

Intra-variety variability in the 'Chemlali' variety cultivated in Southeastern Tunisia by ISSR and SSR markers

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Abstract - The olive tree (*Olea europaea*. L), is a characteristic species of the Mediterranean landscape, this species account numerous varieties that show a wide diversity in their morphology and phenology. The Tunisian olive heritage is also characterized by a rich variety dominated by the presence of two main varieties, Chemlali (Center and South) and Chétoui planted in the North of Tunisia. Various studies of the olive genetic diversity carried out in Tunisia based on molecular or biochemical markers have concerned mainly the different varieties grown in the North and Center of Tunisia. Some varieties have been initiated in the olive cultivated in the South that's why it is necessary to study the different varieties of olive trees grown mainly in Tunisian south-eastern. This work is aimed to study the genetic variability of intra-variety olive Chemlali variety collected from different regions of the Tunisian south-east, based on molecular markers ISSR and SSR. The detected polymorphism rate is 48.74% and 44.44% respectively. These two markers were found to be very effective in the study of the genetic diversity of these different samples tested. The examination of the two dendrograms obtained with two molecular markers shows the tested samples made genetic affinity groups regardless of their geographic origin.

Keywords: *Olea europaea*, genetic diversity, molecular markers, ISSR, SSR.

1. Introduction

Olive (*Olea europaea* L.) is one of the oldest cultivated plants and is an important oil producing crop in the Mediterranean Basin. Trees are extremely long-lived and tolerant to drought, salinity, and almost total neglect and have been reliable producers of food and oil for thousands of years. There are two forms: cultivated olive (var. *europaea*) is clonally propagated by cuttings and grafting, and wild olive or oleaster (var. *sylvestris*) is reproduced from seeds (Khadari et al. 2008). Archeological data suggest that cultivated olive was derived from oleasters through vegetative multiplication of individuals presenting interesting traits such as fruit size and oil content (Khadari et al. 2008). The olive tree is a species with a high degree of cross-pollination that leads to high levels of heterozygosity and genetic polymorphism (Angiolillo et al. 1999; Rallo et al. 2000). Over many centuries, most olive cultivars have been derived by random crosses or mutation. The majority of varieties are highly localized, but there are a few cultivars dispersed over widespread areas. The development of locally specific varietal populations was carried out by sexual reproduction, whereas other cultivars were established and maintained by vegetative means (Lumaret et al. 2004; Breton et al. 2006). Currently, there is much confusion in the identification of olive cultivars. Identification of cultivars is considered a major requirement because of the longevity of the crop and the need to improve efficiency in growing olives and extracting their oil. It is crucial that cultivars be identified using powerful techniques. In the past, olive cultivars were categorized by morphological traits, including tree, fruit, and leaf characteristics (Grati-Kamoun 1999), which are influenced by environmental factors.

Reliance on phenotypic characters has possibly led to great confusion and uncertainty about the current classification of olive varieties in many countries. Recently, molecular techniques based on DNA markers have been shown to provide powerful tools for genetic analysis of olive cultivars (Belaj et al. 2001; Busconi et al. 2003; Pafundo et al. 2005). Among these markers, microsatellites (SSR) have become the most popular in many species (Qin et al. 2012; Guo et al. 2012), and in olive cultivars they have a high potential for resolving issues of synonymies, homonymies, and misnamings (Taamalli et al. 2006; Rekik et al. 2008; Muzzalupo et al. 2009).

2. Materials and Methods

2.1. Plant material

Molecular analysis was performed Twelve Tunisian olive accessions all belonging to the variety 'Chemlali', olive trees were selected from different geographical regions of the South-East Tunisian.



Figure 1. Map of geographic origin of accessions used in this study

The 'Chemlali' accessions analyzed and their origins are reported in Table 1.

Tableau 1. Studied accessions of olive genotypes and their origin

Denomination accessions in targeted locations	Geographic region of production origin	Denomination cultivars in targeted locations
Chemlali Abyath	South East	Zerkine-Mareth-Gabès
Chemlali Douiret	South East	Tataouine
Chemlali Ontha	South East	Tataouine
Chemlali Djerba	South East	Djerba
Chemlali Zarzis	South East	Zarzis
Chemlali el Gorthab	South East	Tataouine
Chemlali Drina	South East	Tataouine
Chemlali –el Alea	South East	Zerkine-Mareth-Gabès
Chemlali Dakhla	South East	Médénine
Chemlali IRA Médénine	South East	Médénine
Chemlali Matmata	South East	Gabès
Chemlali Ben Guerdane	South East	Ben Guerdane

2.2. DNA extraction

Total genomic DNA was extracted from young leaves using the CTAB method according to the protocol of Rekik et al. (2008). DNA so prepared was further purified and resuspended in Tris-EDTA of pH 8.0. The quantity of the DNA was assessed by and the quality by 2 % agarose gel electrophoresis.

2.3. Primers used

The same DNA samples analyzed by ISSR (A1, A2 and A3) were also analyzed using SSR markers. SSR analysis was performed according to Boret and Branchard (2001) using 3 primers. These markers were selected for their high polymorphism in many olive cultivars (Lumaret et al. 2004) and in Tunisian cultivars (Grati-Kamoun et al. 2006).

2.4. ISSR and SSR fingerprinting

Each PCR reaction is performed in a total volume of 25 µl, containing 2 µl of buffer (5x Colorless GoTaq Flexi Buffer) PCR, 2 µl (100 ng) of genomic DNA, 25 mM MgCl₂, and 500U of Taq polymerase. We varied the concentration of 2 mM to 4 mM NTP and the amount of primer 2 to 4 µl and for a stock solution of 10 µM. Amplification was carried out using a thermocycler (DNA-Amp Master cycler gradient eppendorf). The initial denaturation was performed for 5 min at 94 ° C, followed by 45 cycles (1 min at 94 ° C, 45 sec at T_m (45°C) specific primer and 1 min 72°C) and followed by a final extension of 7 min at 72 ° C. For the SSR primer, the program: 95°C for 5 min for denaturation, followed by 40 cycles (30 sec at 94 ° C, 45 sec at 60 ° C and 1 min at 72 ° C) and followed by a final extension of 7 min at 72 ° C (Rekik 2008).

2.4. Data analysis

All analyses were carried out using the program MVSP version 3 (Multi Variate Statistical Package). Polymorphic bands were recorded into a data matrix of presence (1) and absence (0) of bands. This data matrix obtained by scoring the ISSR and SSR profiles both individually as well as collectively were subjected to the calculation of a similarity matrix (S) using Jaccard's coefficients (Jaccard, 1908). Several parameters were considered in this study: The polymorphic bands percentage (PBP), the resolving power (RP) was calculated for each primer according to Backus and Gilbert (1968): $RP = \sum I_b$; $I_b = 1 - (2|0.5 - p|)$; where p: is the proportion of species containing I bands. Genetic relationships among cultivars are represented by a dendrogram based on the distances calculated from the Jaccard index. The dendrogram was constructed using the Unweight Pair Group Method using an Arithmetic average (UPGMA).

3. Results

3.1. ISSR analysis

The analyses of the amplification profiles obtained for each primer are summarized in Table 2.

Tableau 2. Polymorphic bands percentage (PBP), the resolving power (RP) and Average informative bands (AVIb).

	Bands ISSR		% polymorphism	Resolving of power (RP)	Average informative bands (AvIb)	Sizes of bands (PB)
	Total	Polymorphic				
ISSR 1	12	5	41.66%	3.58	0.23	160-550
ISSR 2	8	4	50%	2.89	0.22	230-900
ISSR 3	11	6	54.55%	3.56	0.23	150-800
Total	31	15	M=48.74%	M=3.34	M=0.22	150-900

Three primers were able to generate 31 bands (15 polymorphic), with 48.74% polymorphism and an average of 5 polymorphic bands generated per primer. The size of bands varies between 150 and 900 bp. The percentage of polymorphism ranged from 41.66% to 54.55%. The primer ISSR1 revealed the lowest percentage (41.66%) while the primer ISSR3 detected the highest percentage (54.55%).

The three polymorphic primers also showed variations in the average of informative bands (AvIb) and the resolving power (RP). The average of AvIb ranged from 0.22 to 0.23. The primer ISSR2 showed the lowest value of AVIb (0.22) and also the lowest value of Rp (2.89) so that the primer ISSR3 has the highest value of Rp (3.56).

3.2. Patterns of genetic diversity based on ISSR

Each polymorphic band was recorded into a data matrix of presence (1) and absence (0) of band. This data matrix was converted into a Similarity matrix (S) using Jaccard coefficient (Jaccard, 1908), and the establishment of dendrogram (Figure 2) by unweighted pair group method using an arithmetic average cluster analysis. The similarity degree between the 12 olive accessions based on ISSR markers ranged from 0 to 0.882. This result shows the high degree of intervarietal genetic diversity at 'Chemlali' variety. The smallest genetic distance value of 0.2 was observed between 'Chemlali Djerba' and 'Chemlali Douiret' as well as 'Chemlali Dakhla' and 'Chemlali-el Alea'. The maximum genetic distance ($Dg > 0.8$) was found between different combinations:

- 'Chemlali Matmata' and 'Chemlali Abyath'; 'Chemlali Ben Guerdane' and 'Chemlali Zarzis'; 'Chemlali IRA Médenine' and 'Chemlali Abyath' ($dg = 0.867$).
- 'Chemlali Ben Guerdane' and 'Chemlali el Gorthab' ($dg = 0.882$).
- 'Chemlali el Gorthab' and 'Chemlali Zarzis' ($dg = 0.875$).
- 'Chemlali Ben Guerdane' and 'Chemlali Douiret'; 'Chemlali IRA Médenine' with 'Chemlali Ontha' and 'Chemlali el Gorthab' ($dg = 0.857$).

It should be noted that 'Chemlali IRA Médenine' has the largest genetic distances with relatively all accessions.

The (S) matrix of ISSR markers allowed the distinction of the 12 Tunisian olive accessions and was used to generate a dendrogram (Figure 2). The dendrogram depicts the pattern of relationships between the studied accessions. There is no clear clustering of accessions in relation with their growing area. Nevertheless, four clusters can be defined.

- * Group1 formed by 10 accessions «Chemlali Abyath, Chemlali el Alea, Chemlali Douiret, Chemlali Ontha, Chemlali Drina, Chemlali Djerba, Chemlali Zarzis, Chemlali Dakhla» from Mareth-Gabès, Tataouine, Djerba, Zarzis and Médenine respectively.
- * Group2 contains only «Chemlali el Gordab» from Tataouine.
- * Group3 includes two accessions «Chemlali Matmata» and «Chemlali Ben Guerdane».
- * Group4 contains only «Chemlali IRA Medenine» characterized by high genetic distance with the rest of studied accessions (0.687 and 0.846).

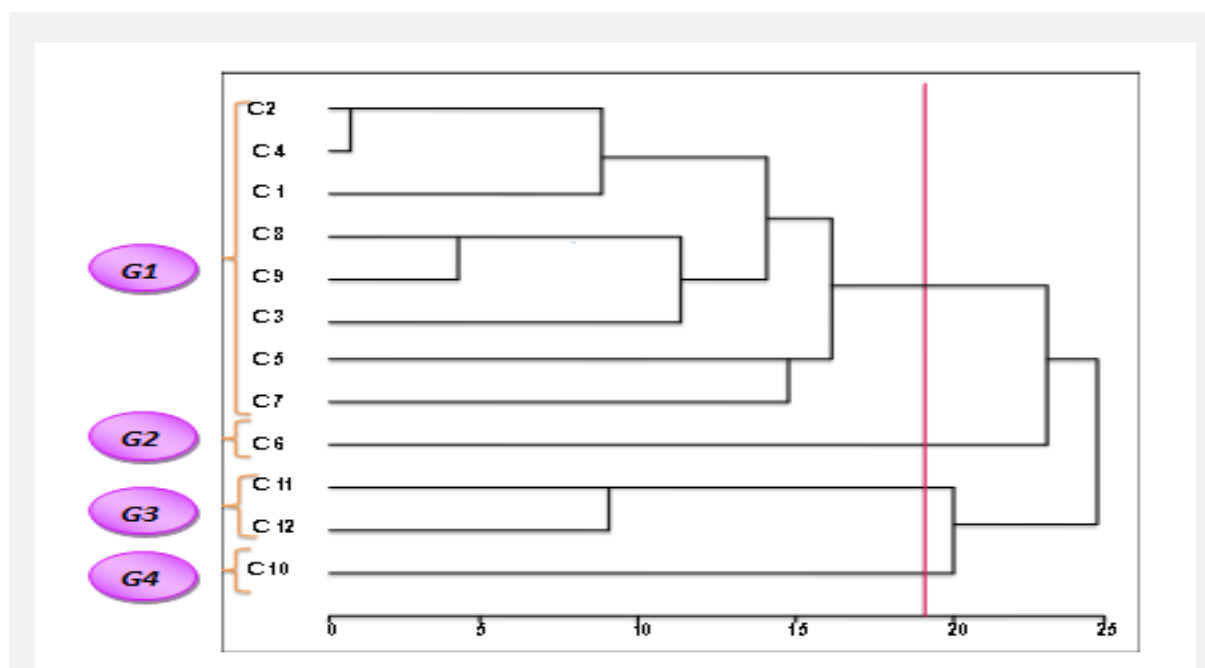


Figure 2. Dendrogram of 12 olive cultivars generated by UPGMA cluster analysis of the genetic similarity values using ISSR data.

3.3. SSR analysis

The analyses of the amplification profiles obtained for each primer are summarized in Table 3.

Table 3. Polymorphic bands percentage (PBP), the resolving power (RP) and Average informative bands (AVIb).

	SSR Bands		% polymorphism	Resolving power (RP)	Average informative bands (AvIb)	Sizes of bands (PB)
	Total	Polymorphic				
UDO.004	1	0	0%	0.91	0.3	150
OUB.2	3	2	66.66%	1.57	0.31	180-700
GAPU.47	3	2	66.66%	1.66	0.83	150-210
Total	7	4	M=44.44%	M=1.38	M=0.48	150-700

Seven different bands were obtained using 3 primers from which 4 were polymorphic with 44.44% polymorphism rate. The molecular size of obtained bands ranged from 150 to 700 bp. The primers OUB.2 and GAPU.47 showed 2 polymorphic bands while UDO.004 showed a monomorphic pattern. The average AvIb ranged from 0.3 to 0.83 while Rp ranged from 0.91 to 1.66 (Table3). The primer UDO.004 showed the lowest AvIb value (0.3) and the lowest Rp (0.91), while the highest AvIb value (0.83) and the highest Rp (1.66) are shown at the primer GAPU.47.

The genetic similarity values based on Jaccard's coefficient ranged from 0 between 'Chemlali Ben Guerdane' and 'Chemlali el Gorthab' ; 'Chemlali el Alea' , 'Chemlali IRA Médenine' and 'Chemlali Matmata' to à 1 between 'Chemlali Dakhla' and 'Chemlali Djerba'.

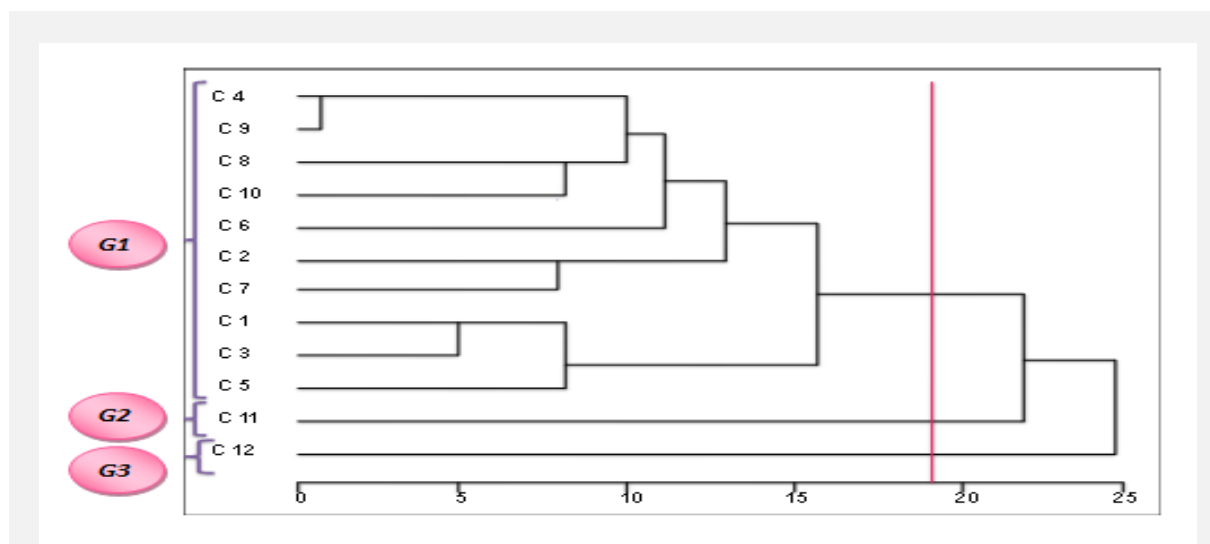


Figure 3. Dendrogram of 12 olive cultivars generated by UPGMA cluster analysis of the genetic similarity values using SSR data

Affinities among cultivars and accessions are shown in the dendrogram (Figure 3), forming three groups. The first group represented by 10 accessions «Chemlali Abyath, Chemlali Douiret, Chemlali Ontha, Chemlali Djerba, Chemlali Zarzis, Chemlali el Gorthab, Chemlali Drina, Chemlali el Alea, Chemlali Dakhla, Chemlali IRA Médenine». The similarity percentage between these accessions is very important (100%). The second group included only the cultivar «Chemlali Matmata». The third main group included the cultivar «Chemlali Ben Guerdane». This extra group characterized by the most important genetic distances over than 0.75 with other cultivars. The geographical origin of cultivars has no influence on the groupings obtained. The studied cultivars constitute different groups have a genetic affinities regardless of their geographic origin.

3.4. Combined analysis

The combined data obtained by ISSR and SSR (29 markers) was used for the construction of a dendrogram using Jaccard's coefficient (Figure 4). The highest value of genetic similarity (0.750) was

between Chemlali Djerba (C4) and Chemlali Douiret (C2). The lowest similarity (0.118) was recorded between Chemlali Ben Guerdane (C12) and Chemlali el Gorthab (C6).

The UPGMA cluster analysis of the cultivars based on combined RAPD and ISSR data gave 4 clusters; the first main cluster contains 10 cultivars «Chemlali Abyath (C1), Chemlali Douiret (C2), Chemlali Ontha (C3), Chemlali Djerba (C4), Chemlali Zarzis (C5), Chemlali Drina (C7), Chemlali el Alea (C8), Chemlali Dakhla (C9)». This group has almost the highest similarity coefficient (0.750). The second cluster includes only «Chemlali el Gordab». This group is characterized by a very significant genetic distances that varies between 0,583 and 0,882. The third group includes « Chemlali Matmata » (C11) and « Chemlali Ben Guerdane» (C12) with an important similarity coefficient (0.571).

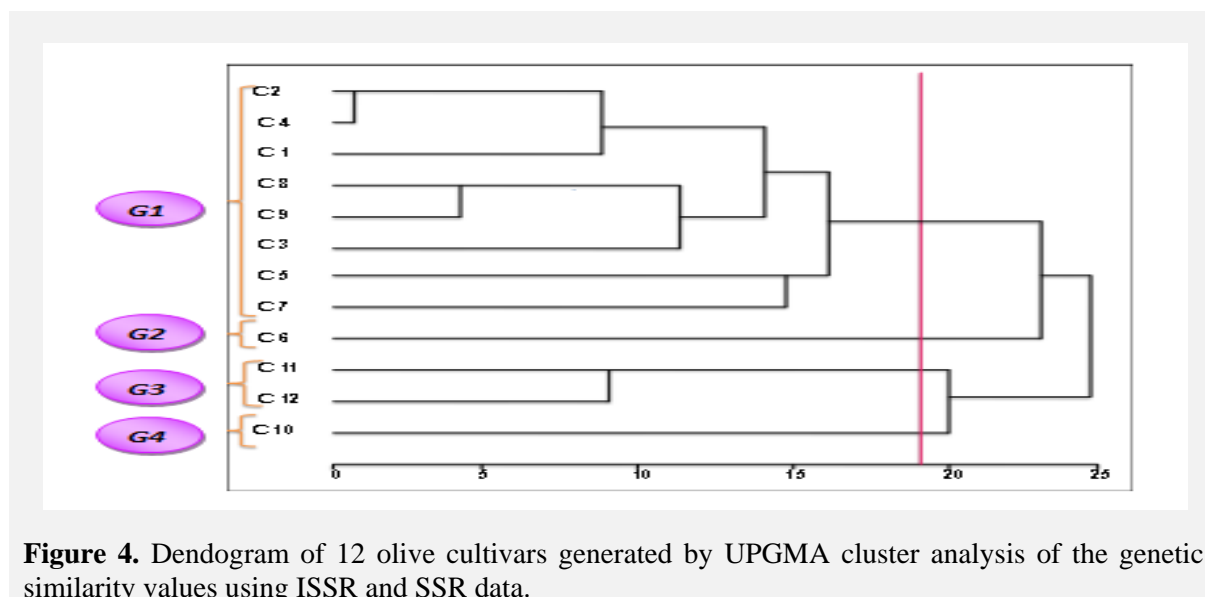


Figure 4. Dendrogram of 12 olive cultivars generated by UPGMA cluster analysis of the genetic similarity values using ISSR and SSR data.

4. Discussion

Recently, the genetic resources of olive trees from different regions were described using PCR-based markers. The studies of polymorphisms, through the ISSR and SSR analysis, proved to be very useful both for olive varietal identification and for intra-varietal ones. Tunisian cultivars were underrepresented. Some research projects were interested to study the genetic variability inter varietal for all variety present in different Tunisian regions, exactly the varieties grown in the North and Center. However, the study of intra-varietal polymorphisms is important since they may have traits that although not considered important in the past, might be important to meet the challenges of modern olive growing (i.e., low vigor, resistance to low temperatures, salinity tolerance, etc.).

In this study, two molecular markers were used to study the genetic relatedness and intra varietal genetic diversity of different accessions belonging to the Chemlali variety grown in different regions in the South East of Tunisia. Differences in the rate of polymorphism yielded by the primers could be explained by differences in the primer sets used, the germplasm analyzed, and in the criteria (faint band/clear band) used for marker selection.

The high polymorphism generated by these markers (ISSR/SSR) indicates that the olive tree is a highly polymorphic species. The high diversity found between olive cultivars is probably due to a diverse germplasmic origin that resulted in a predominant allogamous species with a high degree of out crossing (Zohary and Spiegel-Roy 1975; Bartolini et al. 1998). Additionally, the complexity of the olive genome (Zohary and Spiegel-Roy, 1975) resulted in new cultivars found throughout the Mediterranean amid low breeding pressures (Besnard et al. 2001a; Contento et al. 2002; Martins-Lopes et al. 2007).

There is no clear structure with the geographical cultivation of accessions that has been observed in this study. Nevertheless, with the UPGMA method, some evidence of relationships according to their geographical origin and/or diffusion was observed. For instance, SSR markers grouped 'Chemlali Douiret(C2) , Chemlali Ontha(C3), Chemlali el Gorthab (C6), Chemlali Drina(C7), which are mainly

cultivated in the region of Tataouine, into one cluster, whereas Chemlali Djerba(C4), Chemlali Zarzis(C5), Chemlali el Alea-Mareth(C8) which are cultivated in different South Eastern Tunisian regions respectively Djerba, Zarzis et Mareth (Gabes) were grouped in the same group. These results were in agreement with several studies (Besnard et al. 2001a; Caraffa et al, 2002; Khadari et al. 2003; Martins-Lopes et al. 2007) that found no clear correlation between olive genotypes and the geographical origin. However, a good correlation between the banding patterns of olive accessions and their geographical origin was obtained in other studies using RAPD markers conducted in Jordan (Hassawi and Hadeib, 2004) and the Mediterranean (Belaj et al. 2001, 2003a, 2003b, 2004; Sanz-Cortes et al. 2001), which agreed with both hypotheses of autochthonal origin as well as the limited diffusion of olive cultivars from their zones of cultivation (Belaj 2001; Besnard et al. 2001).

The result obtained in our study is consistent with other results of genetic polymorphism studies in different olive cultivars using molecular markers ISSR for example in Syria and other Mediterranean regions (Besnard et al., 2001, Belaj et al., 2003a), in Maroc and the Western Mediterranean countries (Essadki et al., 2006), in Egypt (Hegazi et al., 2012), in Iran (Noormohammadi et al., 2012), in Portugal (Martins -Lopes et al., 2007), in Italy (Ganino et al. 2007) and in Spain (Belaj al. 2004 and Gomes et al. 2009). Gemas et al. (2003) showed that ISSR is a very efficient and reliable technique to study the variability of the variety intravarietal Portuguese 'Galega'.

Genetic polymorphism rate obtained is moderately high (44.44%) but is still low compared to that obtained by Pasqualone et al., (2001) which is equal to 97.9% in a study of the identification and genetic diversity 30 cultivars olive in Southern Italian.

Recently, it was reported that the analysis of DNA sequences by microsatellite improves the efficiency of the classification and identification of Tunisian olive cultivars (Rekik et al, 2008; Ben-Ayed and al.2014).

SSR microsatellite markers are high discriminative power, and which are particularly interesting to analyze the structure of genetic diversity both at intraspecific level and between species. According to several authors, these SSR markers provide a quick way to generate generic cards and to perform analyzes populations.

The high discrimination capacity of these markers, as determined by unique RAPD/ISSR markers and unique banding patterns generated by different primer combinations, will be useful for management of a germplasm bank, thus providing the nursery industry with a useful tool for certification of their plant material.

In addition, Belhadj et al. (2012) developed a basic collection of olive cultivars based on molecular markers and agronomic traits only 12 Greeks among the 361 cultivars memberships, using the largest collection of world olive germplasm (AIPA Centre Alameda del Obispo, Cordoba, Spain) with two SSR and ISSR.

5. Conclusion

This study confirmed that both ISSR and SSR markers are powerful tools for olive varietal identification, enabling an accurate characterization of all cultivars examined. Such information may prove useful in the selection of optimal varieties and help promote continued progress in olive breeding strategies.

Molecular markers are still the most important and most efficient markers to study the variability and genetic relationships between different plant species.

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