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ARTICLE

Study of intracultivar variation among main Iranian olive cultivars using SSR markers

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ABSTRACT Three Iranian olive cultivars of Geloleh, Shengeh and Rowghani with commercial interest are distributed in 3 provinces in North of Iran. Fifty one accessions belonging to these 3 olive cultivars were screened by 13 microsatellite markers revealing high genetic variability both within and between cultivars. In total, 54 alleles were detected with a mean number of 4.2 alleles per locus. Six unique allelic patterns were observed. Heterozygosity ranged from 0.00 to 1.00 while the mean number of polymorphic information content (PIC) was 0.51. The existence of homonyms, synonyms or mislabeling as well as intracultivar polymorphism was showed by allele differences between olive accessions studied. The phenogram obtained by UPGMA clustering showed variability among as well as between cultivars.
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Olive (*Olea europaea* L.) is a subtropical species typical of the Mediterranean basin and it most likely originated from the Near-East during the Chalcolithic period (5700-5500 years B.P; Zohary and Hopf 1994).

Archaeological findings revealed that olive cultivation in Iran dates back to 2000 years ago (Sadeghi 1992). At present olive cultivars are cultivated mainly in the North of Iran, which is characterized by Mediterranean climatic condition. In the last ten years, olive plantation has grown in Iran and currently, 95000 hectares of olive orchards produce about 6500 tons of olive oil annually. Although a large number of olive accessions are growing in Iran, there have been few reports on morphological, cytogenetical and molecular characteristics of these accessions (Samaee et al. 2003; Hosseini-Mazinani et al. 2004; Noormohammadi et al. 2007; Omrani-Sabbaghi et al. 2007; Sheidai et al. 2007).

Discrimination of varieties based on morphology evaluation is limited by effect of environmental conditions, the need for extensive observations of mature plants and requirement of well-trained staff (Belaj et al. 2001). Therefore, more comprehensive studies using reliable markers are needed to gain a better understanding of the levels of genetic diversity in olive cultivars, which may be of use in the cultivars identification.

Different molecular techniques such as isozymes (Trujillo et al. 1995) Random Amplified Polymorphic DNA (RAPDs; Wiesman et al. 1998; Mekuria et al. 1999; Belaj et al. 2001; Besnard et al. 2001) Amplified Fragment Length Polymorphism (AFLP; Angiolillo et al. 1999) and Simple Sequence Repeat (SSR; Rallo et al. 2000; Sefc et al. 2000; Bandlej et al. 2002) have been increasingly used to characterize the olive cultivars.

Microsatellites are useful because they are abundant, uniformly distributed, highly polymorphic, codominant and amenable to automation (Morgante and Olivieri 1993; Powell et al. 1996; Rafalski et al. 1996). Microsatellite markers have been proven to be very suitable markers for fingerprinting and revealing the genetic diversity in olive cultivars (Cipriani et al. 2002; De la Rosa et al. 2002; Khadari et al. 2003; Belaj et al. 2004; Diaz et al. 2006).

The present study tries to characterize three important Iranian olive cultivars of Geloleh, Shengeh and Rowghani which cultivated in three provinces of Gilan, Zanjan and Ghazvin reporting the available genetic polymorphism both within and between cultivars.

Materials and Methods

Plant materials and DNA extraction

Fifty one accessions belonging to 3 Iranian olive cultivars (Geloleh, Shengeh and Rowghani) were used in the molecular study (Table 1). Naming of cultivars have been based on common morphological traits ('Geloleh' = round fruit) and practical utility (Rowghani = oily cultivar). These cultivars were identified on the basis of morphological characteristics (Sadeghi 1992). Trees were sampled from seven different locations randomly selected in Gilan, Zanjan and Ghazvin

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Table 1. Cultivar accession included in the study with tree code, source of material, geographical diffusion and use of fruit, ordering number (Nr).

Nr	Cultivar accession	Tree **	Source of materials	Geographical diffusion	Use of fruits*
1	GELOLEH	*1136, 1127, 1147*, 1139	Harzevil	Gilan	O
2	GELOLEH	*501, 502, 1158, 389*, 391, 387, 393	Ettka Garden	Gilan	O
3	GELOLEH	*497*	BahramAbad	Ghazvin	O
4	GELOLEH	*1117, 1122*, 1118	Manjil	Gilan	O
5	GELOLEH	*316*, 313, 317	Motahari Garden	Zanjan	O
6	ROWGHANI	*1048, 1050*, 1049	Research Garden	Gilan	O&T
7	ROWGHANI	*209*, 208	BahramAbad	Ghazvin	O&T
8	ROWGHANI	*119, 112, 122*, 123	Vakhman	Ghazvin	O&T
9	ROWGHANI	*367, 376*	Ettka Garden	Gilan	O&T
10	ROWGHANI	332, 331, 321	Motahari Garden	Zanjan	O&T
11	SHENGEH	*363*	Ettka Garden	Gilan	O&T
12	SHENGEH	*1089, 1097, 1090*, 1085 *1086, 1096, 1098*,1119 *263, 259, 1094*, 1103 *1083,1102, 1104, 1082*	Research Garden	Gilan	O&T
13	SHENGEH	*1115*, 1116	Manjil	Gilan	O&T

* O (oil), T (Table olive) and O&T (Oil and Table olive)
** Italic shows that accession which studied by 13 microsattelite markers.

provinces in North of Iran. In order to reduce the number of replication of each genotype, a primary screening was performed on 51 olive accessions by using five high polymorphic SSR markers (ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA16, ssrOeUA-DCA18 and UDO99-043). Representative of those accessions which showed the same allelic profiles involved in further studies while other samples eliminated. Therefore the number of genotypes reduced into 32 for more studies.

Total genomic DNA was extracted from fresh leaves using the CTAB method (Murry and Tompson 1980) with modification described by De la Rosa and coworker (2002).

Microsatellite assay

Thirteen microsatellite markers of ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA11, ssrOeUA-DCA15, ssrOeUA-DCA16, ssrOeUA-DCA18 (Sefc et al. 2000) UDO99-011, UDO99-019, UDO99-043, UDO99-024 (Cipriani et al. 2002) and GAPU59, GAPU71B, GAPU101 (Carriero et al. 2002) were used for studying genetic polymorphism in 32 selected olive accessions. Amplification of microsatellites was performed in PCR reactions in a total volume 20µl, containing 2 ng genomic DNA, 1X supplied PCR buffer (Biotools, Spain) 200µM of each dNTP (Roche), 0.25 unit of Taq DNA polymerase (Biotools, Spain) and 0.2 µM of forward (fluorescently labeled) and reverse primers. The amplifications were carried out on a thermal cycler (Perkin-Elmer-9600) programmed with a denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 20 s, the annealing temperature 50ºC for 30 s and 72ºC for 30 s and final extension at 72ºC for 7 min. Finally, the analysis was carried out on an automatic capillary sequencer ABI 3130 Genetic Analyzer (Applied Biosystems/HITACHI) using fluorescent dyes, and fragment sizes were determined using internal standards.

Data analysis

The peaks present in genotypes were recorded for each of the thirteen microsatellite loci using Genotyper 3.7 computer software (Applied Biosystems). Observed heterozygosity (H_0) was obtained as the ratio of the heterozygous individuals to the total number of genotypes per locus, expected heterozygosity (H_E) (Nei 1987), Polymorphic information content (PIC) (Botstein et al. 1980) and null alleles frequency (r) (Brookfield 1996) were also calculated.

Genetic distances between all pairwise combinations of the accessions were calculated using different similarity measures including Dice and Jaccard's coefficients. Grouping of the genotypes was determined by using different clustering methods including UPGMA (unweighted paired group mean using average), Single linkage and WARD (minimum spherical cluster) methods as well as ordination based on principal coordinate analysis (PCO) (Ingrouille 1986; Chatfield and Collin 1995). Cophenetic correlation was determined for different clustering methods. NTSYS-pc version 2.02 (Rohlf 1998), Cervus version 2.0 (Marshall et al. 1998) softwares were used for statistical analyses.

Results

SSR diversity in three Iranian olive cultivars

The initial screening with five primers (ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA16, ssrOeUA-DCA18 and

Table 2. Allele size, number of alleles, unique alleles and heterozygosity indices for studied cultivars in 13 SSR primers.(H_O) observed heterozygosity,(H_E) expected heterozygosity, (PIC) Polymorphic Information Content.

Locus	Size range	No. alleles	No. unique alleles	No. unique allele patterns	H_O	H_E	PIC	Probability of null alleles
ssrOeUA-DCA3	229/253	6	1	2	0.93	0.80	0.75	-0.0917
ssrOeUA-DCA9	170/207	8	2	2	0.84	0.78	0.74	-0.0395
ssrOeUA-DCA16	122/178	4	0	1	0.61	0.60	0.52	-0.0134
ssrOeUA-DCA18	162/180	4	0	1	0.46	0.43	0.38	-0.0363
UDO99-043	170/216	6	2	5	0.68	0.65	0.60	-0.0456
ssrOeUA-DCA11	142/178	4	0	0	1.00	0.74	0.68	-0.1577
ssrOeUA-DCA15	243/263	3	0	0	0.10	0.46	0.41	+0.6274
UDO99-011	114/130	5	1	1	0.97	0.68	0.62	-.02031
UDO99-019	129	1	0	0	0.000	0.000	0.000	0.000
UDO99-024	166/189	3	0	0	0.56	0.55	0.46	-0.0109
GAPU59	206/216	2	0	0	0.40	0.33	0.27	-0.1117
GAPU71B	121/135	3	0	1	0.65	0.63	0.55	-0.0089
GAPU101	191/217	5	0	0	0.75	0.72	0.67	-0.0205
Total*	-	54	6	13	0.61	0.57	0.51	-

*Numbers of H_O, H_E, PIC are mean values.

UDO99-043) revealed the presence of the same allelic profiles among different trees (replications) of the same cultivars. So, among 51 accessions, 19 trees could be considered as duplications of the plant materials and they were excluded from further analysis. All used thirteen microsatellite markers (except UDO99-019) were polymorphic, revealing the presence of 54 alleles in all 3 cultivars analyzed. The number of alleles in each locus varied from one (UD99-O19) to eight (DCA9) with an average number of 4.2 alleles per locus (Table 2).

Under "Hardy Weinberg" equilibrium, the observed heterozygosity varied from 0.000 in monomorphic locus of UDO99-019 to 1.00 in DCA11 locus with an average value of 0.61. The observed Heterozygosity at all loci was higher than expected heterozygosity except DCA15 locus with high frequency of null alleles (Table 2). The study of allelic polymorphism obtained in the present work allows discrimination of 82% of the olive accessions analyzed by producing unique genotype profiles (Table 2). The UDO43 and DCA9 loci revealed the highest number of unique alleles (2) while some loci did not show any unique alleles (for example DCA16, DCA18 and GAPU59; Table 2). Calculated PIC values ranged from 0.000 to 0.75 in 13 loci with average of 0.51. DCA3 locus showed highest PIC value while DCA19 showed lowest PIC value.

Genetic relationships

Different similarity coefficients determined among the cultivars studied, showed the highest value of similarity (r = 1.0) between three pairs of accessions such as Shengeh-263 and Shengeh-363 and also Rowghani-1050 and Rowghani-209.

Similarity matrices obtained from cultivars studied were employed for elucidating the genetic relationships among olive cultivars by different clustering methods, including UPGMA, single linkage and WARD (Fig 1). The cophenetic coefficients determined for different clustering methods revealed the highest value for UPGMA (r = 0.81) indicating a good fit of the original data to the clustering dendrogram.

Discussion

The high level of variation in average number of alleles per locus (4.2) was observed in olive cultivars. The same result has also been reported by other workers (Rallo et al. 2000; Carriero et al. 2002; De la Rosa et al. 2002; Khadari et al. 2003; Belaj et al. 2004). They have reported average values of 7.5, 6.4, 5.7, 7.4 and 5.2 alleles per locus related to different number of loci in their studies. Variation reported in the number of alleles in olive cultivars by different workers may be related to variation in the loci studied as well as the number of genotypes and their localities (Lopes et al. 2004).

The heterozygosity deficiency has been found in DCA15 locus to be non-significant based on χ^2 test ($p < 0.001$) might be due to the presence of null alleles. A possible explanation of the deficit in amount of observed heterozygosity may be the occurrence of null alleles at this locus (Ishibashi et al. 1996). The presence of null alleles is a consequence of sequence polymorphisms in the flanking regions of the locus due to point mutations or insertion/deletions (Jones et al. 2003). Higher expected values of heterozygosity were also reported by Lopes et al. (2004), which were related to the occurrence of the null alleles.

The study of allelic polymorphism discriminated 82% of the olive accessions analyzed by producing unique genotype profiles. Therefore, the olive cultivars studied can be well discriminated by using the above said indices due to the

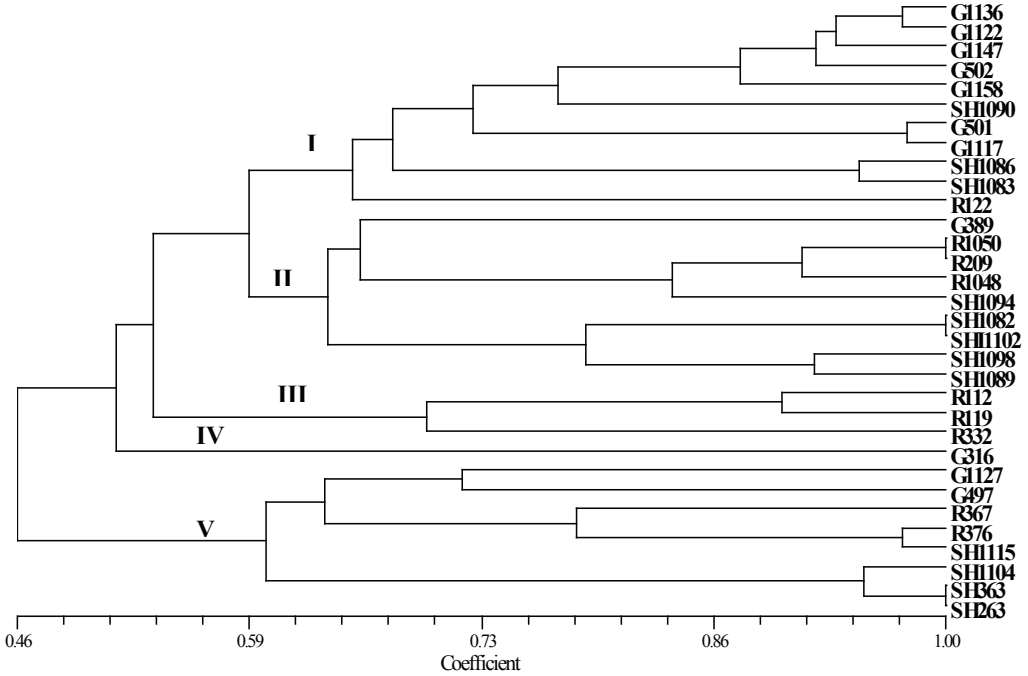

Figure 1. UPGMA dendrogram of Iranian olive cultivars based on Dice's coefficient. Abbreviations: G (GELOLEH), SH (SHENGEH), R (ROWGHANI).

presence of a high amount of genetic variability among these three cultivars.

Calculated PIC values with average of 0.51 are close to the values observed by Bandelj et al. (2004). It has been suggested that PIC values > 0.5 are informative markers while loci with PIC values > 0.7 are suitable for genetic mapping (Bandelj et al. 2004). Therefore in the present study eight loci may be considered informative while four loci (DCA3, DCA9, UDO11 and GAPU101) may be used in genetic mapping of the cultivars studied (Table 2).

By using UPGMA method, five major groups/clusters were identified in cluster analysis. The first major cluster is mainly comprised of Geloleh accessions, three accessions of 'Shengeh' and one accession of 'Rowghani'. The second major cluster consists of two subclusters. Accessions of Geloleh-389, Rowghani-1050, 209, 1048 and 'Shengeh-1094' formed the first subcluster while second subcluster consists of 4 'Shengeh' accessions (Fig. 1). The third major cluster is comprised of accessions of 'Rowghani-112', 'Rowghani-119' and 'Rowghani-332', while one accession of 'Geloleh-316' formed the forth cluster. The fifth major cluster contains two subclusters, some accessions of 'Geloleh', 'Shengeh' and 'Rowghani' form the first subcluster while 3 accessions of 'Shengeh' are placed in the second subcluster. Two accessions 'Rowghani- 376' and 'Shengeh-1115' differed only in one locus (data not shown). These accessions with similar genotypes and different denomination are suggested to be synonymous or mislabeled.

In the present work, all five clusters included accessions are from three Northern provinces of Iran (Gilan, Zanjan and Ghazvin) without geographical separation. This may show material exchanges occurred between these provinces by local gardeners. Meanwhile, it is suggested that olive cultivars might be renamed or misnamed in new locality which were cultivated.

The stability of the groups was also confirmed by partitioning the variants of data sets using PCO. Generally, PCO plot supported the clustering results obtained (Fig. 2).

Generally accessions of 3 olive cultivars studied are distributed in different clusters possibility due to their genetic variability or misnaming. These cultivars are very common in the North of Iran and their denominations are complicated because of morphological similarity. Homonymy is one of the problems in Iranian olive germplasm as much as occur in Mediterranean's cultivars. The main reason may be come from denominating cultivars based on common morphological traits, particularly of fruit or practical use of cultivar like Geloleh which means round fruit and Rowghani because of producing olive oil. Therefore generic names of Iranian olive cultivars specially these 3 main olive cultivar include different genotypes and may be considered homonyms or mislabeling. Discrimination of homonymous cases in olive germplasm has also been reported by using SSRs and other molecular markers by other workers (Belaj et al. 2001; Khadari et al. 2003). Intracultivar variations have also been reported in 'Shengeh' by using morphological characters (Hosseini-Mazinani et al.

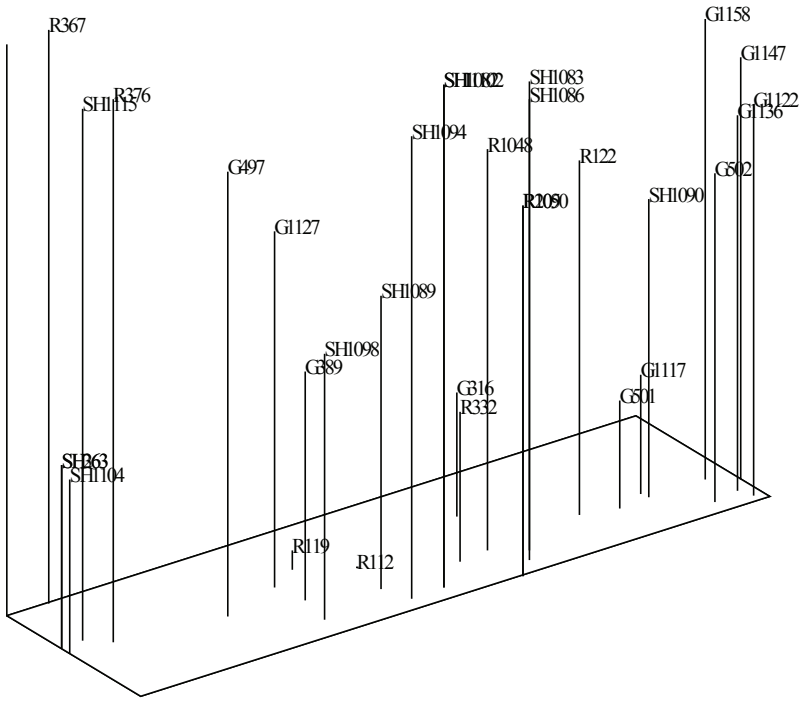

Figure 2. PCO ordination of the studied olive cultivars based on SSR markers.

2004). Omrani-Sabbaghi et al. (2007) also has been reported the existence of intracultivar variation in some Iranian olive cultivars.

In conclusion, this study shows that SSR markers are a powerful tool for cultivar identification and analysis of genetic structure. High information content of the markers enables characterization and discrimination of olive cultivars only with combination a few loci. The most of microsatellite markers used in this study were very informative in the 3 Iranian olive cultivars analyzed. Genetic characterization of a larger number of genotypes will help in identifying potentially synonymous and homonymous cultivars, which will be very useful in germplasm management.

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