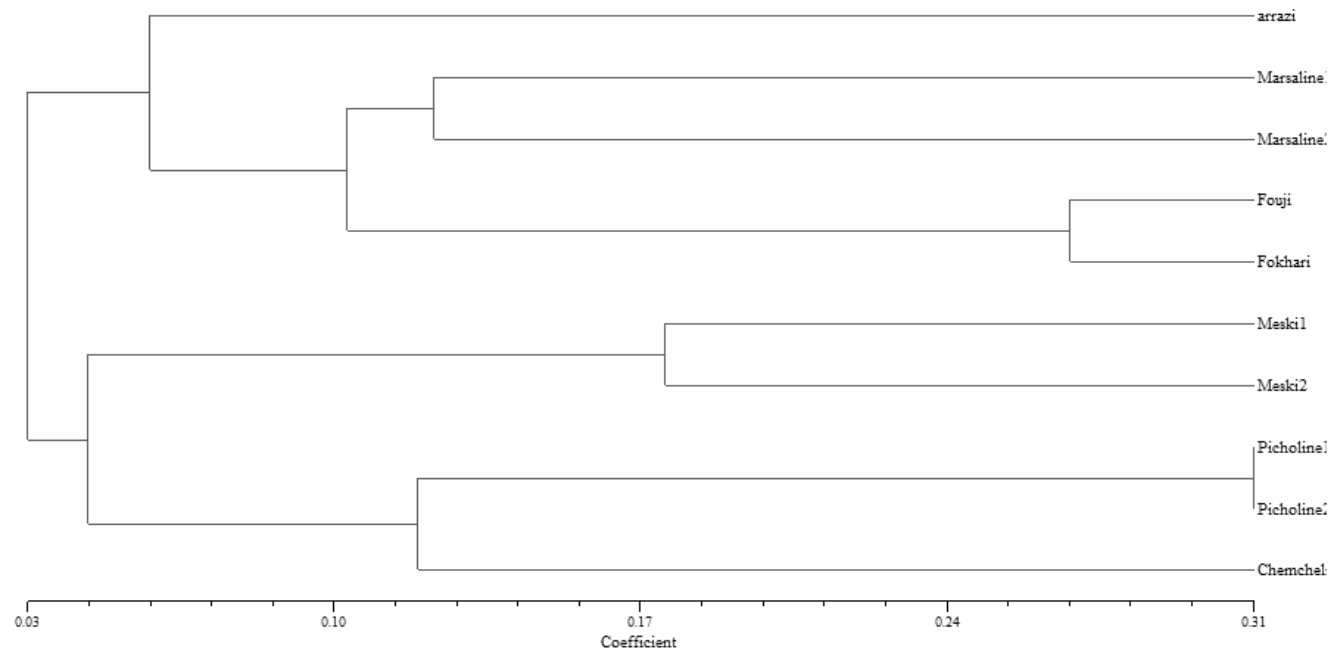

Fig. 2 SSR dendrogram based on Jaccard's genetic distance.

CONCLUSION

It may be concluded from this study that microsatellite markers provide consistent information for cultivar identification and are valuable tools for studying the genetic relationships between Tunisian olive cultivars which might allow us to make future germplasm collections.

It will be necessary to investigate the genetic diversity of much more Tunisian cultivars for future researches and to sample a large number of Mediterranean varieties in order to identify their genetic background in relation to Tunisian cultivars.

REFERENCES

Bartolini G, Petruccelli R (2002) Classification, origin, diffusion and history of the olive.- Food and Agriculture Organisation of the United Nations, Rome, Italy

Belaj A, Satovic Z, Cipriani G, Baldoni L, Testolin R, Rallo L, Trujillo I (2003) Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. *Theoretical and Applied Genetics* **107**, 736-744

Ben Amar F, Msallem M, Khabou W (2006) Clonal selection program of the Tunisian table olive (*Olea europaea* L.) variety "Meski". *Proceedings of the 7th TJASSST*, pp 59-60

Besnard G, Baali-Cherif D, Bettinelli-Riccardi S, Parietti D, Bouguedoura N (2009) Pollen-mediated gene flow in a highly fragmented landscape: consequences for defining a conservation strategy of the relict Laperrine's olive. *Comptes Rendus Biologies* **332**, 662-672

Bracci T, Sebastiani L, Busconi M, Fogher C, Belaj A, Trujillo I (2009) SSR markers reveal the uniqueness of olive cultivars from the Italian region of Liguria. *Scientia Horticulturae* **122**, 209-215

Brito G, Loureiro J, Lopes T, Rodriguez E, Santos C (2008) Genetic characterisation of olive trees from Madeira Archipelago using flow cytometry and microsatellite Markers. *Genetic Resources and Crop Evolution* **55**, 657-664

Carriero F, Fontanazza G, Cellini F, Giorio G (2002) Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). *Theoretical and Applied Genetics* **104**, 301-307

Charafi J, El Meziane A, Moukhli A, Boulouha B, El Modafar C, Khadari B (2008) Menara gardens: a Moroccan olive germplasm collection identified by a SSR locus-based genetic study. *Genetic Resources and Crop Evolution* **55**, 893-900

Cipriani G, Marrazzo MT, Marconi R, Cimato A, Testolin R (2002) Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. *Theoretical and Applied Genetics* **104**, 223-228

Doveri S, Gil FS, Díaz A, Reale S, Busconi M, Machado AC, Martín A, Fogher C, Donini P, Lee D (2008) Standardization of a set of microsatellite markers for use in cultivar identification studies in olive (*Olea europaea* L.). *Scientia Horticulturae* **116**, 367-373

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus* **12**, 13-15

GICA: Groupement des Industries de Conserves Alimentaires (2008) Strategic study on the Tunisian olive branch table. Available online: http://www.gica.ind.tn

Grati-kamoun N, Lamy Mahmoud F, Rebaï A, Gargouri A, Panaud O, Saar A (2006) Genetic diversity of Tunisian olive tree (*Olea europaea* L.) cultivars assessed by AFLP markers. *Genetic Resources and Crop Evolution* **53**, 265-275

Hannachi H, Msallem M, El Gazzah M, Ben Elhadj S (2006) Etude de la variabilité pomologique des olives et de la composition en acides gras des huiles de 15 variétés d'olivier tunisiens (*Olea europaea* L.) cultivées *in situ*. *Revue des Régions Arides* **17**, 43-64

Hannachi H, Breton C, Msallem M, Ben El Hadj S, El Gazzah M, Bervillé A (2008) Differences between native and introduced olive cultivars as revealed by morphology of drupes, oil composition and SSR polymorphisms: A case study in Tunisia. *Scientia Horticulturae* **116**, 280-290

IOOC: International Olive Oil Coucil (1997) Méthodologie pour la caractérisation primaire des variétés d'olivier. Available online: http://www.internationaloliveoil.org

IOOC: International Olive Oil Coucil (2003) Available online: http://www.internationaloliveoil.org

Muzzalupo I, Perri E (2008) Genetic characterization of olive germplasm by molecular markers. *The European Journal of Plant Science and Biotechnology* **2**, 60-68

Mehri H, Hellali R, Mehri R (1990) Mise en évidence de l'auto-incompatibilité chez la variété «Meski». *Annales de l'INRAT* **63**, 16-28

Mehri H, Hellali R (1995) Etude pomologique des principales variétés d'olives cultivées en Tunisie. Document technique, Ed Institut de l'Olivier, 45 pp

Mehri H, Mehri-Kamoun R, Msallem M, Faïdi A, Polts V (2003) Reproductive behaviour of six olive cultivars as pollinizer of the self-incompatible olive cultivar Meski. *Advances in Horticultural Science* **17**, 42-46

Msallem M, Hellali R (2000) Amélioration génétique par croisement de la variété d'olive de table «Meski» synthèse de trois campagnes. *Revue Ezzaitouna* **6**, 59-73

Marshall TC, Slate J, Kruuk L, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7**, 639-655

Nikoloudakis N, Banilas G, Gazis F, Hatzopoulos P, Metzidakis J (2003) Discrimination and genetic diversity among cultivated olives of Greece using RAPD markers. *Journal of the American Society for Horticultural Science* **128**, 741-746

Ouazzani N, Lumaret R, Villemur P (1995) Apport du polymorphisme alloenzymatique à l'identification variétale de l'olivier (*Olea europaea* L). *Agronomie* **15**, 31-37

Owen CA, Bita EC, Banilas G, Hajjar SE, Sellianakis V, Aksoy U, Hepaksoy S, Chamoun R, Talhook SN, Metzidakis I, Hatzopoulos P, Kalaitzis P (2005) AFLP reveals structural details of genetic diversity within cultivated olive germplasm from the Eastern Mediterranean. *Theoretical and Applied Genetics* **110**, 1169-1176

Poljuha D, Sladonja B, Šetić E, Milotić A, Bandelj D, Jakše J, Javornik B (2008) DNA fingerprinting of olive varieties in Istria (Croatia) by microsatellite markers. *Scientia Horticulturae* **115**, 223-230

Pollastri S (2008) Biotechnological advances in olive (*Olea europaea* L.). A review. *Advances in Horticultural Science* **22**, 123-128

Rekik I, Salimonti A, Grati Kamoun N, Muzzalupo I, Lepais O, Gerber S, Perri E, Rebai A (2008) Characterization and identification of Tunisian olive tree varieties by microsatellite markers. *HortScience* **43**, 1371-1376

Sakouhi F, Harrabi S, Absalon C, Sbei K, Boukhchina S, Kallel H (2008) α-Tocopherol and fatty acids contents of some Tunisian table olives (*Olea europea* L.): Changes in their composition during ripening and processing. *Food Chemistry* **108**, 833-839

Sanz-Cortés F, Parfitt DE, Romero C, Struss D, Llàcer G, Badenes ML (2003) Intraspecific olive diversity assessed with AFLP. *Plant Breeding* **122**, 173-177

Sefc KM, Lopes MS, Mendonça D, Rodrigues dos Santos M, Laimer da Câmara Machado M, da Câmara Machado A (2000) A. Identification of microsatellite loci in olive (*Olea europaea*) and their characterisation in Italian and Iberian olive trees. *Molecular Ecology* **9**, 1171-1193

Sneath PHA, Sokal RR (1973) *Numerical Taxonomy. The Principles and Practice of Numerical Classification*, W.H. Freeman and Co, San Francisco, 513 pp

Taamalli W, Geuna F, Banfi R, Bassi D, Daoud D, Zarrouk M (2006) Agronomic and molecular analyses for the characterisation of accessions in Tunisian olive germplasm collections. *Electronic Journal of Biotechnology* **9**, 467-481

Terzopoulos PJ, Kolano B, Bebeli PJ, Kaltsikes PJ, Metzidakis I (2005) Identification of *Olea europaea* L. cultivars using inter-simple sequence repeat markers. *Scientia Horticulturae* **105**, 45-51

Trigui A, Msallem M (2002) Catalogue des variétés Autochtones et types locaux, 159 pp

Trigui A (1996) L'amélioration quantitative et qualitative de la production oléicole en Tunisie: l'incournable nécessité et les perspectives de l'identification et de l'amélioration génétique de l'olivier. *Olivae* **61**, 34-40

Trujillo I, Rallo L, Arus P (1995) Identifying olive cultivars by isozyme analysis. *Journal of the American Society for Horticultural Science* **120**, 318-324

Yamada P, Zarrouk M, Kawasaki K, Isoda H (2008) Inhibitory effect of various Tunisian olive oils on chemical mediator release and cytokine production by basophilic cells. *Journal of Ethnopharmacology* **116**, 279-287