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Genetic Diversity of Olive Germplasm Using RAPD Markers

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Abstract: Ten olive (*Olea europaea* L.) genotypes were collected from Agriculture Research Institute Tarnab at Peshawar and Sang Bhatti at Sawabi, KPK, Pakistan to evaluate their genetic diversity. Genomic DNA was isolated from fresh leaves, using a CTAB extraction method with few modifications. Three RAPD primers OPC-01, OPC-09 and OPE-07 obtained from Biron Technologies were tested by RAPD-PCR. Totally 27 bands were amplified and these amplified fragments ranged in size from 100 to 400 bp. Comparatively, more bands were observed in OPC-09 primer, with 12 bands and least number of bands were observed in OPC-01 primer with 7 bands from the electrophoresis results of the olive genotypes. Among three RAPD primers, only OPC-09 was polymorphic, showing (42%) polymorphism.

Key words: Olive, Genetic Diversity, Germplasm, RAPD Markers

Introduction

The olives (*Olea europaea* L.), a diploid species with 46 chromosomes belongs to the *Oleaceae* family that contained 30 genera and 600 species (Cronquist, 1981). It occurs in two forms, wild and cultivated. The olive tree is native to the coastal areas of the eastern Mediterranean Basin and northern Iran where in the third millennium B.C. its cultivation was started (Loukas and Krimbas, 1983). Olives has a broad genetic patrimony (Bartolini et al., 1998).

Olive plantation is occurring between two belts (30-45^{o} North and South of the equator) around the world, so it is possible to grow olive in Pakistan as it lies in between these belts. In KPK and Baluchistan, wild species of olive occurred in large quantities which show that improved varieties can also be grown here successfully. From the analysis of potential areas of Pakistan with proper ecology for the cultivation of olive shows that it can successfully be grown in tribal areas of the KPK, Swat, Dir, Malakand, Loralai, Khuzdar and Quetta with appropriate management practices. Evaluation of the large number of olive germplasms or

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cultivars to know the extent and distribution of genetic variation between them is essential for successful breeding and conservation program of olive (Zohary, 1994; Green and Wickens, 1989). In the past several attempts have been made for this purpose on the basis of morphological trait analysis, including leaves, flowers or seeds as useful markers, but to avoid the effect of diversity in environmental conditions on olive genotypes could make the genotypic evaluation technique inefficient. Advancement in agriculture biotechnology, such as the discovery of DNA-based techniques, has opened new ways for fast and reliable genotyping of crops or plants. For instance, environmental factors such as agronomic practices or phenologic stages in a plant can not influence DNA-based markers, as the comparison of the organism in this technique is at the molecular level. The uses of these markers are getting great attention currently in the breeding programs of the economically important crops and/or plants, such as olives (Gonzalo et al., 2000).

Random amplified polymorphic DNA (RAPD) markers are used to detect DNA polymorphism without the prerequisite of using predetermined genetic data. RAPD technique is sufficient enough to detect polymorphism, even among the closely related cultivars. Beside the identification of new cultivars, RAPD markers can also estimate the genetic similarity among different genotypes. Moreover, RAPD markers could successfully detect genetic variations and similarities in both oleasters and cultured varieties of olives. Further, these markers could explore the phylogenetic connection between the plants and designate the varieties accurately.

RAPDs can be used for different purposes, for example (i) to differentiation olive cultivars (Khadari et al., 2003), (ii) in the study of inter- or intra-cultivar genetic diversity (Gemas et al., 2004), (iii) to establish genetic relationships between cultivars (Awan et al., 2011) and (iv) to identified genetic similarities and distances between wild olive trees (Sesli and Yegenoglu, 2009; 2010). The objectives of the present study were to evaluate polymorphism in olive germplasm growing in different agro-climatic conditions of Sawabi and Tarnab and optimization of PCR reaction parameters to obtain reproducible DNA bands for different olive genotypes.

Materials and Methods

Plant material

Ten Olive genotypes (Table1) were collected from the Agricultural Research Institute Tarnab, and Sang Bhatti, Sawabi, Pakistan. Young leaves from each of these genotypes were used as a sample for the whole study. The leaves were preserved in plastic zip bags and stored at -20˚C until DNA extraction.

DNA extraction

CTAB method was used to extract genomic DNA from the olive genotypes (Doyle and Doyle, 1990) with few modifications. The fresh young leaves (0.03 g per sample) were crushed in 750µl of extraction buffer PH-8.0 (2% w/v CTAB, 500 mM NaCl, 50 mM EDTA, 100 mM Tris base, 200 µl b-mercapto ethanol) pre-heated to 6°C. Samples were incubated for 10-15 minutes. After that, 30µl of 10/10 b-mercapto ethanol and130µl of 10% SDS was added. Samples were incubated for overnight at 65˚C and then vortexed. Afterwards, 600µl of chloroform-iso amyl alcohol (24:1 v/v) was added and gently agitated for 5-10 minutes to form an emulsion.

Table 1. List of the Olive genotypes and its growing region used in the study.

Code	Olive genotypes	Sampling Areas	District	Latitude	Longitude
1	Azarbiajan	Tarnab	KPK	33° 30' 43N	70° 41' 46E
2	Hamdi	Tarnab	KPK	33° 30' 43N	70° 41' 46E
3	Sevillano	Tarnab	KPK	33° 30' 43N	70° 41' 46E
4	Nabali	Tarnab	KPK	33° 30' 43N	70° 41' 46E
5	Nabali	Sang Bhatti	KPK	34° 07' 11N	72° 28' 19E
6	Coratina	Tarnab	KPK	33° 30' 43N	70° 41' 46E
7	Coratina	Sang Bhatti	KPK	34° 07' 11N	72° 28' 19E
8	Sorino	Sang Bhatti	KPK	34° 07' 11N	72° 28' 19E
9	Frantoio	Sang Bhatti	KPK	34° 07' 11N	72° 28' 19E
10	Gemlik	Sang Bhatti	KPK	34° 07' 11N	72° 28' 19E

Then centrifugation for 10 minutes at 12000 rpm at 25˚C. The supernatant was taken and 100µl of sodium acetate and 750µl of chilled isopropanol was added and mixed well and then incubated at -20˚C for 2 hrs. Again centrifugation for 10 minutes with 12000 rpm at 4˚C and pellet was taken. Washed the pellet with 500µl of wash solution (75% ethanol, 25mM NaCl and 5mM Tris base) or with 70 percent ethanol and then air dry it for 10 minutes. The DNA pellets were dissolved in 30µl of TE buffer (10mM Tris and 1mM EDTA) by incubation at 65˚C for 30 minutes. Later the extracted DNA was stored at -20˚C for long periods (at least for 1 year). DNA quality and concentration in samples were checked by spectrophotometric analysis at 260 and 280 nm and running on 1.5% agarose gel.

RAPD-PCR analysis

Three RAPD primers OPC-01, OPC-09 and OPE-07 were tested for PCR amplification. The PCR reaction was composed of 1x PCR buffer, 2 mM MgCl2, 0.2 mM deoxynucleotide triphosphate (dNTPs), 0.2 ng/µL of each 10-mer primer, 0.2U/µL Taq DNA polymerase and approximately 20-28 ng/µL of template DNA and PCR H_2O to make the final volume up to 25 µL. For PCR process following temperature program was set: 1 cycle of 5 minutes at 95°C for initial strand separation followed by 45 cycles of 30 seconds at 95°C for denaturation, 45 seconds at 35°C for annealing and 45 seconds at 72°C for primer extension. Finally, 1 cycle of 7 minutes at 72°C was used for final extension, followed by 4°C temperature to hold samples until removed from PCR machine.

Agarose gel electrophoresis

Separation of DNA fragments was done by electrophoresis on 1.5 % (w/v) agarose gels in 1X TAE buffer containing 5 µL (10mg/ml) ethidium bromide. Fermentas DNA loading dye was used. Size of fragments was estimated using the 50 bp DNA ladder (Fermentas Gene Ruler™). Amplified products were detected and photographed by placing the gel on UV transilluminator.

Table 2. RAPD-PCR Primers sequences used for the amplification of DNA from Olive genotypes.

Primer	Sequences
OPC-01	(5′TTCGAGCCAG3′)
OPC-09	(5′CTCACCGTCC3′)
OPE-07	(5′AGATGCAGCC3′)

Results

The results showed that the DNA was successfully obtained from all samples. The DNA extraction was conformed on 1.5% agarose gel and by spectrophotometric analysis. Table 3 showed that DNA ranged from 20.01 ng/µl to 28.46 ng/µl while DNA quality was from 1.09 to 1.30. Three RAPD primers OPC-01, OPC-09 and OPE-07 were used to establish the relationship among the olive genotypes. PCR amplification profile of the ten olive genotypes used in the experiment is shown in Fig. 1. Totally 27 bands were amplified and these amplified fragments in most of cases ranged in size from 400 to 100 bp. More number of bands was observed in OPC-09 primer with 12 bands and less number of bands was observed in OPC-01 primer with 7 bands from the electrophoresis results of the olive genotypes. A total of three RAPD primers only OPC-09 was polymorphic (42%)

Table 4 showed that by using OPC-01 primer Azarbiajan, Sevillano, Nabali, Coratina, Sorino, Frantoio and Gemlik showed only monomorphic bands while polymorphic bands were absent, thus giving 100% monomorphism. On the other hand, Azarbiajan, Sevillano, Nabali, Coratina, Sorino, Frantoio and Gemlik showed monomorphic bands when amplified with OPC-09 primer. However, Sevillano, Nabali, Coratina, Sorino and Gemlik genotype also produced polymorphic bands thus giving 58% monomorphism and 42% polymorphism. OPE-07 primer showed only monomorphic bands in Azarbiajan, Sevillano, Nabali (Tarnab), Nabali (Sang Bhatti), Coratina (Tarnab), Coratina (Sang Bhatti), Frantoio and Gemlik while polymorphic bands were absent in these genotypes, so give 100% monomorphism.

Table 3. List of values obtained from spectrophotometric analysis

Genotypes	Absorbance at 260	Absorbance at 280	Quantity (ng/µL)	Quality	Visibility ++ (good) +/- (fair)
1	0.082	0.064	27.29	1.28	++
2	0.080	0.063	26.84	1.27	++
3	0.096	0.078	25.42	1.24	++
4	0.054	0.046	22.87	1.17	++
5	0.089	0.077	22.31	1.16	++
6	0.049	0.043	22.00	1.15	++
7	0.056	0.051	20.01	1.09	++
8	0.095	0.083	21.72	1.14	++
9	0.084	0.065	27.56	1.28	++
10	0.067	0.051	28.46	1.30	+/-

Table 4. Polymorphism of Olive genotypes using different RAPDs primers.

Primer	No. of bands	Polymorphic bands	Monomorphic bands	% Polymorphism	% Monomorphism
OPC-01	7	0	7	0	100
OPC-09	12	5	7	42	58
OPE-07	8	0	8	0	100

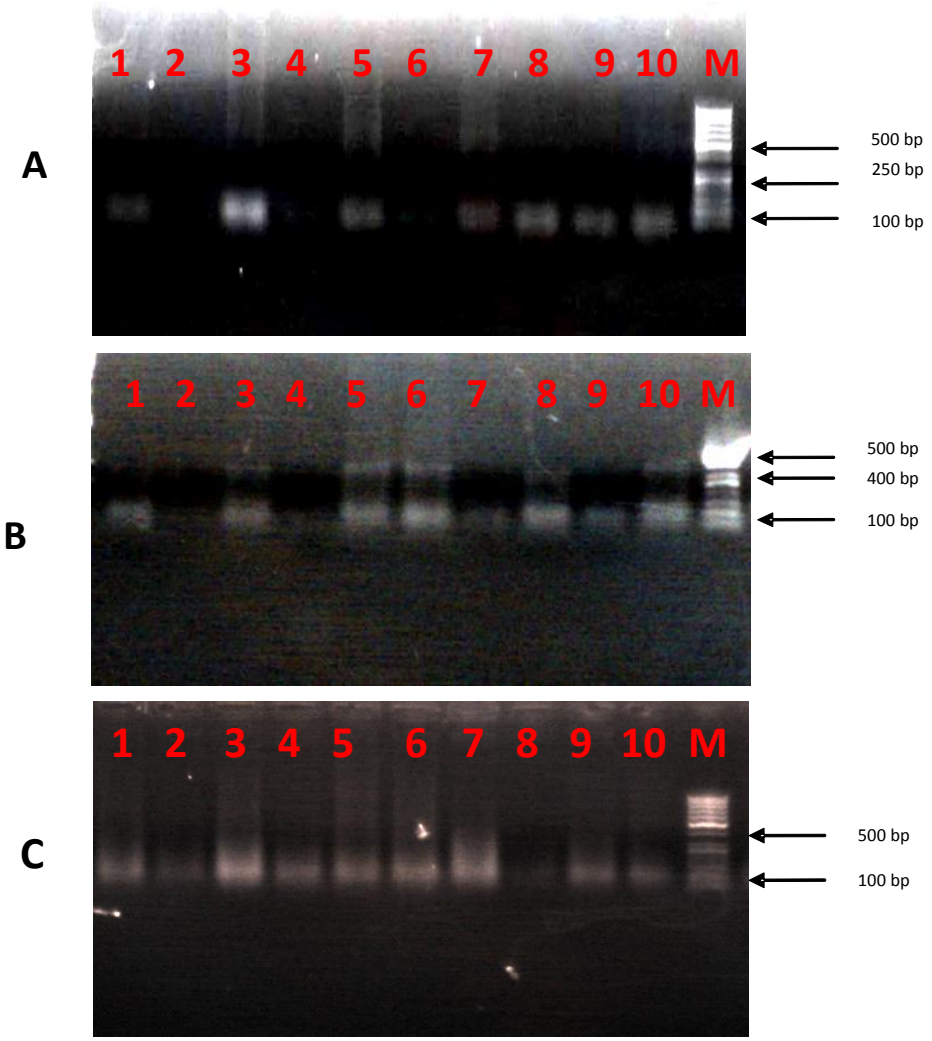

Figure 1. PCR amplification profile of 10 Olive Cultivars using RAPD primers OPC-01 (A), OPC-09 (B) and OPE-07 (C). Numbers correspond to genotypes in Table 1. M, molecular weight markers; numbers on the right represent molecular weights (bp).

Discussion

RAPDs primer was used in different organisms in the late 80's (Williams et al., 1990) and because of their speed and simplicity, it is considered as a very imported tool for the identification of cultivar and genetic similarity studies in plants. The results of our study showed that different sizes of DNA fragments were reproduced from DNA samples by the RAPD- PCR method. Total 27 bands were amplified with size ranging from 100 to 400 bp. By using OPC-09 primer 12 bands were observed in Olive genotypes showing 42% polymorphism and 58% monomorphism was observed from the experiment that OPC-09 primer generated polymorphic bands in olive genotypes. While OPC-01 and OPE-07 primer showed only monomorphic bands in different Olive genotypes.

Belaj et al. (2004) used 46 random primers to search the genetic structure of olive varieties by RAPD technique and showed a higher genetic polymorphism rate. Sesli and Yegenoglu (2009) used 60 random primers to identify genetic similarities and distances among wild olive trees by RAPD-PCR technique. Only 38 primers showed highly polymorphic and continuous scorable bands. Total 167 bands were found and each primer was screened at an average of 4.4 polymorphic bands.

The results obtained from our experiments by using these 3 RAPD primers were not significant. There might be more genetic diversity if more RAPD primers and other DNA-based markers were used. The evaluation capability of bands and non formation of primer artifacts is dependent on the sensitivity of PCR conditions. Suitable primer and primer concentration, purity of obtaining DNA, the number of cycles and denaturation, annealing, extension periods and purity and concentration of DNA included in the reaction mix affect the RAPD analysis and correspondingly its products. As demonstrated in previous studies, RAPD analysis is a useful method for studying the genetic structure in olives (Martins-Lopes et al., 2007).

Conclusions

Totally 27 bands were amplified and these amplified fragments in most of cases ranged in size from 100 to 400 bp. By using RAPD primer OPC-09, high level of polymorphism was observed in olive genotypes. Results obtained from the use of these 3 RAPD primers were not significant. There might be more genetic diversity if other RAPD primers and DNA-based markers were used.

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