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Molecular characterization of olive cultivars grown in Iraq using amplified fragment length polymorphism and simple sequence repeat markers

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In this study, genetic relationships among olive cultivars grown in Iraq were investigated by means of two DNA molecular marker classes: amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR). Total genomic DNA was isolated from young leaves of the selected olive cultivars. Namely 'Arbqween', 'Baashiqi', 'Dahkan', 'Labeeb', 'Khdhier', 'Manzenllo', 'Nepali', 'Qaysi', 'Shami' and 'Sorani'. AFLP-PCR technology was performed using nine selective primer combinations, yielding 250 bands, 145 (58%) of which were polymorphic while 10 SSR primer pairs generated 85 polymorphic bands on a total of 283 fragments (30%). According to AFLP markers, the unweighted pair-group method with an arithmetic average (UPGMA) ordered olive cultivars into two main clusters irrespective of their origin at similarity level of 0.48. Similarly, UPGMA based on 85 polymorphic SSR loci ordered the olive cultivars into two main clusters at similarity level of 0.54. The combination of AFLP and SSR markers was then performed, thus generating two groups with 50% similarity. Genetic similarity index estimated by both DNA markers used in this study proved three cultivars to be very close to each other ('Qaysi', 'Baashiqi' and 'Dahkan') as they always clustered together in the resulting dendrograma. The scatter diagrams of the first two (PC1 and PC2) of the Principal Component Analysis (PCA) confirmed the results obtained by the two marker classes. The results of this research confirmed AFLP and SSR to be useful tools in genetic relationships among olive cultivars, in creating a molecular database for Iraqi olive cultivars, in breeding strategies and in correct cultivar identification.

Key words: *Olea europaea*, genetic diversity, amplified fragment length polymorphism (AFLP), sequence repeat (SSR) markers.

INTRODUCTION

Olive *(Olea europaea* L.) is one of the most ancient cultivated fruit tree species in the Mediterranean basin. It is a predominant allogamous species showing a high degree of out crossing, which leads to considerable levels of heterozygosity and DNA polymorphism among individuals (Angiolillo et al., 1999; Rallo et al., 2000). The wide genetic patrimony and the large number of synonyms and homonyms in olive require precise

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methods for discrimination of cultivar identification and classification. Different techniques have been used to evaluate olive diversity. Morphological, agronomical or biochemical characterization have been adopted for variability evaluation (Barone et al., 1994; Barranco et al., 2000; Ouazzani et al., 1993; Trujillo et al., 1995). The introduction of DNA markers provides a good discriminatory system, independent from environmental conditions. AFLP markers have been widely used in genetic analysis because a substantial number of polymorphic bands can be generated with only a few primer combinations, confirming their importance in studies of genetic diversity among cultivated olives, wild forms and related species (Angiolillo et al., 1999; Baldoni et al., 2000; Belaj et al., 2003; Carolyn et al., 2005) and in genetic map construction (Rosa et al., 2003; Amal et al., 2010; Khadari et al., 2010). Nowadays, Simple Sequence Repeat (SSR) has been proven to be very suitable markers for cultivar identification and identity typing in olive as they are transferable, highly polymorphic and co-dominant markers (Rallo et al., 2000; Carriero et al., 2002; Cipriani et al., 2002; Khadari et al., 2008; Charafi et al., 2009).

The objectives of this study were: 1) to identify DNA fingerprints of 10 olive cultivars grown in Iraq by AFLP and SSR markers; 2) to verify their efficiency in discriminating among olive genotypes and 3) to assess the amount of genetic diversity presents in all 10 olive cvs.

MATERIALS AND METHODS

DNA extraction

A total of 10 well-defined reference olive cultivars grown in Al-Latifia Research Station, Ministry of Agriculture of Iraq, were considered in this study: ‘Arbqween’, ‘Baashiqi’, ‘Dahkan’, ‘Labeeb’, ‘Khdhier’, ‘Manzenllo’, ‘Nepali’, ‘Qaysi’, ‘Shami’ and ‘Sorani’. Total genomic DNA was extracted from young and healthy leaves according to Benito et al. (1993) with some modifications. After purification, the resultant DNA was quantified and its integrity was determined after 1% agarose gel electrophoresis as described by Samboork et al. (1989).

AFLP analysis

AFLP analysis was carried out as proposed by Vos et al. (1995), with a few modifications. A quantity of 250 ng of genomic DNA from each cultivar was digested at 37°C for 3 h with 10 U each of two restriction enzymes, *Tru* 9I (recognition site 5-T↓TAA3-) and *Pst*I (recognition site: 5-CTGCA↓G 3-), in 20 µL final volume of reaction mixture containing 1x one-for-all buffer.

After the digestion, specific adapters were ligated with the restricted DNA fragments by adding 3 µL of a solution containing 50 pmol of *Tru* 9I-adaptor and 5 pmol of *Pst*I-adaptor, 0.3 U of T_4 DNA-ligase, 10 mM rATP in 1x one-for-all buffer. Incubation was continued at 37°C for 3 h. After ligation, the reaction mixture was diluted to 1:4 using sterile distilled water. Preselective amplification was performed in a reaction volume of 20 µL containing 50 ng of each of the two primers (*P00* and *M00*) corresponding to the *Tru* 9I and *Pst*I adaptors, respectively. Preamplification product was diluted to a ratio of 1:4 then 2 µL were used as a template for selective amplification. Selective amplification was conducted using nine *Tru* 9I and *Pst*I primer combinations (Table 1). Primer ends were labelled with different colours (6FAM, NED or HEX) in order to facilitate automating genotyping. Amplification was performed using a thermocycler (PE Applied Biosystems PCR 9600) programmed for 36 cycles with the following cycle profile: a 30 s DNA denaturation step at 94°C, a 30 s annealing step and a 1 min extension step at 72°C.

The starting annealing temperature was set at 65°C, with a decrease of 0.7°C at each subsequent cycle for the next 12 cycles (touchdown PCR), reaching the constant 56°C for the remaining 23 cycles. For final analysis, 2 mL of amplified DNA (diluted to 1/10) and 8 mL of (Rox) at 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp DNA standard size (Applied Biosystems)were loaded into 96-well plates. Genotyping was carried out using an automatic DNA analyzer (Model 3100 Prism; Applied Biosystems). Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis.

SSR amplification

A total of 15 olive SSR primer pairs were tested according to Cipriani et al. (2002). PCR reactions were performed in a total reaction mixture of 20 µl containing: 50 ng of total genomic DNA (2 µL) as template, 1 µL/PCR buffer (Roche, Manhim Germany), 0.2 mM of dNTP PCR mix (Roche), 0.625 U of *Taq* DNA polymerase (Roche) and 0.2 mM of each primer using forward primer end labeled (6FAM, NED or HEX) (VBC Genomics Bioscience Research GmbH., Vienna, Austria) (Table 2). M13 tailing sequence 5- TTT CCC AGT CAC GAC GTT 3- was attached with the primer sequences in order to be suitable for automated genotyping. Amplifications were performed using a thermocycler (Applied Biosystems, Carlsbad, CA) under the following conditions: a denaturation step of 2 min at 94° C followed by 35 cycles of 20 s at 94°C, 50 s at 55°C and 50 s at 72°C, with a final extension step at 72°C for 7 min. Amplification product quality was first verified by electrophoresis according to their molecular weight using 1.4% agarose gels and then detected by staining with ethidium bromide as described by Sambrook et al. (1989). The DNA profiles were visualized on an ultraviolet transilluminator and documented by using a gel documentation system (Alpha Innotech Imaging Station; Cell Biosciences, Santa Clara, CA).

For final analyses, 2 ml of amplified DNA (diluted to 1/10) and 8 ml of Rox DNA standard size (Applied Biosystems) were loaded into 96-well plates. Genotyping was carried out using an automatic DNA analyzer (Model 3100; Applied Biosystems). Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis.

AFLP and SSR data analysis

Electropherogram data were analyzed by using Foundation Data Collection Software (Genetic Analyser Data Collection Version 2.0; Applied Biosystems). Allele size scoring was performed by Gene Mapper software (Version 3.7; Applied Biosystems). Primer efficiency was calculated by dividing the number of bands generated by a primer by the total number of bands generated by all primers. Polymorphism percentages were calculated by dividing the number of polymorphic bands amplified by a primer by the total number of bands amplified using the same primer. Discrimination power for each primer was calculated by dividing the number of

Table 1. Number of fragments amplified, polymorphic bands, primer efficiency and discrimination power of the nine primer combinations used for AFLP analysis in ten olive cultivars grown in Iraq.

Primer combination	Total fragment (number)	% Primer Efficiency	Polymorphic fragment (number)	% Polymorphism	% Discrimination power
M54-cct\P19-ga	37	14.8	19	51	13.10
M54-cct\P38-act	25	10	10	40	6.90
M54-cct\P40-agc	33	13.02	16	48	11.03
M54-cct\P41-agg	25	10	16	64	11.03
M55-cga\P19-ga	36	14.04	26	72	17.93
M55-cga\P38-act	18	7.2	9	50	6.21
M55-cga\P40-agc	30	12	18	60	12.41
M55-cga\P41-agg	24	9.06	18	75	12.41
M55-cga\P35-aca	22	8.8	13	59	8.97
Total	250		145		
Mean	27.7		16.11		11.11

Table 2. The repeat motifs, number of fragments amplified, polymorphic bands, primer efficiency and discrimination power of the ten primers used for SSR analysis in the ten olive cultivars grown Iraq.

Marker	Repeat motif	Total fragment (number)	% Primer efficiency	Polymorphic fragment (number)	% Polymorphism	% Discrimination power
UDOO9-4	(AC)10	25	8.8	5	20.0	5.9
UDOO9-5	(AC)9	27	9.5	11	40.7	12.9
UDOO9-6	GT)5(AT)6G(GT)9	36	12.7	8	22.2	9.4
UDOO9-7	(GT)21	20	7.1	6	30.0	7.1
UDOO9-8	(AC)13	40	14.1	6	15.0	7.1
UDOO9-9	(AG)16	14	4.9	2	14.3	2.4
UDOO9-11	CT)7(CA)10(CT)2(CA)2 CT(CA)2CT(CA)9	63	22.3	18	28.6	21.2
UDOO9-12	(GT)10	30	10.6	3	10.0	3.5
UDOO9-14	(GT)10	11	3.9	10	90.9	11.8
UDOO9-15	(TG)12	17	6.0	16	94.1	18.8
Total		283		85		
Mean		28.3	10.0	8.5	30.04	10.00

polymorphic bands amplified by a primer by the total number of polymorphic bands obtained.

A rectangular binary matrix was created for each marker classes by giving 1 for band presence and 0 for band absence at a certain molecular weight. Base on this matrix, the Jaccard similarity matrix (Jaccard, 1908) was used for cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) to study the genetic relationships among the cultivars. The two data sets obtained with SSR and AFLP markers were combined after doing a mantel test using random permutations.

This test revealed significant correlation (71%) between them at P<0.0001. The mantel test, major allele frequency, heterozygosity, gene diversity, and polymorphism information content (PIC) estimation were done using a software package (PowerMarker Version 1.31; Liu and Muse, 2005). The number of observation for a marker locus is the number of nonmissing genotypes observed in the sample. Availability is defined as1−*Obs*/*n*, where *Obs* is the number of observations and *n* is the number of individuals sampled. Gene diversity is the expected heterozygosity which is defined as the probability that two randomly chosen alleles from the population are different. A closely related diversity measure is the polymorphism information content (PIC) (Botstein et al., 1980). The phylogenetic diagram was drawn by PAST software Version 1.91 (Hammer et al., 2001). Principal coordinate analysis (PCA) was performed according to Euclidean similarity index using the PAST software.

RESULTS

AFLP analysis

A total of 250 scorable bands were generated from nine selective AFLP primer combinations (Table 1). The number of amplified fragments per variety varied from 18

Table 3. Number of fragments amplified, polymorphic bands, primer efficiency and discrimination power of AFLP, SSR and both AFLP + SSR markers analysis in ten olive cultivars grown Iraq.

Marker	Total fragment (number)	% Primer efficiency	Polymorphic fragment (number)	% Polymorphism	% Discrimination power
AFLP	250	46.9	145	58.0	63.1
SSR	283	53.1	85	30.0	36.9
AFLP + SSR	533	50.0	230	43.2	

Table 4. Major allele frequency, gene diversity and PIC estimated by AFLPs, SSRs and both AFLP + SSR markers in the ten olive cultivars.

Marker	Major Allele Frquency	Sample Size	Allele number	Number of observations	Availability	Gene diversity	PIC
AFLP	0.757	10	2.0	9.8	0.98	0.335	0.271
SSR	0.505	10	4.1	8.3	0.83	0.594	0.545
AFLP+SSR	0.755	10	2.1	9.5	0.95	0.336	0.272

to 37 with an average of 27.7 fragments per primer combination. Among these fragments scored across all the cultivars, 145 bands (58%) were polymorphic for at least one of the cultivars. The primer combination M55/ P19 amplified 26 polymorphic bands (72% polymerphism) showing the highest primer efficiency (14.4%) and discrimination power (17.93%). While the primer combination M55 /P38 amplified only nine polymorphic bands (50% polymorphism) with only 6.21% discrimi-nation power. The same primer combination showed the lowest primer efficiency (7.2%) as shown in Table 1.

SSR analysis

A total of 283 bands were scored for all SSR primers (Table 2). SSR primer pair generated from 11 to 63 bands with an average of 28.3 fragments per primer pair. Among 283 fragments scored across all the cultivars, 85 bands (30%) were polymorphic for at least one of the cultivar. The primer pair UDOO9-11 amplified 18 polymorphic bands (28.6% polymorphism) showing the highest primer efficiency (22.3%) and a discrimination power (21.2%) while the primer UDOO9-12 amplified only three polymorphic bands (10% polymorphism) with only 3.5% discrimination power. The primer pair UDOO9-14 showed the lowest primer efficiency (3.9%) (Table 2). The summary of the above results is shown in Table 3. A total of 533 scorable bands were generated from both AFLP and SSR primers. Among these fragments scored across all the cultivars, 230 bands (43.15%) were polymorphic. SSR primers generated more than 283 bands with the highest primer efficiency (53.1%) compared with AFLP primers which generated 250 bands with 46.9% primer efficiency. However, SSR gave less polymorphic (85 bands) than AFLP which gave 145 bands.

Accordingly, AFLP revealed higher discrimination power (63.3 vs. 36.9%) with respect to SSRs and higher polymorphism (58 vs. 30%), while the combination of AFLP+SSR reduced the polymorphism to 43.2% (Table 3) as expected. Major allele frequencies of the selected loci for the two marker classes are shown in Table 4. It was higher using AFLPs (0.757) than SSRs (0.505) or AFLPs+SSRs (0.755).

AFLPs recorded higher observa-tion number (9.8) with 0.98 availability compared with SSRs, which recorded 8.3 with 0.83 availability or 9.5 (AFLP+SSR) with 0.95 availability. Nevertheless, the average Nei's gene diversity (Nei, 1973) detected by primers showed different results. Genetic diversity value was higher in SSR markers (0.594) showing higher polymorphism infor-mation content (PIC) (0.545) com-pared with AFLP mar-kers (0.271) showing (0.271) PIC or AFLP+SSR (0.272) with PIC value of 0.272 (Table 5).

Cultivars cluster analysis

According to AFLP analysis, UPGMA ordered the olive cultivars into two main clusters irrespective of their origin at similarity levels of 0.48 (Figure 1A). The first cluster (A) consisted of the cultivar ‘Shami’ and the second cluster (B) consisted of two sub-clusters at a similarity level of 0.54. The first one (B1) consisted of two sub-clusters (B1a) with the cultivar ‘Nepali’ and (B1b) which consisted of two sub-clusters (B1b1) with four cultivars ‘Labeeb, Dahkan, Qaissy and Baashiqi’ and (B1b2) with three cultivars: ‘Mazenllo, Khdeir and Sorani’ and the laste cluster is (B2) which consisted of the cultivar ‘Arbqween’. UPGMA method also ordered the olive cultivars according to 85 polymorphic SSR loci, into two main clusters at similarity level of 0.54 (Figure 1B). The first

Table 5. PC1 and PC2 percentages for the PCA analysis estimated by AFLPs, SSRs and both AFLP + SSR markers for ten olive cultivars grown in Iraq.

Marker	PC 1%	PC 2%	PC1+PC2
AFLP	18.89	17.90	36.79
SSR	32.87	24.57	57.44
AFLP+SSR	25.82	14.75	40.57

cluster (A) consisted of two sub-clusters (A1) with the cultivar 'Arbqween' and (A2) with two cultivars 'Nepali and Shami'.

The second cluster (B) consisted also of two sub-clusters at similarity levels of 0.60. The first (B1) consisted of four cultivars 'Mazenllo, Sorani, Khdeir and Labeeb' and (B2) which consisted of three cultivars 'Qaissy, Baashiqi and Dahkan'. Cultivars cluster analysis according to UPGMA methods for both AFLP+SSR polymorphic loci, ordered the olive cultivars into two main clusters at a similarity levels of 0.50 (Figure 1C). The first cluster (A) consisted of the cultivar 'Shami' and the second cluster (B) consisted of two sub-clusters at similarity levels of 0.58. The first one (B1) consisted of two sub-clusters; the first B1a consisted of the four cultivars 'Labeeb, Dahkan, Qaissy and Baashiqi' and B1b, consisted of three cultivars 'Mazenllo, Khdeir and Sorani'. The second was B2, consisted of the remaining two cultivars 'Arbqween and Nepali'.

Principal component analysis

In order to confirm the results of genetic relationships among olive cultivars, data were analyzed by multivariate PCA. The scatter diagram of the first two (PC1 and PC2) based on 145 AFLP bands revealed 36.79% of the variation among cultivars in which PC1 was 18.89% while PC2 was 17.90% as shown in Table 5 and Figure 2A. Results exhibit the same two clusters as shown in the dendrogram. Data also were analyzed using PCA based on 85 SSR polymorphic loci which represent 57.44% of the total variation in which PC1 recorded 32.87% and PC2 was 24.57%. The two axes exhibited similar clusters of cultivars as shown in the dendrogram (Figure 2B). AFLP+SSR markers recorded 40.57% variation when analyzed by PCA (Figure 2C). PC1 reached 25.82% and PC2 reached 14.75% (Table 5).

DISSCUSION

Results obtained from this study demonstrate the efficiency of the two marker classes for assessment of genetic diversity of olive cultivars grown in Iraq. SSR primers exhibited the highest primer efficiency (53.1%) compared with AFLP primers which showed 46.9% primer efficiency giving less polymorphic bands than AFLP. Genetic diversity value was higher in SSR markers with higher polymorphism information content (PIC) compared with AFLP markers or AFLP+SSR. The higher level of polymorphism detected in olive cultivars by SSR markers compared with AFLPs revealed the discriminating capacity of the former. This result is in accordance with previous studies where SSRs were compared with other marker systems (Powell et al., 1996; Russell et al., 1997; Pejic et al., 1998). The hyper variability observed at SSR loci was expected because of the unique mechanism by which this variation was generated; replication slippage and unequal crossing over are thought to occur more frequently than single nucleotide mutations and insertion/deletion events, which generate the polymorphisms detectable by AFLP and RAPD analyses (Powell et al., 1996; Milbourne et al., 1997). The co-dominant nature of these markers permits the detection of a high number of alleles per locus and contributes to higher levels of expected heterozygosity being reached than would be possible with RAPDs and AFLPs.

However, this result also depends on the studied species. In barley (Russell et al., 1997) and in tetraploid potato (McGregor et al. 2000), for example, AFLPs scored a higher level of expected heterozygosity (also called diversity index) than SSRs and RAPDs. Molecular markers were used to estimate genetic relationships among olive cultivars from different countries around the world. The high level of polymorphism observed in this study for the two marker systems is consistent with the results from previous studies carried out on olive cultivars using different molecular markers (Wiesman et al., 1998; Angiolillo et al., 1999; Baldoni et al., 2000; Rallo et al., 2000; Belaj et al., 2001; Besnard et al., 2001; Sanz-Cortés et al., 2001) reflecting great diversity within the cultivated olive germplasm (Bartolini et al., 1998). Genetic similarity index estimated by the two DNA marker classes used in this study indicates that cultivars are very close to each other (Qaysi, Baashiqi and Dahkan) since they were always in the same group in the dendrogram with different proportions. 'Baashiqi' is known as an Iraqi olive cultivar, with high oil content and grows well under Iraqi environment so it is a potential source for breeders.

Our findings confirm the utilization of SSR markers for segregation analysis studies. It is well known that 'Shami' resulted from crossing between 'Arbqween' and 'Nipali' since they were in the same group in the dendrogram estimated by SSR loci.

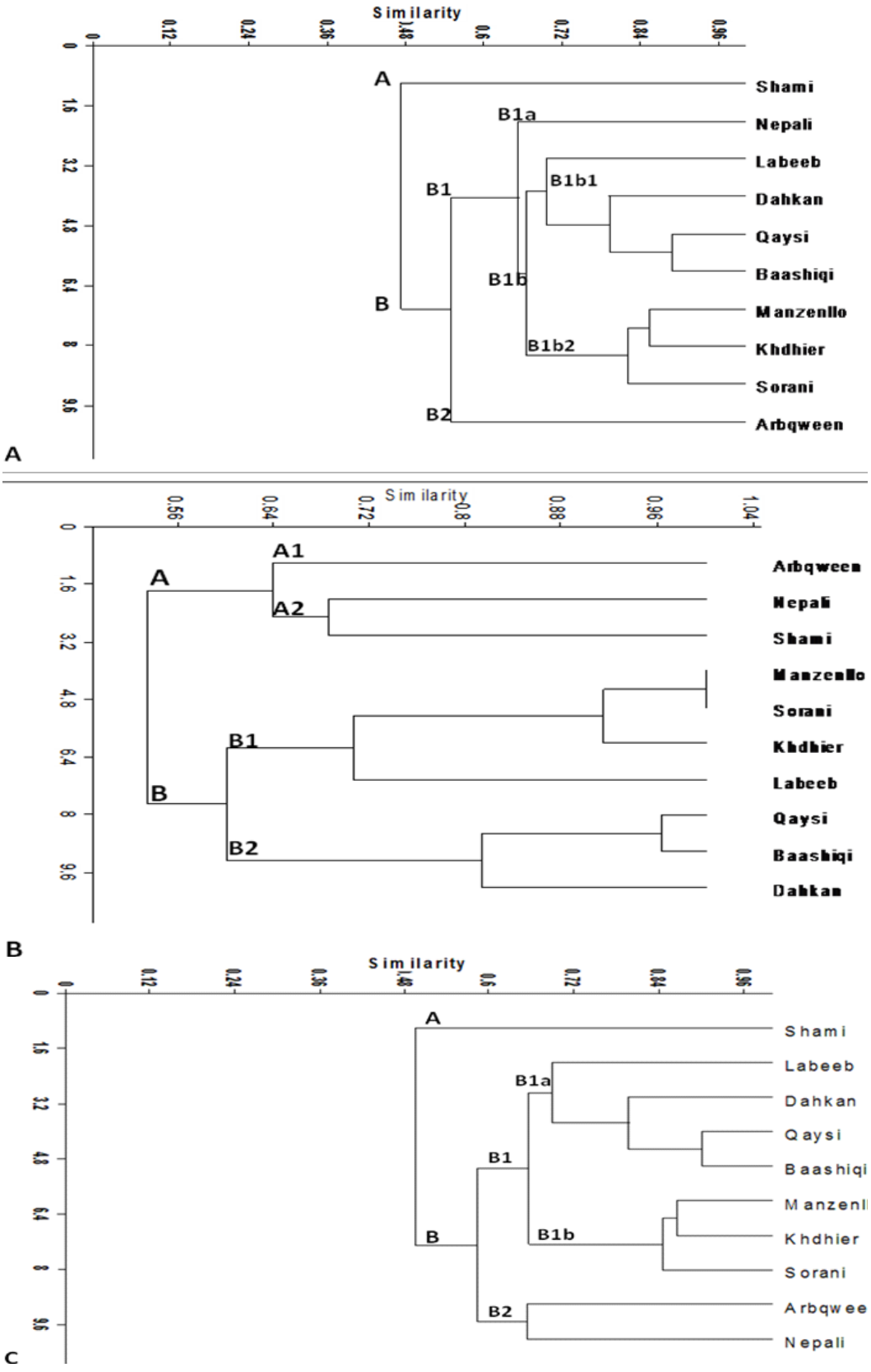

Figure 1. Genetic relationships among the 10 olive cultivars grown in Iraq estimated by A) AFLP analysis, B) SSR analysis and C) AFLP+SSR.

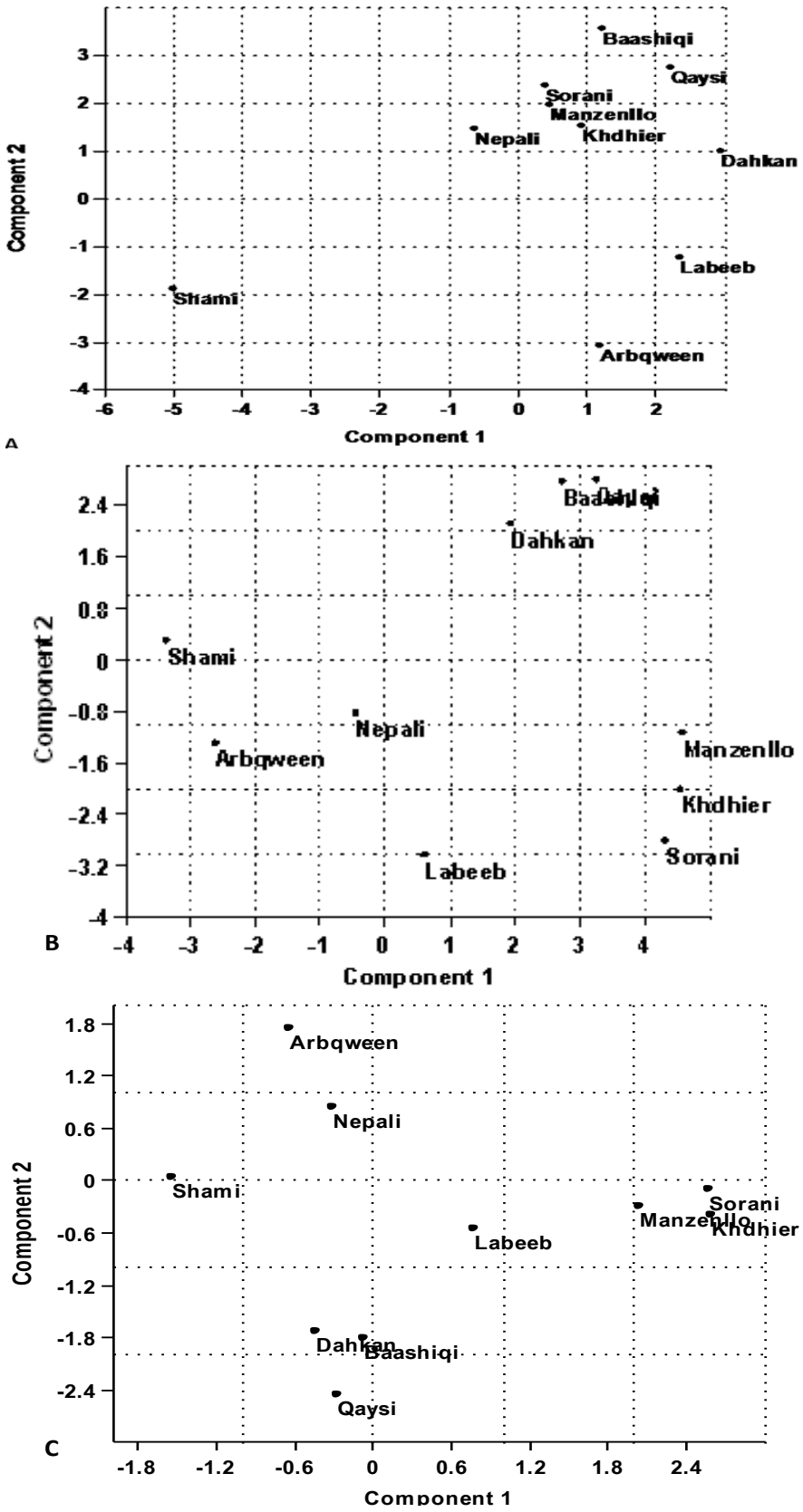

Figure 2. Principal component analysis (PCA) of 10 olive cultivars grown in Iraq as revealed by A) AFLP analysis, B) SSR analysis and C) AFLP+SSR.

In conclusion, AFLPs are highly efficient in detecting genetic relationship among olive cultivars, while the co-dominant nature of SSRs markers makes them very suitable for segregation and genome mapping in olive trees.

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