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Genetic variation in critically endangered plant *Amsonia orientalis* Decne

Özlem Aksoy, Tuba Erbulucu, Fazıl Özen, Asuman Deveci

Kocaeli University, Faculty of Science and Literature, Department of Biology, Umuttepe Campus, 41380 Kocaeli, Turkey

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Abstract

Amsonia orientalis Decne. (Apocynaceae) is a medical plant which has very restricted distribution only in west of Turkey and east of Greece on the world. Because of containing various alkaloids this species has anticancerogenic and antitumour effects. RAPD-PCR and SDS-PAGE were used to investigate the pattern of genetic variation among four known natural populations of A. orientalis. Polymorphic bands were obtained with 34 primers and pair wise genetic distance between samples was calculated using POPGENE package version 1.32. Genetic distances were calculated for all the species studied at both RAPD-PCR and SDS-PAGE methods. According to RAPD-PCR results the lowest genetic identity value (0.2881) was found between populations Ömerli and Gaziosmanpaşa, while the highest genetic distance value (1.2443) was found between populations Gaziosmanpaşa and Ömerli. The dendogram which is obtained from the SDS-PAGE analyses, allows two main groups to be distinguished. The first group contains Ömerli population and the second group has Gaziosmanpaşa, Paşaalanı and Adnanmenderes populations. The observed genetic variations showed that Ömerli and one of Balıkesir population should be considered in any planned in situ or ex situ conservation programs for this critically endangered plant species..

***Corresponding Author:** Özlem Aksoy ✉ ozlem.aksoy@kocaeli.edu.tr

Introduction

Long-term survival and evolution of species depend on the maintenance of sufficient genetic variation within and among populations to adapt to new selection pressures as those exerted by environmental changes (Barret and Kohn, 1991). Genetic diversity maintained in a plant species would be influenced by many processes, such as the long-term evolutionary history and the characteristics of the species, including genetic drift, gene flow, mode of reproduction and mating system (Hamrick and Godt, 1989). Understanding of the genetic variation within and between populations is therefore required for the establishment of effective and efficient conservation strategies for endemic and endangered plant species (Hamrick and Godt, 1996). In the past few years, an increasing number of plant species' natural populations have been small or isolated due to habitat destruction or fragmentation (Shrestha *et al.*, 2002). More and more researches have been focused on the importance and feasibility of appropriate strategies for conservation of the endangered plants (Hogbin *et al.*, 1998; Jian *et al.*, 2006). Knowledge of the levels and distribution of genetic variation is essential to understand the population dynamics, adaptation and evolution and is efficient in devising conservation strategies of endangered plants (Zawko *et al.*, 2001; Premoli *et al.*, 2000). Random amplified polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) was considered as one of the simplest and most cost-effective techniques and has been widely used extensively to analyze genetic diversity of population genetics and conservation genetics (Artyukova *et al.*, 2004; Wang *et al.*, 2006). This technique is simple, PCR-based, require no prior sequence information, and a large number of putative loci may be screened quickly. RAPD markers are considered to be a rapid tool for assessing the genetic diversity at the molecular level (Williams *et al.*, 1990; Williams *et al.*, 1993). Their analysis provides a high resolution discrimination of samples and can be carried out on small amounts of DNA (Carter and Sytsma, 2001). Some limitations of RAPDs, such as poor reproducibility in early RAPD analyses, has been largely overcome through improved, strictly applied laboratory techniques and scoring procedures (Nybom and Bartish, 2000). If used carefully, results obtained from RAPDs are reliable (Jolner *et al.*, 2004), as shown by Collins *et al.* (2003) for Taxus species and hybrids. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a biochemical method and is most widely used due to its validity and simlicity for describing genetic structure of plant collections. Seed storage proteins have been used as genetic markers in analyses of genetic diversity within and between species, genetic resources conservation and breeding, genome relationship, as a tool in crop improvement (Ghafoor *et al.*, 2000)

A. orientalis Decne [Syn. Rhazya orientalis (Decne.) A. D.C.], a dicotyledon, is a medicinal plant which has very restricted distribution only in Turkey and Greece in the world (Tutin *et al.*, 1998; Davis, 1978). There is no record about the populations of this species in Greece since 1973 (Zahariadi, 1973). Therefore it is doubtful if A.orientalis populations are stil exist naturally in Greece. Tutin *et al.*, (1998) reported the presence of A. orientalis in three localities in Turkey, these are Apolyont (Lake Uluabat), Menekşe Deresi (Istanbul) and Hıdırlık Tepe, Balıkesir. However the plant was not encountered in these localities in the study by Özen, (2006), only small populations were reported in Gazi Osman Paşa, Adnan Menderes, and Paşaalanı Districts in Balıkesir. A. orientalis is placed in “critically endangered” (CR) category according to the Red Data Book of Turkish Plants and placed among the plants which must be protected Europe-wide by European Council in the plant list according to Bern Convention (Ekim *et al.*, 2000). So the habitat of A.orientalis has been badly destroyed and individual number decreased rapidly because of human overexploitation, which might be the main cause inducing endangered status of the rare resources. A. orientalis, like the other members of Apocynaceae has medical and economical importance as well as it contains many glycosides and glycoalkaloids. The plant has also antimicrobial

activity. As mentioned in literature, these biochemical substances in the plant structure can be used in treatment of various diseases, especially in heart disease and cancer. A. orientalis, commonly called as "blue star" or "oriental Rhazya", is also cultivated as an ornamental plant in some gardens in Europe because of its attractive star like blue-purple flowers.

Literature on A. orientalis, which is critically endangered in nature according to IUCN categories, is very limited in number. Nevertheless, anatomy, morphology, palinology of A. orientalis have been investigated and it was found that extracts of this plant have strong antimicrobial activity against microorganisms (Akyalçın *et al.*, 2006). Furthermore, it is known to have anti-tumor and anti carcinogenic effects because of its various alkaloids (Dabine Lengyel *et al.*, 1986; Rahman and Zaman 1988; Rahman *et al.*, 1989). In addition, it has been shown that A. orientalis prefered soils with sandy-loamy, saltness, slightly alkalina, mid-calcerous, poor organic material, and with very rich iron and magnesium (Özen, 2006). The main aim of this study was to explore the levels of genetic variability within populations, identify levels of genetic differentiation among populations, and provide baseline information for establishment of conservation strategies for A. orientalis .

Materials and methods

Plant material

The natural populations of A.orientalis were sampled throughout its distribution region after analysing records in Flora of Turkey (Table 1.). A.orientalis populations were only reported from the west of Turkey and east of Greece in 1968, since then there were no records (Tutin, 1968). As the populations of A.orientalis had a very restricted distribution in Turkey and also in the world, four different populations that could only be found and collected from Balıkesir (Adnanmenderes, Paşaalanı, Gaziosmanpaşa) and İstanbul (Ömerli) (Figure 1.) were used as material. General appearance of A.orientalis is shown in Figure 2. Fresh leaves were collected randomly from 10 plants in each population and preserved in freezer box. The sampled leaves were immediately stored at -86 °C in plastic bags until DNA extraction. The distances between sampled plants were from 1 to 5 m, depending on the population size.

Table 1. Populations of A.orientalis sampled for RAPD analysis.

Population code*	Locality	Altitude(m)	Sample size
Ö	İstanbul, Ömerli	154	5
A	Balıkesir, Adnan Menderes	258	5
P	Balıkesir, Paşa Alanı	128	5
G	Balıkesir, Gaziosmanpaşa	180	5

The code for each population corresponds to the codes in figures and tables.

Table 2. Characteristics of RAPD primers used for generating RAPD markers of A.orientalis sampled from four natural populations.

Primer No	*Primer*	*Sequence 5'-- 3'*	*Tm (°C)*	GC percentage (%)
1	*OPA 01*	*CAGGCCCTTC*	*34*	*70*
2	*OPA 02*	*TGCCGAGCTG*	*34*	*70*
3	*OPA 03*	*AGTCAGCCAC*	*32*	*60*
4	*OPA 04*	*AATCGGGCTG*	*32*	*60*
5	*OPA 05*	*AGGGGTCTTG*	*32*	*60*
6	*OPA 06*	*AGGGGTCTTG*	*32*	*60*
7	*OPA 07*	*GAAACGGGTG*	*32*	*60*
8	*OPA 08*	*GTGACGTAGG*	*32*	*60*
9	*OPA 09*	*GGGTAACGCC*	*34*	*70*
10	*OPA 10*	*GTGATCGCAG*	*32*	*60*
11	*OPC 05*	*GATGACCGCC*	*34*	*70*
12	*OPC 06*	*GAACGGACTC*	*32*	*60*
13	*OPC 08*	*TGGACCGGTG*	*34*	*70*
14	*OPC 09*	*CTCACCGTCC*	*34*	*70*
15	*OPC 10*	*TGTCTGGGTG*	*32*	*60*
16	*OPC 11*	*AAAGCTGCGG*	*32*	*60*
17	*OPC 12*	*TGTCATCCCC*	*32*	*60*
18	*OPC 13*	*AAGCCTCGTC*	*32*	*60*
19	*OPC 14*	*TGCGTGCTTG*	*32*	*60*
20	*OPC15*	*GACGGATCAG*	*32*	*60*
21	*OPC 16*	*CACACTCCAG*	*32*	*60*
22	*OPC 18*	*TGAGTGGGTG*	*32*	*60*
23	*OPC 19*	*GTTGCCAGCC*	*34*	*70*
24	*OPC 20*	*ACTTCGCCAC*	*32*	*60*
25	*OPN 01*	*CTCACGTTGG*	*32*	*60*
26	*OPN 02*	*ACCAGGGGCA*	*34*	*70*
27	*OPN 03*	*GGTACTCCCC*	*34*	*70*
28	*OPN 04*	*GACCGACCCA*	*34*	*70*
29	*OPN 05*	*ACTGAACGCC*	*32*	*60*
30	*OPAF 05*	*CCCGATCAGA*	*32*	*60*
31	*OPAF 06*	*CCGCAGTCTG*	*34*	*70*
32	*OPAF 10*	*GGTTGGAGAC*	*32*	*60*
33	*OPAF 11*	*ACTGGGCATC*	*32*	*60*
34	*OPAF 14*	*GGTGCGCACT*	*34*	*70*

Table 3. Nei's original measures of genetic identitiy and genetic distance.

Pop Name	Ö	A	P	G
Ö	****	0.3729	0.4576	0.2881
A	0.9865	****	0.6610	0.5085
P	0.7817	0.4140	****	0.5593
G	1.2443	0.6763	0.5810	****

Table 4. The lenght between the populations.

Between	*And*	*Length*
3	*Ö*	*50.20866*
3	*A*	*18.77441*
2	*Ö*	*10.73546*
1	*A*	*20.69879*
1	*P*	*20.69879*
2	*G*	*31.43425*

Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

Genomic DNA was extracted from 0.1-0.2 g powdered leaf tissue by using a Qiagen DNeasy Plant Mini Kit. The quantity and quality of DNA were determined with a spectrophotometer Shimadzu UV-mini 1240. A set of 40 random 10-mer primers was purchased from Thermo Scientific (Table 2). PCR amplifications were performed in a 25 µl reaction mixture containing 10 ng of template DNA, 1X Taq polymerase buffer and 1 U of Taq polymerase, and 2.5 mM MgCl2, 1 µM dNTP, 1 mM primer. Amplifications were performed in a TC-3000 Thermal Cycler. The cycle programme included an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 30°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 5 min. The PCR products were separated on a 1 % agarose gel containing ethidium bromide (0.5 µg/mL) and digitally photographed with the UVP GelDoc-It 310 Imaging System. A 1 kb DNA ladder was used as size marker (Fermentas). A negative control with no DNA template was also included in each PCR amplification in order to verify the absence of contamination.

Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Leaf total protein isolation was done as described by Saraswati and Matoh (1993). Following leaf protein extraction the Bradford Assay method was used to analyze the concentrations of proteins present in the leaf (Bradford, 1976). The total SDS-PAGE was carried out following the Laemli's method (1970). Proteins on the gel were fixed and stained with Coomassie G-250 overnight according to Demiralp *et al.* (2000).

Data analysis

Since RAPD-PCR markers are dominant, it was assumed that each band represents the phenotype at a single biallelic locus (Williams *et al.*, 1990). A binary matrix was produced by scoring each amplified fragment as present (1) or absent (0) from each individual. Only clear and distinct bands were scored. Bands having the same gel mobilities were assumed to be homologous. The matrix was used to produce an input file and analyzed using the software program POPGENE 1.32 (Yeh *et al.*, 1999). In order to examine the genetic relationship of the populations, the UPGMA tree was constructed based on the genetic distance among different populations, modified from NEIGHBOR procedure of PHYLIP version 3.5.

Results and discussion

For the RAPD-PCR analysis the four known natural populations of A. orientalis were used. 40 random primers were tested and 118 polymorphic bands were obtained from 237 clear and reproducible bands with 34 primers (Figure 3). Dendrogram obtained from RAPD-PCR results allowed two main groups to be distinguished. The upper group was Ömerli population. The lower group was Adnanmenderes, Paşaalanı and Gaziosmanpaşa populations (Figure 4). The ovearall values for mean observed number of alleles (n_a) and mean effective number of alleles (n_e) were 2.000 and 1.661, respectively. Assuming the Hardy-Weinberg equilibrium, the average gene diversity was 0.394 (H_t) at the species level. The mean value of the

Shannon's information index (I) was 0.582 at the species level. Nei's genetic distance between populations varied from 0.2881 to 1.2443. The lowest genetic distance value (0.2881) was found between populations Ömerli and Gaziosmanpaşa, while the highest (1.2443) between populations Gaziosmanpaşa and Ömerli (Table 3). An UPGMA dendrogram was constructed based on Nei's genetic distance (Figure 4). The lenght between the populations is shown in Table 4.

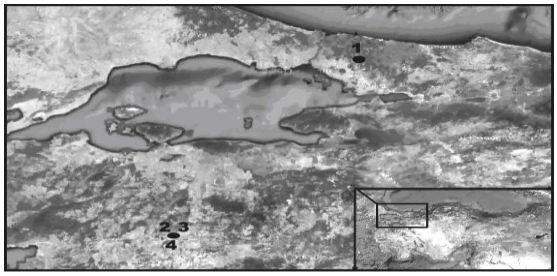

Fig. 1. Geographic location of four A.orientalis populations sampled from Turkey. İstanbul, Ömerli (1), Balıkesir, Adnan Menderes (2), Balıkesir, Paşa Alanı (3), Balıkesir, Gaziosmanpaşa (4).

Fig. 2. General view of *A. orientalis*.

Genetic markers are important tools in the determination of genetic diversity, which is the first step for breeding projects and the protection of genetic resources (Bardakçı, 2001). Rare endangered plants may have high diversity, which attributed to the following factors: insufficient length of time for reduction of genetic diversity following a natural reduction of population size and isolation; recent fragmentation of a once continuous genetic system by human activity (Zawko *et al.*, 2001; Maguire and Sedgley, 1997). Generally, geographically restricted species exhibit lower levels of genetic variation than widely distributed species (Hamrick and Godt, 1996). The breeding systems, life forms and seed dispersal mechanisms of plant species have been regarded as the main factors affecting levels of genetic diversity, genetic divergence, and genetic structure within and among plant populations (Hamrick and Godt, 1996; Loveless and Hamrick, 1984). Outcrossing perennial species commonly have higher levels of genetic diversity than selfing and clonal plants (Hamrick and Godt, 1996). The population history and habitat type are another important factors shaping the degree of genetic diversity and differentiation (Shikano *et al.*, 2010). High to moderate levels of genetic differentiation among populations is a common pattern in endemic or narrowly distributed plant species (Nybom, 2004; Cruse-Sanders and Hamrick, 2004). Due to the technical simplicity and speed of the RAPD-PCR method, it has been successfully used for the generation of genetic similarities and phylogenetic analysis (Surgun *et al.*, 2012). In their recent review on estimates of genetic diversity obtained by RAPD-PCR markers, Nybom and Bartish, (2000) compiled mean GST values of 0.59, 0.19 and 0.23 for selfing, mixed mating and outcrossing plant species, respectively. Gillies *et al.*, (1999) studied Mahogany populations (Swietenia macrophylla, Meliaceae), a species with wind-dispersed seeds like A. polyneuron, through RAPD markers and observed a Shannon index of diversity of 0.45. As is concluded, populations with high genetic diversity should be firstly protected in conservation biology (Marshall and Brown, 1975). So Ömerli population of A.orientalis should be protected first. But populations of Balikesir in our study, though with lower diversity should also be paid more attention to in avoiding its extinction. Small populations are more prone to be extinct for environmental

fluctuation (Goodman, 1987) and lose a large of genetic variation because of genetic drift.

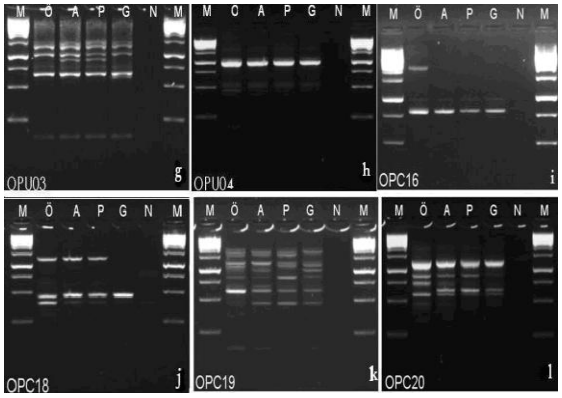

Fig. 3. Examples of the RAPD band profiles of four natural populations of A.orientalis produced by different primers. OPN 01 (a), OPN 02 (b), OPU 02 (c), OPN 03 (d), OPN 04 (e), OPU 05 (f), OPU 03 (g), OPU 04 (h), OPC 16 (i), OPC 18 (j), OPC 19 (k), OPC 20 (l), M: marker 1.0 kb, N: negative control.

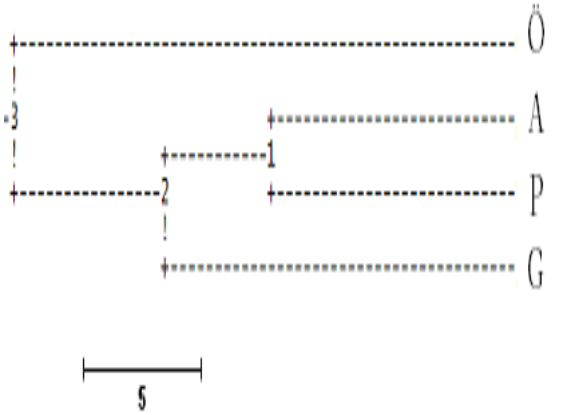

Fig. 4. UPGMA dendrogram based on Nei's genetic distance, showing the genetic relationships between 4 populations of *A. orientalis*.

RAPD-PCR and SDS-PAGE were used to investigate the pattern of genetic variation among some species of Alyssum L. (Brassicaceae); a nickel hyperaccumulator plant growing in Turkey. The genotypes of Alyssum sp. showed genetic similarity values that ranged from 33 to 83 percent. (Babaoğlu *et al*., 2004). Okumuş and Gülümser, (2004) analyzed seed storage proteins of 7 landrace accessions of four Vicia species collected in Turkey by SDS-PAGE. Smilarly Sammour, (1991) showed phylogenetic relationships between species of Vicia by using SDS-PAGE method. In our study protein profiles of four known natural populations of A. orientalis were obtained by SDS-PAGE (Figure 5) and protein density graphics were shown in figure 6. The dendrogram, allowed two main groups to be distinguished (Figure 7). The first group included Ömerli and Gaziosmanpaşa populations, the second group included Paşaalanı and Adnanmenderes populations and their samples were grouped in the same cluster. There was a significant genetic distance between Ömerli and Gaziosmanpaşa populations. According to the genetic relations obtained from leaf protein banding patterns, the species can be put in order as; Ömerli, Gaziosmanpaşa, Paşaalanı and Adnanmenderes.

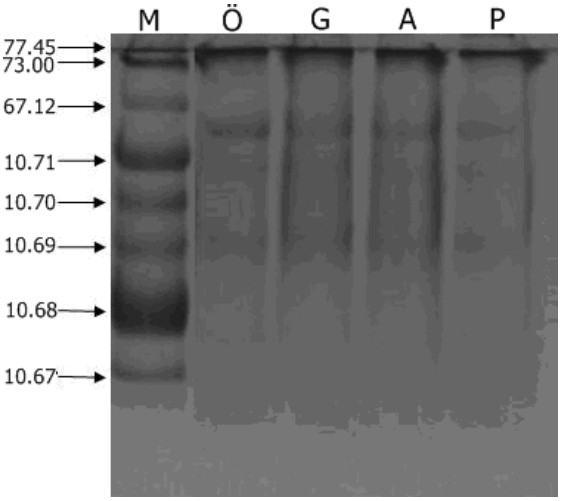

Fig. 5. Protein profiles of 4 populations of A.orientalis (M: Marker, Ö: Ömerli population, G: Gaziosmanpaşa population, A: Adnanmenderes population, P: Paşaalanı population).

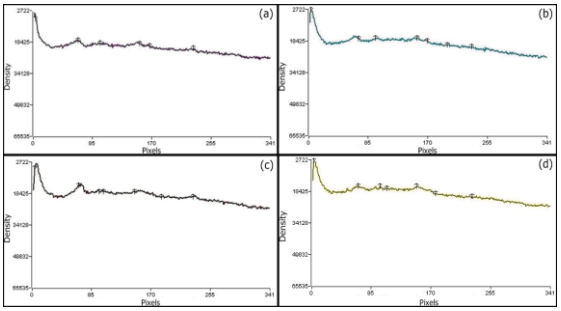

Fig. 6. Protein density graphics of 4 populations of A.orientalis . a: Ömerli population, b: Gaziosmanpaşa population, c: Adnanmenderes population d: Paşaalanı population.

RAPD-PCR and SDS-PAGE analyses showed that there was a genetic variability among four known natural populations of A. orientalis. SDS-PAGE and RAPD-PCR were useful methods as an addition to the morphological investigations in the determination of genetic variation in critically endangered plant A. orientalis. Moreover, populations should be conserved separately because the mixing of populations may give rise to outbreeding depression, loss of adaptation or breakup of coadapted gene complexes (Waser, 1993), which should be avoided during A. orientalis conservation. The results obtained from this study were the first to describe population genetic parameters for this species, and will provide valuable baseline information for future population studies and management actions.

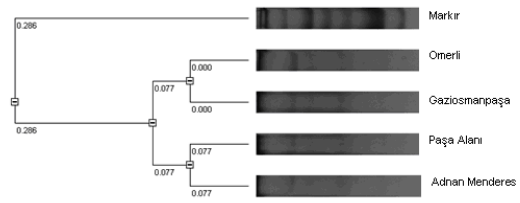

Fig. 7. The genetic relationships between 4 populations of A.orientalis according to the protein profiles

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