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Full Length Research Paper

Molecular analysis of genetic diversity in olive cultivars

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The present study was conducted to study the genetic diversity in the available cultivars of olive flora in Pakistan. On an average, 15.5 alleles were amplified using Randomly Amplified Polymorphic DNA (RAPD) molecular primers. Mean genetic distance estimates ranged from 0.158 to 0.512 (G.D = 15.8 to 51.2%). Size of scorable fragments ranged from approximately 250 to >2000 bp. A high level of genetic dissimilarity (GD= up to 51%) was recorded among Ferugenia genotype only. Entries were grouped in clusters using cluster analysis. On the basis of dendrogram, most diverse genotypes Ferugenia and Coratina were identified, that can be used in future olive propagation program.

Key words: Olive, randomly amplified polymorphic DNA (RAPD) molecular primers, genetic diversity, Pakistan.

INTRODUCTION

Pakistan is facing a huge deficit in production and in edible oil to meet the demand and large quantities are imported every year for local consumption at the expense of precious foreign exchange. Several non traditional oilseed crops have been promoted, but they are not competitive enough to replace the established crops. It is therefore imperative to introduce a new crop that can be grown on the marginal lands addressing the issue of income generation breaking the vicious cycle of poverty and natural resource degradation. In Pakistan, millions of wild olive trees belong to the type *Olea cuspidata,* which are growing in Federally Administered Tribal Area (FATA), Khyber Pakhtunkhwa, Potohar region and Northern Balochistan. The presence of such large number of trees indicates that the agro-climatic conditions of these areas are conducive for olive cultivation. Similarly, different olive varieties belonging to *Olea europaea* sub-species *sativa* were introduced into the different part of the country, which are successfully bearing fruits. In Pakistan 668278 ha suitable areas are available for olive plantation in which up till now, only 480 ha areas is covered by olive plantation.

Olives have not been traditionally grown in Pakistan for the edible oil production, although few grooves existed both in the plain and hilly areas, the oldest cultivation being established in the 1970s. Due to the recent establishment of olive cultivation, very little information is available about the appropriate cultural practices to obtain economic yields under the diverse agro-climatic conditions. The great variation in the climatic requirements of each variety and the climatic conditions in the olive growing areas in Pakistan make it imperative to conduct a scientific study for identifying appropriate varieties and best or proper time and interval of cultural practices for each location. The use of RAPD primers (Williams et al., 1990) has given environment independent markers. This procedure has been performed in olive to identify varieties and used to determine the relationships between varieties (Claros et al., 2000). The aim of the present study is to identify the genetically diverse species for cultivation and propagation in different part of Pakistan.

MATERIALS AND METHODS

Plant material

In this experiment, molecular genetic relationships between 5 olive local and exotic cultivars were analyzed at the National Agriculture Research Council, Islamabad. These Olive cultivars were selected on the basis of phenotypic variation and yield potential from the research stations of the Pakistan Oil Development Board.

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DNA extraction

Young leaves of these varieties were used for DNA extraction. DNA extraction was carried out using a micro-prep DNA extraction protocol (Fulton et al., 1995). After isolation, concentration of total genomic DNA of each genotype was monitored by visual assessment of band intensity in comparison with lambda DNA molecular standards of known concentrations, that is, 100, 300 and 500 ng using 0.8% agarose gel. DNA of each genotype was diluted to a working concentration of 20 ng/µl for PCR analysis.

PCR analysis

A modified RAPD method based on Williams et al. (1990) was used for the amplification of DNA. To establish RAPD protocols for the olive, PCR analysis was performed by changing and checking the concentrations of total genomic DNA from 5 to 50 ng/20 µl reaction volume, $MgCl_2$ from 1.5~3.0 mM, dNTP mixture from 100~400 µM each, random primer from 0.1 to 1.0 µM and *Taq* DNA polymerase from 0.2 to 1.25 units. After standardization of PCR, the 20 µl reaction mixture contained 1 × PCR buffer [10 mM Tris HCl (pH 8.3), 50 mM KCl], 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate (dNTP), 0.4 µM of 10-mer primer (Operon Technologies Inc., Alameda, CA), 1 unit AmpliTaq Gold DNA polymerase and approximately 20 ng of template DNA. The reaction mixture was overloaded with one drop of mineral oil to prevent evaporation. DNA amplification was performed in a DNA thermal cycler - 480 (Perkin Elmer Cetus, Norwalk, USA) programmed to 1 cycle of 5 min at 94°C for initial strand separation followed by 45 cycles of 1 min at 94°C for denaturation, 1 min at 36°C for annealing and 2 min at 72°C for primer extension. Finally, 1 cycle of 7 min at 72°C was used for final extension, followed by 4°C temperature to hold samples until removed from PCR machine. The reproducibility of amplification products was checked twice for each experiment (Lavee and Weisman, 1999).

Primer selection

Initially, three cultivars were used to optimize the RAPD protocols and select the suitable primers which exhibit polymorphisms among these cultivars. Altogether, 45 arbitrary decamer oligonucleotides, belonging to kit OPA, OPB, OPC, OPF, OPH, OPI, OPJ and OPK from Operon Technologies Inc. (Alameda, California, USA), were tested as single primers to identify the most promising ones for detecting polymorphism. However, finally the data of 18 primers (OPA-05, OPA-09, OPA-10, OPB-13, OPB-14, OPC-03, OPC-10, OPF-01, OPF-10, OPH-13, OPI-16, OPI-17, OPJ-01, OPJ-13, OPK-08, OPK-11, OPK-12, OPK-13,) were used and compiled to examine the genetic diversity and relationship among 5 olive genotypes.

Electrophoresis of amplified products

After amplification, 3 µl of gel loading dye buffer (0.02% Bromophenol blue, 0.02% xylene cyanol FF, 50% glycerol and 1% SDS) was added directly to the reaction tubes and spun for few seconds in micro-centrifuge, after mixing with the entire reaction mixtures. Aliquots of 15 µl of amplification products plus loading dye were then loaded in 1.5% agarose gels for electrophoresis in 1 x TBE (10 mM Tris-Borate, 1 mM EDTA) buffer and run at 100 V for 40 min to separate the amplified products. Lambda *Bst*PI fragment was used as marker. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/ml) for 40 min and photographed under UV light using black and white film # 667 (Polaroid, Cambridge, Mass., USA) (Newburry and Ford, 1993).

Data analysis

Photographs from ethidium bromide stained agarose gels were used to score the data for RAPD analysis. Each DNA fragment amplified by a given primer was treated as a unit character and the RAPD fragments were scored as present (1) or absent (0) for each of the primer-cultivar combinations. Bands were scored from top of the gel (band number 1) to the bottom. The left lane of the gel was considered as lane-1. Since DNA samples consisted of a bulk sample of DNA extracted from 5 to 10 seedlings, a low intensity for any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNA. Therefore, intensity of the bands was not taken into account and the fragments with the identical mobility were considered to be the identical fragments. The presence and absence of bands was scored in a binary data matrix. Pair-wise comparisons of the accessions based on the presence or absence of unique and shared amplification products were used to generate similarity co-efficients. Estimates of genetic dissimilarity were calculated among all pairs of the cultivars (Nei and Li, 1979):

Genetic Distance (GD) = $1- d_{xy}/d_x+d_y-d_{xy}$

Where:

d_x = number of scored fragments of individual ‘x’,
d_y = number of scored fragments detected in individual ‘y’ and
d_{xy} = number of shared fragments between individuals ‘x’ and ‘y’.

The resulting genetic distances were used to evaluate the relationships among germplasm accessions, advanced breeding lines and improved cultivars with cluster analysis using an unweighted pair-group method with arithmetic averages (UPGMA) and then plotted in the form of a dendrogram. All computations were carried out using the computer program NTSYS, version 2.1 (Applied Biostatistics Inc., USA) (Wiesman et al., 1998).

RESULTS AND DISCUSSION

The amplification profile obtained by using random primers, the five olive cultivars were used in the present study which showed various banding pattern for the loci detected. The amplified fragments in most of cases ranged in size (estimated using molecular size marker 1 kb DNA Ladder, Gene Link, USA) from 250 to >2000 bp. The results are in agreement with published reports (Abbas et al., 2009; Abbas et al., 2008) where similar size range of amplified fragments were observed. On an average 15.5 alleles were amplified by using RAPD primers. Mean genetic distance estimates ranged from 0.158 to 0.512 (G.D = 15.8 to 51.2%). Ozkaya et al. (2009) reported similar ranges of genetic distances in olive cultivars. The bivariate data and dissimilarity coefficient matrices of five olive cultivars based on the data of 18 RAPD primers using UPGMA method (Nei and Lie, 1979) were used to construct separate dendrograms using computer program “STATISTICA 7”. For the dendrogram constructed from data using RAPDs, the genotypes were grouped in 2 main groups (A and B) (Figures 1 and 2). Group A consisted of 2 genotypes and group B comprised of 3 genotypes only. Group B was the largest comprising of 3 genotypes. Based on the dendro-gram analyses, Coratina and Ferugenea were most

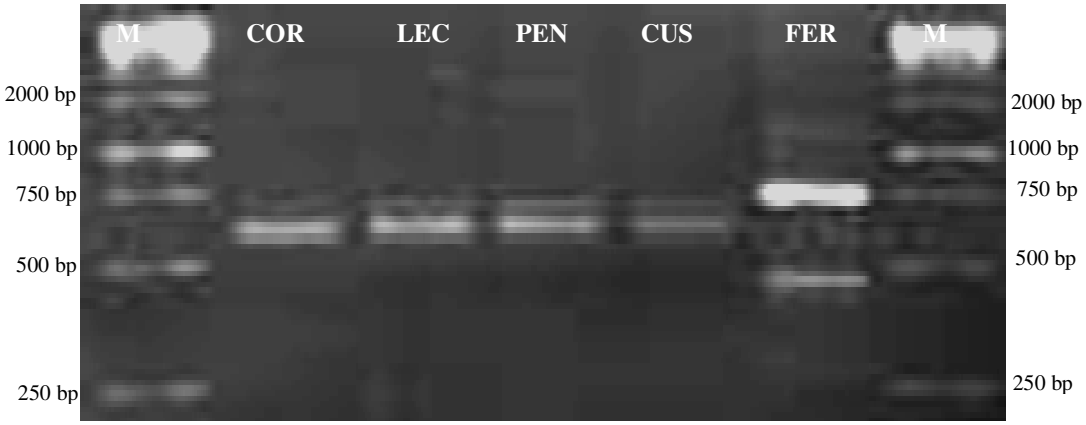

Figure 1. PCR amplification profile of 5 Olive cultivars using RAPD primer OPK-13.

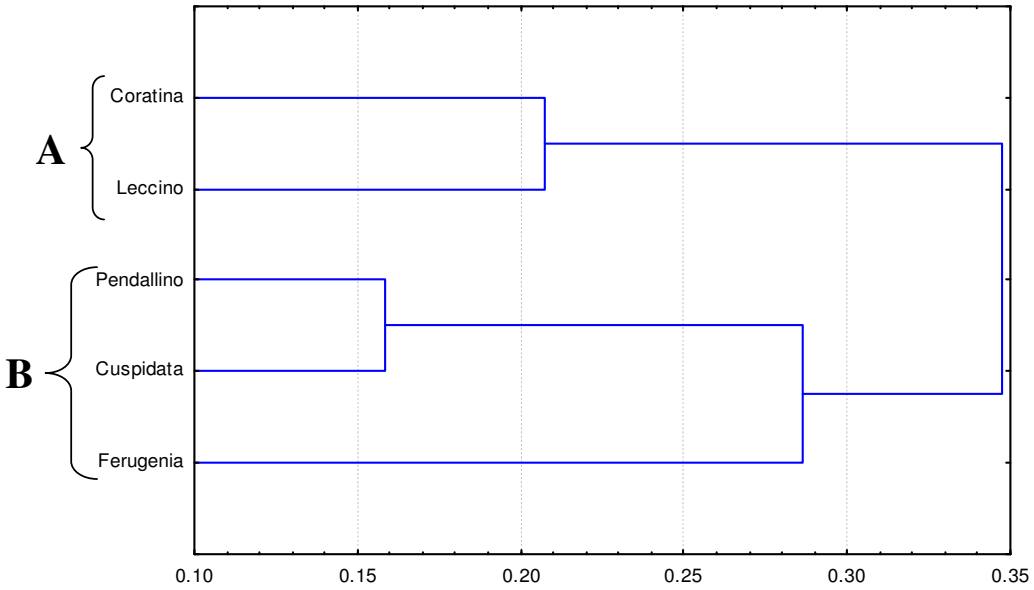

Figure 2. Dendrogram constructed for 5 Olive cultivars.

Table 1. Average estimates of genetic distances among 5 Olive Cultivars.

Cultivars	Coratina	Leccino	Pendallino	Cuspidata	Ferugenea
Coratina					
Leccino	0.20732				
Pendalino	0.32927	0.24390			
Cuspidata	0.36585	0.25610	0.15854		
Ferugenea	0.51220	0.37805	0.32927	0.24390	

distantly related from one another.

In genetic diversity analyses, the comparisons among the 2 genotypes (Coratina and Ferugenea) showed high estimate of genetic distance (GD = 51.2%). This finding was further strengthened by average genetic diversity analyses (Table 1), where the two genotypes showed higher levels of genetic dissimilarity with the rest of the genotypes used during the present studies. Polymerase chain reaction (PCR) based assays are easy, cheap, fast and do not require any sequence information on the

targeted genome and hence, have been extensively used to study genetic polymorphism at the molecular level. The technique has been employed to detect the genetic differences among varieties/advanced lines/germplasm accessions etc (Belaj et al., 2003). The present findings strengthened the previous findings (Ozkaya et al., 2004), where similar ranges of genetic distances were estimated.

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