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DNA Fingerprinting and Cultivar Identification of Olive (*Olea europaea* L.) using SSR markers

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Abstract

Background: Genetic diversity and population structure of the olive varieties cultivated in Pakistan are yet to be explored.

Methods: In present study, we studied population structure and genetic diversity and developed DNA fingerprints of 13 olive varieties using 63 Simple Sequence Repeat markers.

Results: Collectively 618 alleles were amplified among which 582 were polymorphic and 36 were monomorphic. High allelic diversity per locus was found among 63 SSR markers, i.e., one for GAPU-12 to 23 for UDO099-008 and GAPU-47 with an average 9.80 alleles per locus. On the basis of cluster analysis genotypes were grouped into two clusters. Cluster I contained varieties Manzanilla, Sohawa-selection, Koroneki, Bulkasar-selection, Arbequina, Arbosona, Chugtai-selection, whereas cluster II was comprised of varieties Bari-Zatoon, Coratina, Gemlik, Frontaio, Pendolino and Ottobratica. UDO-24 marker alone identified seven olive varieties. Similarly, DCA-07 and EMO-02 identified six olive varieties each.

Conclusion: The findings of this manuscript will be helpful for future studies related to DNA fingerprinting and genetic diversity assessment for choice of SSR markers and identification of olive varieties.

Introduction

The olive (*Olea europaea* L.) is an ancient fruit species cultivated in Mediterranean basin since pre-historic times. It is the first tree cultivated ever. Subsequently, it gained significant importance in all the civilization established around Mediterranean Sea shore [1]. The Olea genus comprises of 35 species varying from trees to ever green shrubs. *Olea europaea* L. is only cultivated species of *Olea* genus grown worldwide for production of olive oil. The cultivated olive is diploid species grown in subtropical areas and comprises of several subspecies based on geographical origins and morphological appearance [2]. Olive oil possesses excellent sensory, functional and nutritional properties. Virgin olive possesses high stability and health constituents i.e. phenolic compounds and monounsaturated fatty acids (MUFAs) [3].

A variety of climates are favorable for olive cultivation i.e. Mediterranean basin coastline to interior regions having cooler climate [4]. In Pakistan favorable climatic conditions, i.e. 250-1000 mm annual precipitation and −10-40 °C temperature fluctuations for olive cultivation exists in Punjab, Western hills of Baluchistan, lower hills of Azad Kashmir, Waziristan, Afghan border, Chitral, Dir and Swat [5]. Government of Punjab took special initiative to enhance olive cultivation in Punjab and strengthen the position of Pakistan in top olive producing countries. Approximately 09 million olive plants are planted in Potohar valley and it is being re-named as olive valley [6].

Many olive varieties were exported from olive producing countries and being marketed by different industries from public and private sector. Simultaneously presence of local cultivars with ambiguous naming, patchy distribution and presence of varietal clones has complicated identification of olive cultivars [7]. Previously morphological traits including fruit and leaf characteristics were used for characterization of olive cultivars [8]. However morphological traits are highly influenced by environmental factors, plant phenology and age [9, 10]. Isozymes were also used at times for identification of plant varieties on the basis of unique profiles. However, sensitivity to developmental and environmental factors and lack of specificity among Isozymes remained a noticeable cause for discouraging their use in variety identification [11].

The availability of molecular tools to detect differences among varieties and clones provided reliable and rapid solutions to these problems [12]. PCR based DNA markers for identification of olive varieties are now available i.e. randomly amplified polymorphic DNA, RAPDs [13-15] and simple sequence repeat markers, SSR [16-20]. SSR markers have advantage over other PCR based markers due to uniform genome coverage, reproducibility, codominance, ease of genotyping and high level of polymorphism [21].

The present study was designed for the development of a DNA database for the identification of 13 olive varieties using SSR markers. Further, genetic diversity and population structure was also studied.

Methods

Plant Material

The research work was conducted at Agricultural Biotechnology Research Institute, Ayub Agricultural Research Institute, Faisalabad . Thirteen olive varieties were used for DNA fingerprinting and SSR analysis. Among thirteen varieties nine were commercial cultivars imported from different countries whereas four were local selections. Genomic DNA of 13 olive varieties was extracted using modified CTAB method [22]. Nanodrop spectrophotometer (ND 2000, Thermo Scientific, USA) was used for quantification of each DNA samples. Finally, the concentration was adjusted to 20 ng/µl for PCR. DNA quality was determined using 0.8% agarose gel prepared with 0.5X TE buffer. 50ng of genomic DNA was loaded from each sample and gel was stained with ethidium bromide after running to check the quality of DNA under UV light in Gel Documentation System.

Polymerase Chain Reaction and Gel electrophoresis

Sixty-three polymorphic SSR markers were selected from previously published papers and got synthesized from Gene Link company (USA). 25 µL of PCR reaction volume was used including 60-80 ng genomic DNA, 0.6 µM of forward and reverse primers and 12 µl of green master mix followed by volume make up with D_3H_2O. The PCR profile was as follows i.e. 94°C for 5 min, followed by 35 cycles each of 94°C for 45 seconds, annealing temperature for 1min, 72°C for 1 min followed by 72°C for 7 min and hold at 8°C. 6% Polyacrylamide (19:1 acrylamide: bis-acrylamide) gel was used to separate PCR products on vertical Gel Electrophoresis system (Biocom Direct, UK) 4 µL of PCR product was run for each samples followed by silver nitrate staining and visualization on Gel Documentation System [22].

Data analysis

A binary data system was used for scoring the PAGE gels. Presence and absence of bands was labeled as 1 and 0 respectively. Size of unknown DNA fragments was determined by comparison with 50 bp DNA ladder. Data was analyzed using NTSyspc 2.2 software for cluster analysis using UPGMA approach. Further allelic diversity parameters i.e. polymorphism rate, number of alleles and polymorphic alleles were calculated for each marker. Polymorphic information content (PIC) was calculated following Powell *et al.* [23] matrix.

The genetic structure of thirteen olive varieties was assessed using model-based Bayesian clustering approach through STRUCTURE v. 2.3.4 [24]. Genotyping data of 63 microsatellites was used to determine population's structure of various olive varieties. Population structure analysis was performed using following Parameters: no admission model; K ranging from 1 to 6; 10,000 Burn-in period; hypothetical populations' number (k) (03), number of in-iteration burns (10, 000), number of Markov chain Monte Carlo simulations (100000). Most likely number of clusters were determined by plotting LnP (K) values against ΔK values using Evanno's Test [25].

Results

Polymorphism features

Out of 63 SSR markers, four i.e., GAPU-11 and GAPU12, GAPU-89 and GAPU-90 were monomorphic and amplified 17 alleles collectively. The results showed that these are highly conserved regions in all the varieties studied . A total of 618 alleles were amplified out of which 36 were monomorphic and 582 were polymorphic with an average of 9.8 alleles per locus (Table 1). Alleles per SSR varied from 23 both for UDO099-008 & GAPU-47 to 1 for GAPU-12. Similarly, polymorphic alleles per SSR varied from 0 for (GAPU-11, GAPU-12, GAPU-90 and GAPU-92) to 23 (GAPU-47 and UDO-099-008) (Table 1). Polymorphism rate was 100% for 47 SSR markers whereas it was 0 for four markers i.e. GAPU-11, GAPU-12, GAPU-90 and GAPU-92 (Table 1). Polymorphic Information Contents (PIC) was also high for GAPU-47 & UDO099-008 (0.95).

Allele size profiling and cultivar identification

We have successfully applied SSR markers for genetic characterization of olive varieties cultivated in Punjab Pakistan. All the varieties in our study were uniquely identified indicating that no two varieties were similar to each other (Table 2). Eighteen SSR markers can identify 13 olive varieties under study. The SSR markers UDO-24 individually identified seven olive varieties (Arbequina, Coratina, Koroneiki, Bari-Zatoon, Pendolino, Manzanilla and Frontaio) (Fig 1).

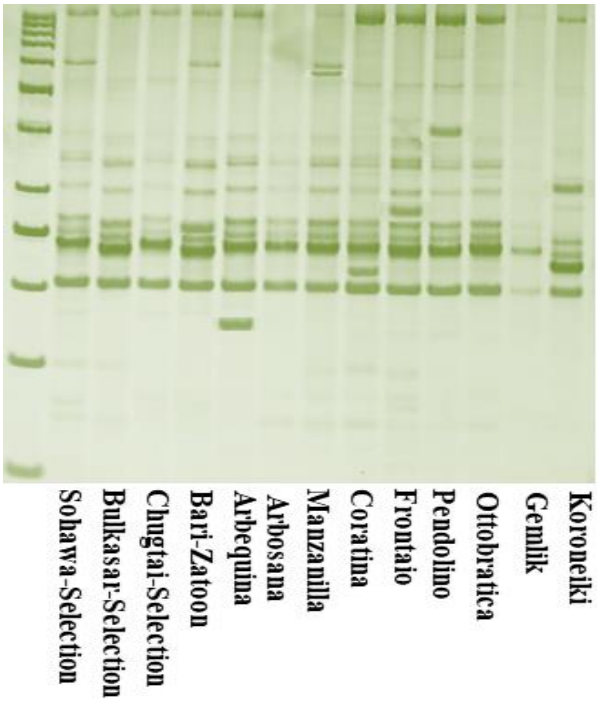

Figure 1: Representative gel image of UDO-24 SSR markers showing 14 polymorphic alleles and DNA Fingerprints for seven olive genotypes i.e., Arbequina, Coratina, Koroneiki, Bari Zatoon, Pendolino, Manzanilla and Frontaio, Lane 1 indicates 50 Bp ladder.

Similarly, DCA-07 and EMO-02 each distinguished six varieties. DCA-4, DCA-5, UDO-4, UDO-6, UDO8, UDO-12, UDO-14, UDO-25 and EMO-88 discriminated three varieties each. Ten SSR markers identified Arbosana. Similarly, Arbequina and Koroneiki were recognized by nine SSR markers. Seven SSR markers diagnosed Sohawa-Selection, Bulkasar-Selection, Manzanilla & Coratina (Table 2) and so on.

Structure and clustering analysis and genetic relationships of olive varieties

The overall allelic variation of 63 SSR markers exhibited high genetic diversity among 13 cultivated olive varieties. The genetic similarity coefficient varied from 0.55 to 0.73 indicating that all varieties were different from each other at 53 % loci whereas at least two varieties share 73% loci in common. Pendolino and Ottobratica were closely related varieties whereas Ottobratica and Showa-Selection were distantly related varieties (Table S3).

Cluster and structure analysis grouped the 13 olive varieties to two major clusters. Cluster I possessed 07 varieties (Sohawa-selection, Manzanilla, Koroneki, Bulkasar-selection, Chugtai-selection, Arbequina, Arbosona) whereas Cluster II exhibited 06 varieties (Bari-Zatoon, Coratina, Gemlik, Frontaio, Pendolino and Ottobratica) (Fig 2). Structure analysis revealed highest peak value for delta K at K=2 indicating that at least 02 distinct population exists among selected varieties . Sohawa-selection, Bulkasar-selection, Chugtai-selection, Arbequina, Arbosona, Manzanilla and Koroneki indicated by red and green colours represent sub-population I. Similarly, Coratina, Bari-Zatoon, Frontaio, Pendolino, Ottobratica and Gemlik were placed in sub-population 2 . Expected heterozygosity between two populations did not varied significantly. Koroneki in sub-population I and Coratina & Bari-Zatoon in sub-population II have different genetic makeup as compared with other genotypes (Fig. 3). Results of the cluster and structure analysis were complementary to each other (Fig 2 and Fig 3).

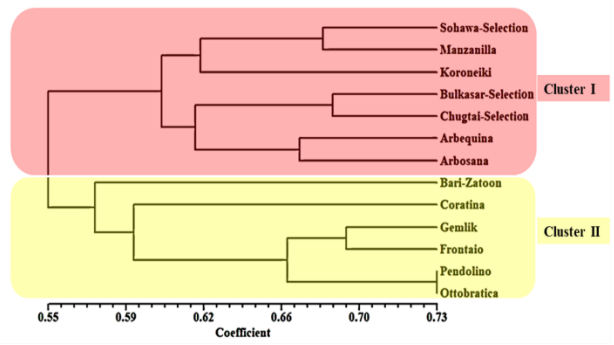

Figure 2: Cluster analysis using unweighted Pair Group Method with Arithmetic Average (UPGMA) method of thirteen olive genotypes.

Cluster and structure analysis also supported the hypothesis that varieties belonging to same geographic origin were placed together. Different varieties originated from Spain i.e. Arbequina, Arbosona and Manzilla were grouped together in the cluster analysis. Similarly, Coratina, Frontaio, Pendolino and Ottobratica were all originated from Italy and share the same cluster (Fig 2).

Marker Name	Polymorphic Status	No of Alleles	Polymorphic Alleles	Polymorphism Rate (%)	PIC
DCA-1	Polymorphic	5	5	100	0.77
DCA-3	Polymorphic	13	13	100	0.92
DCA-4	Polymorphic	16	16	100	0.93
DCA-5	Polymorphic	8	8	100	0.87
DCA-7	Polymorphic	11	11	100	0.90
DCA-8	Polymorphic	14	13	93	0.88
DCA-10	Polymorphic	8	8	100	0.86
DCA-11	Polymorphic	9	9	100	0.87
DCA-13	Polymorphic	5	5	100	0.78
DCA-14	Polymorphic	5	5	100	0.79
DCA-15	Polymorphic	4	4	100	0.64
DCA-17	Polymorphic	4	4	100	0.71
GAPU-11	Monomorphic	8	0	0	0.88
GAPU-12	Monomorphic	1	0	0	0.00
GAPU-14	Polymorphic	6	6	100	0.83
GAPU-19	Polymorphic	5	5	100	0.79
GAPU-47	Polymorphic	23	23	100	0.95
GAPU-59	Polymorphic	11	11	100	0.90
GAPU-62	Polymorphic	5	3	60	0.77
GAPU-71A	Polymorphic	10	10	100	0.89
GAPU-71B	Polymorphic	11	11	100	0.90
GAPU-82	Polymorphic	3	3	100	0.67
GAPU-89	Polymorphic	12	12	100	0.89
GAPU-90	Monomorphic	5	0	0	0.80
GAPU-92	Monomorphic	3	0	0	0.67
GAPU-103	Polymorphic	13	13	100	0.92
GAPU-108	Polymorphic	3	3	100	0.65
UDO99-003	Polymorphic	7	7	100	0.83
UDO99-004	Polymorphic	7	7	100	0.84
UDO99-005	Polymorphic	6	5	83	0.81
UDO99-006	Polymorphic	17	17	100	0.93
UDO99-008	Polymorphic	23	23	100	0.95
UDO99-009	Polymorphic	13	13	100	0.92
UDO99-011	Polymorphic	10	10	100	0.89
UDO99-012	Polymorphic	13	13	100	0.91
UDO99-014	Polymorphic	12	12	100	0.90
UDO99-015	Polymorphic	5	5	100	0.77
UDO99-017	Polymorphic	6	5	83	0.74
UDO99-019	Polymorphic	9	8	89	0.84
UDO99-020	Polymorphic	6	5	83	0.81
UDO99-022	Polymorphic	6	4	67	0.82
UDO99-024	Polymorphic	16	14	88	0.92
UDO99-025	Polymorphic	13	13	100	0.90
UDO99-026	Polymorphic	12	12	100	0.91
UDO99-027	Polymorphic	12	12	100	0.91
UDO99-028	Polymorphic	21	21	100	0.94
UDO99-031	Polymorphic	6	6	100	0.76
UDO99-034	Polymorphic	6	5	83	0.71
UDO99-035	Polymorphic	14	14	100	0.91
UDO99-036	Polymorphic	20	19	95	0.94
UDO99-039	Polymorphic	9	8	89	0.85
UDO99-041	Polymorphic	10	10	100	0.90
UDO99-042	Polymorphic	15	15	100	0.91
UDO99-044	Polymorphic	8	8	100	0.87
EMO-2	Polymorphic	15	15	100	0.92
EMO-3	Polymorphic	14	13	93	0.92
EMO-13	Polymorphic	7	7	100	0.85
EMO-30	Polymorphic	12	12	100	0.90
EMO-88	Polymorphic	11	11	100	0.89
EMO-90	Polymorphic	9	6	67	0.88
EMO-L	Polymorphic	11	11	100	0.89
OR-2	Polymorphic	12	12	100	0.91
OR-8	Polymorphic	4	1	25	0.74

Table 1: Polymorphic statuses, number of alleles, number of polymorphic alleles, polymorphism rate and polymorphic information contents (PIC) of 63 olive SSRs.

Genotypes	DNA Fingerprints (base pairs)
Sohawa-Selection	DCA-07 (195), DCA-10 (200), UDO-12 (175), UD0-14 (160), UDO-42 (160), GAPU-103 (215) and EMO-2 (200)
Bulkasar-Selection	DCA-07 (210), UD0-11 (150), UD0-19 (177), UD0-28 (150), UDO-36 (225), UDO-42 (165) and GAPU-71A (240)
Chugtai-Selection	UDO-11 (220), UDO-12 (230), UDO-28 (265), UDO-36 (235), GAPU-71A (250) and GAPU-103 (135)
Bari-Zatoon	UD0-24 (210) and EMO-02 (255)
Arbequina	DCA-07 (145), DCA-10 (280), UD0-09 (140), UDO-011 (135), UDO-24 (130), UDO-28 (245), GAPU-71A (235), EMO-02 (240) and EMO-L (195)
Arbosana	DCA-04 (238), DCA-05 (245), DCA-10 (175), DCA-19 (275), UDO-28 (275), UDO-36 (150), UDO-42 (200), GAPU-47 (225), GAPU-103 (185) and EMO-02 (230)
Manzanilla	DCA-05 (220), DCA-07 (175), UDO-12 (170), UDO-14 (100), UDO-24 (485), UDO-36 (195) and EMO-02 (300)
Coratina	DCA-05 (380), DCA-13 (160), UDO-011 (110), UDO-12 (205), UDO-24 (165), GAPU-71A (255) and EMO-02 (390)
Frontaio	UD0-24 (225) and EMO-L (75)
Pendolino	DCA-05 (320) and UDO-09 (145), UDO-24 (300)
Ottobratica	DCA-05 (340), DCA-13 (190) andUDO-09 (150)
Gemlik	UDO-14 (95) and EMO-L (110)
Koroneiki	DCA-04 (420), DCA-05 (225), DCA-10 (225), UDO-011 (170), UDO-014 (225), UDO-24 (255), GAPU-47 (220), GAPU-103 160) and EMO-L (205).

Table 2: DNA Fingerprints of 13 Olive varieties.

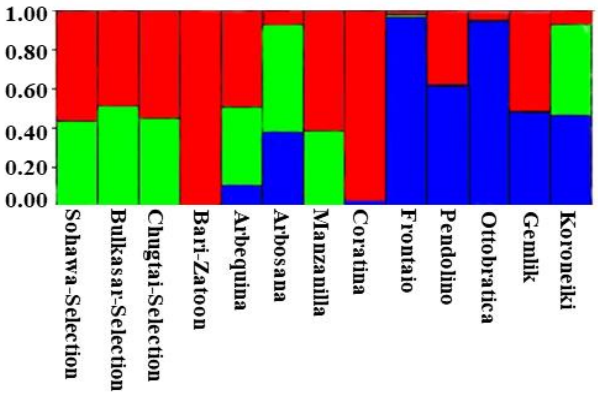

Figure 3: Structure analysis of 13 olive varieties, Parameters: no admission model; K = 02; 10,000 Burn-in period; 100000 Rep. Red and green color indicates sub-population 1, blue & red indicates sub-population 2.

Discussion

DNA fingerprinting helps the seed regulating bodies in controlling the marketing of false seed especially in case of fruit trees. In fruit plant it is difficult to predict true to type plants at the time of purchase it took almost 03-05 years to identify the variety at the time when its starts bearing fruit. However, DNA fingerprinting has potential for variety identification at the seedling stage. We have successfully applied 63 SSR markers for genetic characterization of 13 olive varieties cultivated in Punjab Pakistan. The SSR markers used in this study were obtained from Carriero *et al.* [26], de la Rosa *et al.* [27], Sefc *et al.* [16], Aggarwal *et al.* [28] and Cipriani *et al.* [18] studies (Table 1). The basis for selection of SSR markers were discriminating power, degree of polymorphism, amplification of a single locus and allowing distinguishing between cultivars.

A detailed DNA fingerprinting profile was provided for identification of 13 olive varieties. All the varieties in our study were uniquely identified indicating that no two varieties were similar to each other (Table 2). UDO-24 was highly informative SSR marker which can individually identify seven olive varieties (Fig 1). Similarly, six varieties were identified by DCA-07 and EMO-02 each (Table 2). These results were in accordance with Sakar *et al*. [29] who identified that DCA-11 as most informative SSR marker for distinguishing of olive varieties.

The genetic similarity coefficient varied from 0.55 to 0.73 indicating that all varieties were different at 53 % loci whereas most closely related varieties, i.e., Ottobratica and Showa-Selection shared 73% loci in common (Fig 2). Cluster and structure analysis grouped the 13 olive varieties to two major groups. Cluster I possessed 07 varieties whereas Cluster II exhibited 06 varieties. Results of the cluster and structure analysis were complementary to each other (Fig 2 and Fig 3). Both analyses supported the hypothesis that varieties belonging to same geographic origin were grouped together. Different varieties originated from Spain i.e. Arbequina, Arbosona and Manzilla were grouped together in the same cluster. Similarly, Coratina, Frontaio, Pendolino and Ottobratica were all originated from Italy and were clustered together (Fig 2). These trends were observed because varieties originated from the same geographical area shares common ancestors and have similar genetic makeup. Previously Aksehirli-Pakyurek *et al*. [30] also reported similar results while studying genetic relationship of Greek and Turkish olive varieties.

Four SSR markers out of 63 SSR, i.e. GAPU-11 and GAPU-12, GAPU-89 and GAPU-90 were highly conserved among the studied varieties and were monomorphic as previously observed for GAPU-59 by Bracci *et al*. [4]. We amplified an average of 9.8 alleles per locus which are very high than previously reported results Noormohammadi *et al*. [31] and Ercisli *et al*. [12] except Sakar *et al*. [29] which reported 14.57 alleles per locus.

GAPU-47 and UDO099-008 were most informative SSR markers with 23 polymorphic alleles each. Whereas previous studies of Sakar *et al*. [29] reported DCA-11 as most discriminative SSR marker with 15 alleles. SSR markers that possess high allelic diversity and high PIC value are useful for genetic diversity assessment and DNA fingerprinting studies [32].

DNA fingerprinting was completed for thirteen olive varieties cultivated in Punjab Pakistan. SSR markers used in this study were found useful for identification of thirteen olive varieties. Among 63 surveyed markers GAPU-47 and UDO099-008 were most informative markers amplifying 23 alleles each. Information generated in this study will be useful for DNA fingerprinting and genetic diversity assessment studies of olive varieties in future. Further, it will also be helpful in easy identification of the olive cultivars in cost effective manner.

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Author Contributions

The experiment was planned by all the authors of this manuscript. Funding was acquired by all the authors in the CGS grant system of PARB. S.J. and R.S. conducted all the experimental work, S.J. and R.S. have drafted the manuscript and performed statistical analysis of the data. S.J. and R.S., M.Z.I and S.U.R. critically reviewed the manuscript. S.J. and R.S. are corresponding to the Journal for submission and revision process.

Competing Interest

Authors declare no conflict of interest in this work.

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