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Full Length Research Paper

Genetic variation among Iranian oaks (*Quercus* spp.) using random amplified polymorphic DNA (RAPD) markers

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Quercus **is one of the most important woody genera of the Northern hemisphere and considered as one of the main forest tree species in Iran. In this study, genetic relationships in the genus** ***Quercus*****, using random amplified polymorphic DNA (RAPD) was examined. Five species, including:** ***Quercus robur, Quercus macranthera, Quercus infectoria, Quercus magnosquamata*** **and** ***Quercus libani*** **were collected from Northwest forests of Iran and analyzed. Each tree was judged as a genuine type of each species according to the morphological structures. 10 RAPD primers reproducibly and strongly generated 104 discrete markers, ranging from 100 to 3000 bp. 100 of these were polymorphic (96%), with an average of 10.4 markers per primer. Jaccard`s similarity coefficient was calculated, and a dendrogram constructed, based on unweighted pair group method analysis (UPGMA). Cluster analysis of RAPD data, using the NTSYSpc 2.02 resulted in two clusters. Genetic analysis represented relatively high interspecific genetic distance, ranging from a minimum of 0.298 between** ***Q. libani*** **and** ***Q. magnosquamata*** **to a maximum of 0.625 between** ***Q. macranthera*** **and** ***Q. magnosquamata*****. The range in distance coefficient indicated high variation among species for** ***Quercus*** **genus in Northwest forests of Iran. This result suggests that RAPD marker is useful for** ***Quercus*** **genetic diversity analysis.**

Key words: *Quercus*, genetic diversity, random amplified polymorphic DNA (RAPD), jaccard, polymorphis.

INTRODUCTION

The genus *Quercus* (oak) is one of the most diversified groups of temperate trees with more than 500 species distributed worldwide (Tovar-Sanchez and Oyama, 2004; Olfat and Pourtahmasi, 2010). This genus is native to the northern hemisphere, and includes deciduous and evergreen species extending from cold latitudes to tropical Asia and the America (Olfat and Pourtahmasi, 2010). Hybridization and hybrid zones are common among oaks (Ishida et al., 2003; Gonzalez-Rodriguez et al., 2005). Many *Quercus* species combine sexual reproduction, through acorn production and clonal growth, through root suckering. The progeny vary in response to many factors such as: ecology, selection and genetic drift (Alfonso-Corrado et al., 2005).

Since Darwinian time, botanists have used oaks as a model genus for studying evolutionary processes and speciation. Oaks have characteristics such as high levels of phenotypic plasticity, interspecific gene flow and genetic variation, which significantly contributed to the genesis of several hundreds of species, subspecies and ecotypes (Neophytou et al., 2010). Indeed, these characteristics influence the biological success of the genus *Quercus*. However, these properties also pose difficulties in classification of this genus, estimating genetic differentiation among species and genetic architecture of populations.

In Iran, Zagros forests have been classified as semi arid which cover five million hectares, including 40% of Iran`s forests (Talebi et al., 2003), and *Quercus* is the most abundant tree species in these forests (Erfanifard et al., 2009). Zagros forests cover a vast area of the country,

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Abbreviations: RAPD, Random amplified polymorphic DNA; **UPGMA,** unweighted pair group method analysis.

country, stretching from Piranshahr (West Azerbaijan Province) in the Northwest of the country to the vicinity of Firoozabad (Fars Province), having an average length and width of 1300 and 200 km, respectively. A definitive ruling on taxonomic relationship is still in question in this genus (Kashani and Dodd, 2002).

A probable high frequency of genetic exchange between the involved taxa, hybridization and introgression, incomplete sorting of polymorphisms during relatively rapid and recent speciation processes are factors that cause the neutral genetic variation among oak species (Gonzalez-Rodriguez et al., 2005). Oaks generally have high levels of within-population genetic variation and low differentiation among population (Gomory et al., 2001).

Despite the ecological and economic importance of *Quercus*, natural populations are rapidly disappearing, due to changing of climatic conditions, global warming, illegal logging, forest fires and deforestation, associated with conservation of lands for agriculture, fruticulture and pasture (Saenz-Romero and Tapia-Olivares, 2003). Long generations, inadequate seed production relative to demand, the impossibility of storing seeds for long periods, and hardly vegetative propagation, pose difficulties in forestry and *Quercus* improvement programs (Valladares et al., 2006). Protection and conservation of high value forest genetic resources requires information on the patterns of genetic variation among and within populations (Saenz-Romero et al., 2003). This aim can be achieved by molecular genetic markers that have provided a powerful and reliable new tool for breeders to search for new sources of genetic diversity, polymorphisms and relationship of populations of different origins (Baig et al., 2008).

Identification of plant genotypes through DNA molecular markers seems to be more beneficial than traditional morphological markers because it allows direct access to the hereditary material. On the other hand, morphological characteristics vary in response to ecological factors (Alfonso-Corrado et al., 2005; Ranjan and Aparajita, 2010). Among numerous molecular markers used for this purpose, random amplified polymorphic DNA (RAPD) markers, based on the polymerase chain reaction (PCR) with random primers, are commonly used.

The methodology of this analysis and its procedure are described in several good reviews (Bardakci, 2001; Liu and Cordes, 2004). *Quercus* species and populations of Iran have not been examined before with RAPD markers. The scope of this research was the evaluation of the possibility of using RAPD markers to assess genetic variation among five species of this genus from different parts of Zagros Mountains territory in West Azerbaijan.

MATERIALS AND METHODS

Five *Quercus* species including: *Quercus robur, Quercus infectoria, Quercus libani, Quercus magnosquamata and Quercus macranthera,* distributed in temperate deciduous Zagros Forests in West Azerbaijan, were selected for the isolation of DNA. Each tree was judged as a genuine type of each species, according to the morphological structures of leaves, cupules, acorns and trichomes. Each tree was originated from seed, with 50 to 120 years of age. For each tree, three to five young intact leaves were collected for the molecular analysis. The leaves were immediately frozen in liquid nitrogen and then transferred to a -80°C freezer until further analysis.

Total genomic DNA isolation

Total DNA was extracted using a DNeasy Plant Mini kit (Qiagen). The concentration and quality of each DNA sample were estimated by comparing the intensity of bands of the analyzed DNA with a 100 to 3000 bp DNA ladder, after electrophoresis in 1% agarose gel. A genetic analysis was performed, using the RAPD methodology (Williams et al., 1990; Welsh and McClelland, 1991), through PCR. A total of 18 RAPD primers were purchased from commercial source Cinnagen, Tehran and tested to find specific diagnostic markers for each species.

PCR was carried out in Master cycler gradient (Eppendorf). The reaction volume was 25 µl. The reaction mixture was prepared using a PCR-Master kit (Cinnagen, Tehran) that contained dNTP, PCR buffer, $MgCl_2$ and Taq DNA polymerase enzyme. The amplification included preliminary denaturation (3 min, at 94°C) and 35 cycles as follows: denaturation (30 s, at 94°C), annealing (40 s, at 40°C), polymerization (1.30 min, at 72°C), and an additional final step of DNA synthesis (10 min, at 72°C). Each amplification reaction was conducted with one unique primer. The PCR products were electrophoresed at 80 V for 2 h on 1.5% agarose gels and photographed under ultraviolet light, after staining with ethidium bromide, through a Gel Logic 212 Pro Imaging System (Carestream, USA). A 100 to 3000 bp DNA ladder (Fermentas) was included in all gels as a reference, to estimate the size of the amplified fragments. To assess the reproducibility of PCR products, the DNA from each species was first amplified independently three times with each primer, and the presence/absence of each band was then scored in all individuals.

Data analysis

Amplified fragments were recorded as absent (0) or present (1) in all individuals for each fragment. These two possible states were considered as the molecular phenotypes, resulting from the expression of two alleles at a single locus, one dominant and one recessive; the dominant being the one that determines the presence of the band. The frequency of the two alleles at each locus can be inferred from the frequency of presence and absence of the band (Gonzalez-Rodriguez et al., 2005). Molecular diversity among species was assessed by calculating the percentage of polymorphic fragments (P%). Estimates of genetic similarity were calculated according to Jaccard's coefficient (Jaccard, 1908). A cluster analysis, using an unweighted pair group method with arithmetic averages (UPGMA) was performed. The analysis was plotted in the form of a dendrogram. All computations were carried out using the NTSYS-Pc, Version 2.02 package (Rohlf, 1993).

RESULTS

As a result, 10 primers were selected out of 18 RAPD oligonucleotides screened, as they generated clear and scorable bands with considerable polymorphism (Table1). The reproducibility of RAPD markers amplifications was

Table 1. List of the RAPD primers used in the present study.

Primer	Sequence	C+G (%)
OPB-01	5´-GTTTCGCTCC-3´	60
OPB-02	5´-TGATCCCTGG-3´	60
OPB-03	5´-CATCCCCCTG-3´	70
OPB-04	5´-GGACTGGAGT-3´	60
OPB-05	5´-TGCGCCCTTC-3´	70
OPD-05	5´-TGAGCGGACA-3´	60
OPD-08	5´-GTGTGCCCCA-3´	70
OPD-11	5´-AGCGCCATTG-3´	60
OPA-03	5´-AGTCAGCCAC-3´	60
OPA-04	5´-AATCGGGCTG-3´	60

Table 2. Primary analysis of PCR amplification products using selected primers.

Primer	Number of bands	Number of polymorphic bands	Polymorphism (%)	Molecular weight range (bp)
OPB-01	9	9	100	350 to 3000
OPB-02	4	4	100	100 to 1200
OPB-03	9	9	100	100 to 2000
OPB-04	10	10	100	300 to 2000
OPB-05	14	12	85.7	100 to 2000
OPD-05	11	11	100	200 to 2000
OPD-08	8	7	87.5	200 to 2000
OPD-11	12	12	100	100 to 1500
OPA-03	16	15	93.75	200 to 2000
OPA-04	11	10	90.9	300 to 2000
Total	104	100	96	100 to 3000

determined by performing separate runs of PCR with different preparations of DNA. No significant differences were observed in different experiments although; occasional variation in the intensities of individual bands was detected.

Bands with the same mobility were considered as identical fragments, receiving equal values regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data of that region were not included in the analysis. A list of the main characteristics displayed by the RAPD primers used in this study is presented in Table 2. Each primer generated a specific RAPD profile. Figure 1 exemplifies a pattern of RAPD DNA profile obtained with OPB-04, OPB-05, OPD-05, OPD-08 and OPD-11 primers, respectively. Using 10 RAPD primers, 104 bands were totally produced (an average of 10.4 bands per primer), 100 of which resulted to polymorphic (96%). The number of bands varied from four (OPB-02) to 16 (OPA-03). The size of the amplified fragments extended from 200 to 3000 bp. The percentage of polymorphism ranged from 85.7 to 100% (Table 2).

Estimates of genetic relationships were obtained from Jaccard's similarity coefficient data, which ranged from a minimum 0.375 between *Q. macranthera* and *Q. magnosquamata* to a maximum 0.702 between *Q. libani* and *Q. magnosquamata*. The genetic similarity matrix derived from RAPD data are presented in Table 3. Based on Jaccard's similarity coefficient, a dendrogram (Figure 2) was generated by adopting UPGMA. The five species of *Quercus* could be grouped into two clusters: Cluster I comprised of two species, *Q. libani* and *Q. magnosquamata*; *Q. robur, Q. infectoria* and *Q. macranthera* were grouped in cluster II.

DISCUSSION

The present study offers an optimization of RAPD primers screening for the evaluation of genetic relationships among five *Quercus* species collected from the Zagros Mountains in West Azerbaijan. *Quercus* is the most abundant tree species in this area (Erfanifard et al., 2009). RAPDs have all the advantages of a PCR-based marker, with added benefits: (1) Primers are commercially available and do not require any prior knowledge of the target DNA sequence or gene organization, (2) simplicity, (3) broad applicability and (4)

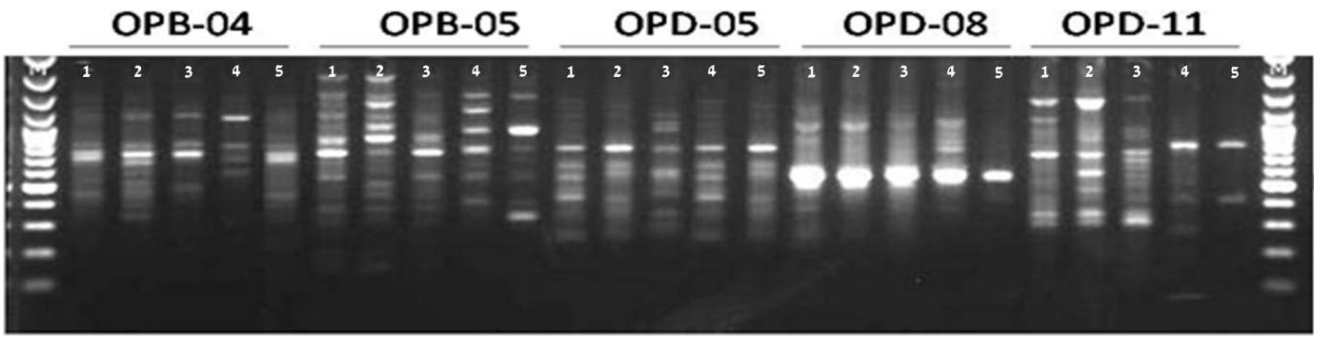

Figure 1. RAPD DNA profile using OPB-04, OPB-05, OPD-05, OPD-08 and OPD-11 primers, respectively. M, DNA ladder 100 to 3000 bp. Numbers 1, 2, 3, 4 and 5 represent *Q. robur, Q. infectoria, Q. libani, Q. macranthera and Q. magnosquamata*, respectively.

Table 3. Similarity matrix values on RAPD data among five *Quercus* species.

Species	*Q. robur*	*Q. macranthera*	*Q. infectoria*	*Q. libani*	*Q. magnosquamata*
Q. robur	1.000				
Q. macranthera	0. 596	1.000			
Q. infectoria	0.615	0.558	1.000		
Q. libani	0.519	0.385	0.538	1.000	
Q. magnosquamata	0.413	0.375	0.567	0.702	1.000

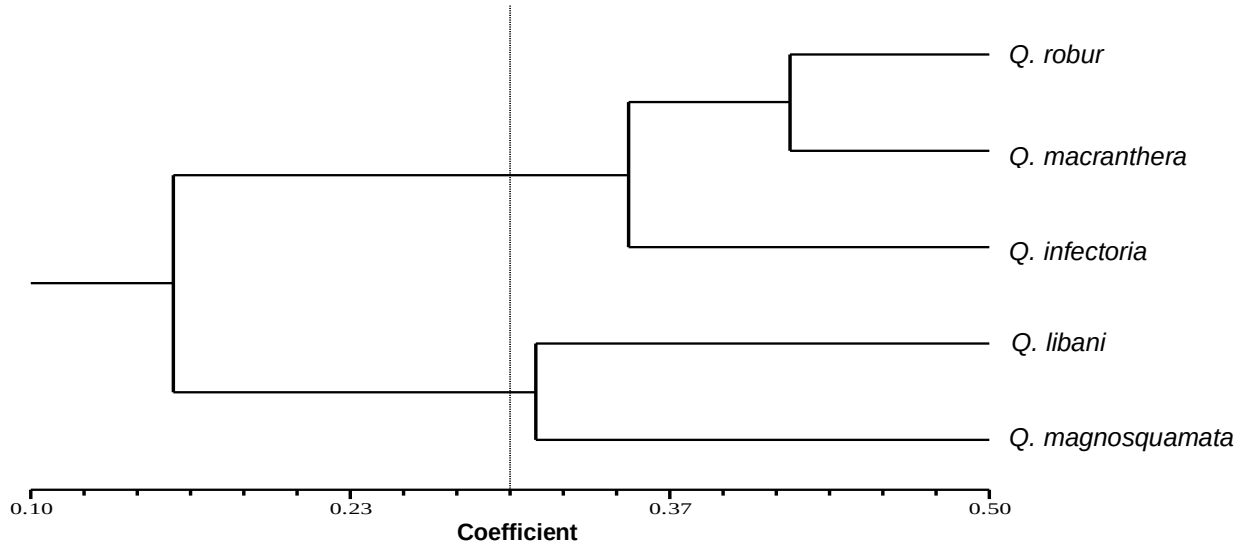

Figure 2. Dendrogram derived from UPGMA cluster analysis using Jaccard's coefficient of RAPD data.

low cost (Baig et al., 2008; Bardakci, 2001).

Despite the shortcomings, such as the difficulty of demonstrating mendelian inheritance of the loci, the inability to distinguish between homozygotes and heterozygotes, and problems with reproducibility in amplification of RAPD markers and data scoring (Liu and Cordes, 2004), the RAPD methodology proved to be an efficient method in assessing genetic diversity (Sekena et al., 2010).

The result shows that RAPD marker system could efficiently distinguish five species of *Quercus,* and reveal the molecular relationships among them. Numerous

publications have appeared concerning genetic variability in various oak species in the world using RAPD markers (Lee et al., 1997; Yakovlev and Kleinschmidt, 2002; Tovar-Sanchez and Oyama, 2004; Gonzalez-Rodriguez et al., 2005). All these studies have provided ample evidence of intra specific variability in phenotypic characters of one important species, *Q. robur*.

Based on bioecological, morphological and phenol-logical traits, pedunculate oak varieties were identified and described (Yakovlev and Kleinschmidt, 2002). In Europe, RAPD markers were applied for comparing *Q. robur* and *Q. petraea* (Muir et al., 2000; Ponton et al., 2001; Kremer et al., 2002; Kelleher et al., 2005). Outside the European continent, studies have been conducted in North America, comparing *Q. grisea* and *Q. gambelii* (Sanchez de Dios et al., 2006), *Q*. *douglasii* and *Q. lobata* (Craft et al., 2002), *Q. crassifolia* and *Q. crassipes* (Tovar-Sanchez and Oyama, 2004), and *Q. affinis* and *Q. dentata* (Ishida et al., 2003). To our knowledge, no report has been recorded on differentiating species of *Quercus* based on genetic markers in Iran, although this genus is the most important broadleaf forest tree both ecologically, and because of its valuable timber (Valladares et al., 2006).

Oaks are renowned for posing problems in defining species boundaries which leads to taxonomic problems in differentiating species (Kashani and Dodd 2002), interspecific hybridization, gene flow; and shared ancestral polymorphisms are the most common reasons for the pattern of variation (Tovar-Sanchez and Oyama, 2004). Due to the recent processes concerning degradation of oak forests, the conservation of the oak gene pool has become urgent (Yakovlev and Kleinschmidt, 2002).

We hope the result of this study can provide an important input into determining efficient management strategies for conservation of valuable gene pool of *Quercus* in Northwest forests of Iran. This study will also be a start point for future investigation aimed at defining the level of intra and inter-specific genetic diversity, distinguishing hybrids among species.

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