African Journal of Biotechnology Vol. 8 (10), pp. 2076-2082, 18 May, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Analysis of genetic variation among accessions of critically endangered *Rhaponticoides iconiensis* and *Rhaponticoides mykalea* based on RAPD and SDS-PAGE markers

Leyla Açık[1*], Fatma Öztürk[1], Mecit Vural[1], Osman Tugay[2] and I.Sefa Gürcan[3]

[1]Gazi University, Faculty of Science, Department of Biology, Ankara, Türkiye.
[2]Selcuk University, Faculty of Science, Department of Biology, Konya, Türkiye.
[3]Ankara University, Faculty of Veterinary, Department of Biostatistics, Ankara, Türkiye.

Accepted 2 March, 2009

Rhaponticoides iconiensis **(Hub.-Mor.) M.V.Agab and Greuter is a rare and endangered endemic species of the Konya region of Turkey. One related taxon, *Rhaponticoides mykalea* (Hub.-Mor.) M.V.Agab and Greuter, is morphologically similar but occurs in different geographical locations. This study has been conducted on the biology of this threatened plant in order to understand better the factors that should be included in the development of conservation practices. The genetic variations were studied using RAPD markers and SDS-PAGE profiles of total seed proteins for three *R. iconiensis* populations and two *R. mykalea* populations. The analyzed *R. iconiensis* populations belonged to different soil types (calcareous and volcanic). The analyzed *R. iconiensis* and *R. mykalea* populations belonged to different bioclimatic zones. A genetic diversity within populations was detected both by SDS-PAGE and RAPD for *R. iconiensis* populations. The level of variation did not differ with respect to soil type for the species studied. Populations collected from the same soil types carried more polymorphisms than those grown in different zones. The genetic diversity was revealed more clearly for all populations by RAPD than through analyzing proteins. Differentiation between ecological groups was higher than that revealed within groups. Conservation programs should take into account the level of genetic diversity within population revealed by these markers according to soil types.**

Key words: *Rhaponticoides iconiensis,* endangered, SDS-PAGE, RAPD.

INTRODUCTION

Rhaponticoides vaill (Asteraceae) is a genus comprising 32 species occurring from Portugal and Morocco in the West to Mongolia in the East (Hellwig, 2004). Most of them are either narrow endemics or have very disjunct areas (Wagenitz, 1986). In Turkey, the genus *Rhaponticoides* is represented with 5 species in the last descriptions (Wagenitz 1975; Davis et al., 1988; Güner et al., 2000; Greuter, 2003; Eren, 2007). Four (*Rhaponticoides iconiensis, Rhaponticoides amasiensis, Rhaponticoides mykalea and Rhaponticoides hierroi*) are endemic to Turkey. *R. iconiensis* and *R. mykalae* are in the CR (critically endangered) threatened category. All of the *Rhaponticoides* species are known as “peygamber çiçegi” in Turkish. The vernacular name of the restricted endemic *R. iconiensis* is “tülüşah or gaşak” (Wagenitz, 1975; Davis et al., 1988; Güner et al., 2000; Ekim et al., 2000). Each species is represented by two subpopulations. The main population of *R. iconiensis* is located in the Akisse and Ortakarahisar districts between Bozkır and Seydişehir in Konya. The other populations disappeared for 10 years from northern site of Karadağ, Karaman due to overgrazing by sheep. *R. mykalea* is inhabited between Davutlar and Yatağan on the roadside macchie and olive garden site. Other small population (c90 individuals) of *R. mykalea* survives around Uluborlu.

*Corresponding author. E-mail: leylaacik@gmail.com. Tel.: + 90 312 2021185. Fax: + 90 312 2122279.

Table 1. Random oligonucleotide primers used for RAPD analysis.

Primer	Primer sequence (5' to 3')	Primer	Primer sequence (5' to 3')
A1	CAGGCCCTTC	OPB 04	AAGTCCGCTC
A2	TGCCGAGCTG	OPB 05	TGCGCCCTTC
A7	GAAACGGGTG	OPB 08	GTCCACACGG
B4	GGACTGGAGT	OPA07	GAAACGGGTG
B6	TGCTCTGCCC	OPO 04	AAGTCCGCTC
B7	GGTGACGCAG	OPO 06	CCACGGGAAG
B18	CCACAGCAGT	OPA17	GACCGCTTGT
SC1023	GGCTCGTACC	OPW 06	AGGCCCGATG
SC1079	CGCCACGTTC	OPC 02	GGTCTACACC
OPR 03	ACACAGAGGGT	OPB 10	CTGCTGGGAC
OPW 10	TCGCATCCCT	OPU 16	CTGCGCTGGA
M13	GAGGGTGGCGGTTCT	OPI 18	TGCCCAGCCT

Dam in Isparta far from the main population in the restricted area.

In the recent years, several molecular techniques have been used for germplasm characterization, variety identification, marker development and identification, molecular diagnostics, phylogenic studies and diversity analysis. Because of its simplicity, rapidity and reliability, the RAPD technique has been used extensively for diversity analysis in many crop species. As this approach utilizes primers of arbitrary sequences, it may be used for different species without previous knowledge of their genomic DNA sequences (Sharma and Jana, 2002).

Since the edible portion of the pulse is the seed, the variation of seed storage proteins has been one of the major concerns for pulse breeders and geneticists. The electrophoresis of seed storage proteins is a method to investigate genetic variation and to classify plant varieties (Sangwan et al., 2003).

The main advantages of seed protein electrophoresis with respect to the conventional morphological approach are independence of the growing season, no need for plant cultivation, availability of material the year around, relative speed of the examination, ease of storing material, and the small sample size needed (Steward and Porter, 1995).

In this study, using RAPD and SDS-PAGE of total seed proteins, *R. iconiensis* and the related taxon *R. mykalea* were studied. The main aim was assess the genetic diversity in endangered *R. iconiensis* species. Additionally, we wanted to determine the variation between the taxon *R. iconiensis* and the related taxa *R. mykalea*.

MATERIALS AND METHODS

Plant material

Seeds were collected from the localities shown below:

R. mykalea - Muğla, Yatağan, 500 m, 15.07.2006, *M.Vural* 9781.
R. mykalea - Isparta, Uluborlu, 1100 m, 16.08.2006, *M.Vural* 9846.
R. iconiensis - Konya, Seydişehir-Bozkır, 1110 m, 13.07.2006, *M.Vural* 9771.
R. iconiensis - Konya, Bozkır-Akisse, 1120 m, 10.09.2006, *O.Tugay* 4410.
R. iconiensis– Konya, Seydişehir-Bozkır, 1050 m, *H.Duman* 6046.

DNA isolation and RAPD amplification

The twenty seeds of each population were grown in a growth chamber to generate leaf tissue for DNA isolation. The young leaves of *R. mykalea* M.Vural 9846, *R. mykalea* M.Vural 9781, *R. iconiensis* O.Tugay 4410, *R. iconiensis* H.Duman 6046 and *R. iconiensis* M.Vural 9771 were used for the DNA isolation. 20 samples representing each population were used (except Duman 6046 which is a herbarium specimen).

Plant DNA extraction was carried out using the CTAB method with minor modifications (Clark, 1997; Acik et al., 2004). DNA was isolated from liquid nitrogen frozen tissue. Fresh young leaves (0.05 g) were ground using mortar. The ground sample was added to 1 ml extraction buffer (2% (w/v) CTAB; 100 mM Tris-Cl buffer (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, (1% 8w/v) PVP-40] and incubated at 65°C for 90 min. The homogenate was mixed with 500 µl 24:1 chloroform: isoamyl alcohol (v/v) and mixed well by gentle inversion. Following centrifugation at 10,000 rpm for 10 min, the upper aqueous layer was transferred to a fresh tube containing 600 µl of isopropanol. The mixture was then allowed to sit at room temperature for 40 min. After centrifugation at 5,000 rpm for 3 min, the pellets were washed twice with 76% ethanol. The pellets were allowed to sit overnight at room temperature and resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA pH 8.0).

The selected 40 arbitrary primers were purchased from Operon Technologies (Alameda, California). Amplification reactions were performed using the protocol reported by Williams et al. (1990). 24 primers with reproducible results were selected for calculations (Table 1). At least three or four samples representing each taxon were used for amplification. Amplification was repeated twice for each sample. For the DNA amplification, a Biometra thermocycler (T Personel) was programmed for 45 cycles at at 96°C for 30 s, 35°C for 30 s and 72°C for 30 s, for denaturing, annealing and primer extension, respectively (Williams et al., 1990). 15 µl of reaction products were separated alongside molecular weight markers (100 bp DNA ladder) by electrophoresis, on 1.5% agarose gels containing ethidium bromide. The gels were photographed under UV light, and the amplification patterns were examined (Maniatis et

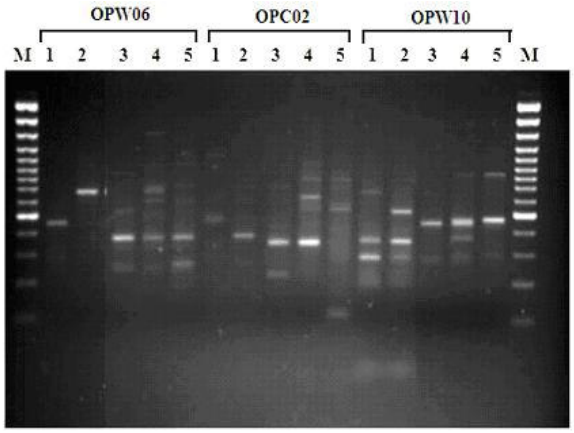

Figure 1. RAPD profiles of *Rhaponticoides* species generated with the random primers. M-100 bp DNA Ladder,1- *R. mykalea* M Vural 9846, 2- *R. mykalea* M Vural 9781, 3- *R. iconiensis* O Tugay 4410, 4- *R. iconiensis* H Duman 6046, 5- *R. iconiensis* M Vural 9771.

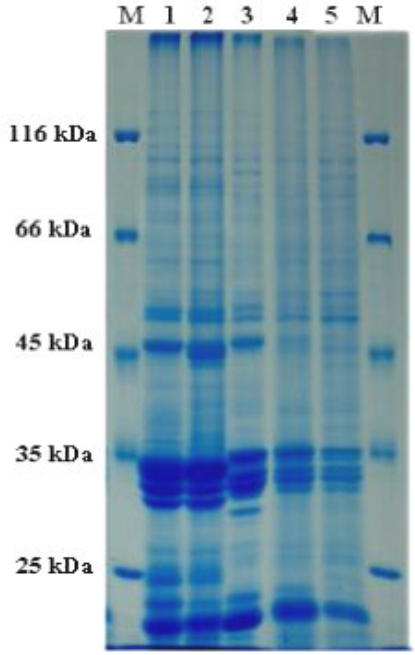

Figure 2. Protein profiles of two *Rhaponticoides* species. M-Protein Marker, 1- *R. mykalea* M Vural 9846, 2- *R. mykalea* M Vural 9781, 3- R. iconiensis O Tugay 4410, 4- *R. iconiensis* H Duman 6046, 5- *R. iconiensis* M Vural 9771.

al., 1989). RAPD images were scored used for the analysis of the amplification products. Only the reproducible bands in multiple runs were considered in this study.

Seed protein analysis

Protein extraction was performed according to Saraswati and Matoh (1993). Electrophoresis was carried out following the Laemmli method (1970). Each run included known molecular weight marker proteins (Fermentas). Proteins on the gel were fixed and stained with Comassie Brilliant Blue G-250 as described by Demiralp et al. (2000). Molecular weights of protein bands were estimated by their relative mobilities.

Cluster analysis

Bands on RAPD and SDS-PAGE gels were scored as either present (1) or absent (0) for all subspecies studied. Common band analysis was conducted using the computer program POPGEN. A UPGMA cluster analysis was performed on the dissimilarity matrix using the genetic distance method modified from the NEIGHBOR procedure of PHYLIP Version 3. The values for genetic distance were then used in a UPGMA cluster analysis to generate a dendrogram (Nei, 1972). The statistical significance within *R. iconiensis* population was tested using the molecular variance (AMOVA) approach used in Arlequin (Excoffier and Schneider, 2005).

RESULTS AND DISCUSSION

R. iconiensis and *R. mykalae* are in the CR threatened category. The M.Vural 9771, O.Tugay 4410 and H.Duman 6046 populations of *R. iconiensis* grow in calcerous soil. The population survived in north skirt of Karadağ, Karaman in volcanic substrate. One of the *R. mykalea* inhabits an area between Davutlar and Yatağan on the roadside in disturbed macchie and olive garden site. The other small population of *R. mykalea* survives around the Uluborlu Dam in Isparta. They are from two different ecological and bioclimatic zones.

The species have survived in a very small area in Bozkır, Konya and consisted of only a few plants with some growing across the water canal. Therefore the locations of different populations were not far from each other. The screens of all 20 individuals from each population with the 24 RAPD primers generated a total of 98 reliable fragments, none of which were polymorphic bands. The banding profile of three of the individuals representing different locations is given in Figure 1. The approximate size of the fragments ranged from 100 to 1500 bp. The banding profile of seed storage proteins is also given in Figure 2. The genetic distances between two species are calculated (Table 2) and the combined dendrogram is constructed by UPGMA (Figure 3). According to the data obtained from RAPD-PCR and SDS-PAGE results, the genetic distances between *R. mykalea* and *R. iconiensis* is 70% and *R. iconiensis* O.Tugay 4410 to *R. iconiensis* H.Duman 6046 and *R. iconiensis* M.Vural 9771 are 42 and 64%, respectively. That of *R. iconiensis* M.Vural 9771 to *R. iconiensis* H.Duman 6046 is 34%.

We studied genetic relationships among *R. iconiensis* populations to relate morphological to molecular data in order help conservation biologists to be effective in saving endangered native Turkish *Rhaponticoides* species. As far as *R. iconiensis* is concerned, we found significant genetic differentiation using seed protein and RAPD analysis. Statistical significance within the *R. iconiensis* population was tested using the molecular

Table 2. Genetic distance data between two *Rhaponticoides* species (RAPD and SDS-PAGE data).

Species	*R. mykalea* (M.Vural 9846)	*R. mykalea* (M.Vural 9781)	*R. iconiensis* (O.Tugay 4410)	*R. iconiensis* (H.Duman 6046)	*R. iconiensis* (M.Vural 9771)
R. mykalea (M.Vural 9846)	****				
R. mykalea (M.Vural 9781)	0.19	****			
R. iconiensis (O.Tugay 4410)	0.40	0.43	****		
R. iconiensis (H.Duman 6046)	0.67	0.70	0.42	****	
R. iconiensis (M.Vural 9771)	1.05	1.09	0.64	0.34	****

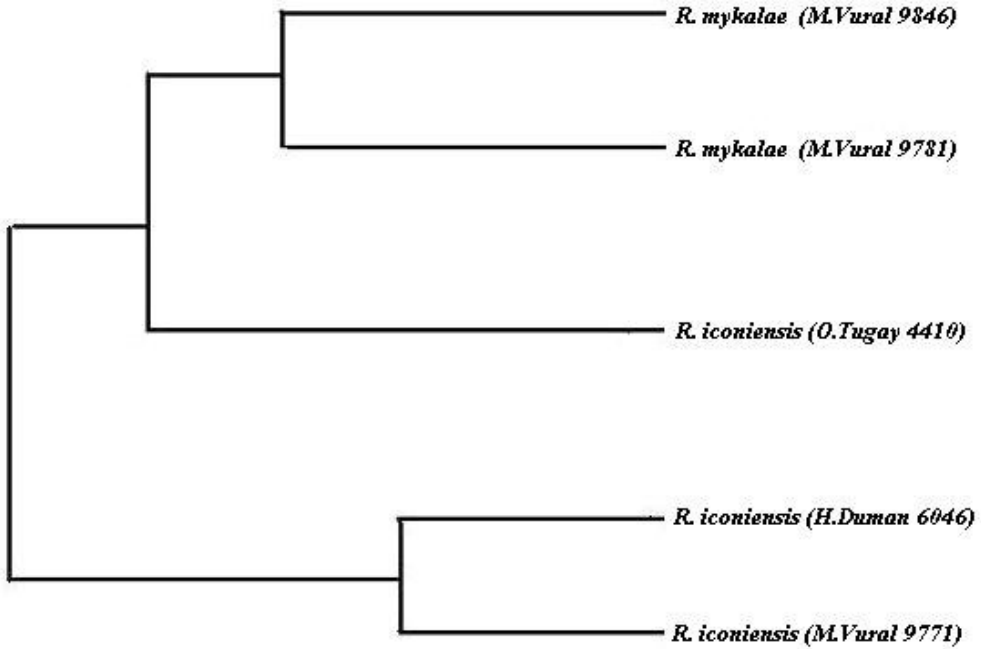

Figure 3. Dendrogram obtained by analysis combined data of RAPD and SDS-PAGE marker data from two *Rhaponticoides* species.

variance (AMOVA) approach used in Arlequin. The covariance components are used to compute fixation index (Table 3). According to the results, there is a large genetic differentiation between the population accessions H.Duman 6046 and O.Tugay 4410, F_{ST} 0.19. We also showed a large genetic differentiation between accession M.Vural 9771 and O.Tugay 4410, F_{ST} 0.20. Although, the sample accessions M.Vural 9771 and H.Duman 6046 were from different altitudes, there is a very little differentiation between them, F_{ST} 0.04, indicating that genetic variation is reduced between these populations.

The UPGMA dendrogram obtained from the cluster analysis of genetic distances from RAPD and protein analysis showed that the accessions O.Tugay 4410 and M.Vural 9771 displayed that greatest differentiation (Table 2). The accessions H.Duman 6046 and M.Vural 9771 are clustered together in the same group and surprisingly, O.Tugay 4410 is grouped together with another taxon, *R. mykalea*. We suggest that more attention must be paid to population O.Tugay 4410 which presents the highest genetic variability. Borba et al. (2007) suggested that *in situ* conservation proposals should put priority on protecting populations with the highest number of individuals. They also added that a larger number of populations need to be protected in order to maintain a large amount of the genetic diversity of the species.

When we used only the results of RAPD-PCR, the po-

Table 3. Population pairwise F_{ST} comparisons.

Species	*R. mykalea* (M.Vural 9846)	*R. mykalea* (M.Vural 9781)	*R. iconiensis* (O.Tugay 4410)	*R. iconiensis* (H.Duman 6046)	*R. iconiensis* (M.Vural 9771)
R. mykalea (M.Vural 9846)	0.0000				
R. mykalea (M.Vural 9781)	0.0396	0.0000			
R. iconiensis (O.Tugay 4410)	0.0537	0.0614	0.0000		
R. iconiensis (H.Duman 6046)	0.1734	0.1543	0.1948	0.0000	
R. iconiensis (M.Vural 9771)	0.1589	0.1713	0.2013	0.0411	0.0000

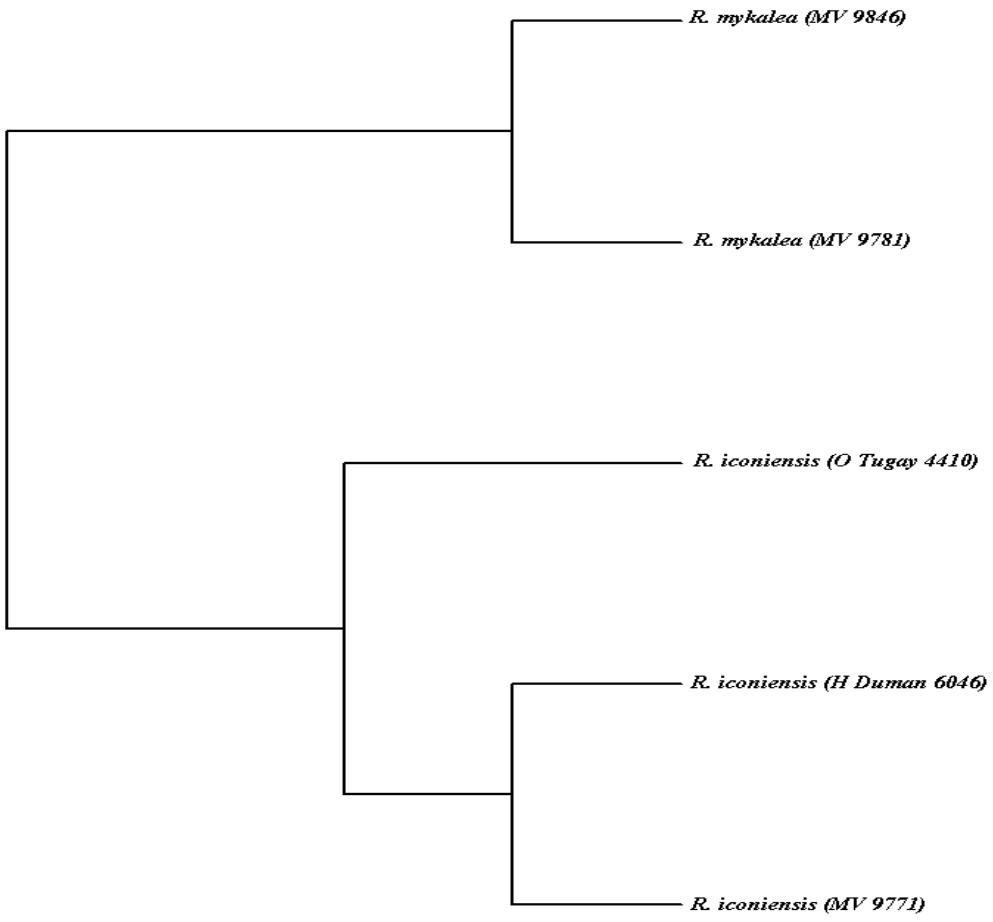

Figure 4. Dendrogram obtained by combined analysis of RAPD data from two *Rhaponticoides* species. Analysis was performed with UPGMA.

pulations of *R. iconiensis* are clustered in the same group and populations of *R. mykalea* are clustered separately from *R. iconiensis* (Figure 4). The genetic distances between two species are shown in Table 4. These finding supports morphological observation of two plant species.

One of the richest reservoirs of the biological diversity in the world is Turkey. In the Flora of Turkey, there are 10754 taxa, 3708 of which are endemic (34.5%). But, recently, the numbers have changed. Five new species are added every month to the Turkish plant lists. Now-

Table 4. Genetic distance data between two *Rhaponticiodes* species based on RAPD data

Species	*R. mykalea* (M Vural 9846)	*R. mykalea* (M Vural 9781)	*R. iconiensis* (O Tugay 4410)	*R. iconiensis* (H Duman 6046)	*R. iconiensis* (M Vural 9771)
R. mykalea (M Vural 9846)	****				
R. mykalea (M Vural 9781)	0.26	****			
R. iconiensis (O Tugay 4410)	0.45	0.46	****		
R. iconiensis (H Duman 6046)	0.72	0.77	0.45	****	
R. iconiensis (M Vural 9771)	0.97	1.03	0.72	0.47	****

adays, there are more than 11150 taxa and 4000 of them are endemic. In Turkey, 2053 of the native plant species are rare or threatened. 740 species are critically endangered (CR). 680 species are endangered (E), and 633 species are vulnerable (VU). These plants are in danger of extinction in the near future unless action is taken to reverse their decline. Fortunately, botanists in Turkey track the status of over 4000 native plants most in need of conservation efforts as elements of natural diversity.

One of the main factors endangering these plants is reduction in their habitat stemming from the development of housing, off road traffic and water canals. Plants became extinct because of naturally changing physical and biological conditions in this area. In addition, human activities and human population increase also caused many plants to be extinct. These activities are habitat alteration and economic exploitation, the increase of invasive native grazers, and the effects of environmental pollution. Conservation biologists must be as effective in saving endangered native species. The area for saving endangered plant species are botanical gardens and natural reserves. Protected collections of seeds in seeds banks can also help stop species loss, but the best and most effective protection would be to maintain native species in the wild (www.epa.qld.gov.au/nature_conservation/endangered_plants; www.dnr.state.md.us/wildlife/espaa.asp).

We should carry on research and effective management, so that no more threatened plant species become extinct and the future of rare and threatened plants should be secure from human activities and natural disasters. In the light of these results, conservation of the diversity of *R. iconiensis* would rationally be achieved by targeting whole diverse populations instead of collecting a few samples from each population (Kaundun and Park, 2002). Individuals from populations containing more polymorphisms should be secured so that important genotypes are saved. In this respect, it is noteworthy that O.Tugay 4410 is the most diverse population. Because of the high level of genetic diversity found, reintroduction of individuals cultured *in vitro* from seeds from the populations at altitudes of 1100 m should be done to construct new areas for endangered *R. iconiensis*.

ACKNOWLEDGEMENT

This study was supported by grants of DPT project no. 1998K121480.

REFERENCES

Açık L, Ekici M, Çelebi A (2004). Taxonomic relationships in *Astragalus* L. sections *Hololeuce* Bunge and *Synochreati* DC. (Fabaceae): evidence from RAPD-PCR and SDS-PAGE of seed proteins. Ann. Bot. Fenn. 41: 305-319.

Borba EL, Funch RR, Ribeire PL, Smitdt EC, Silva-Pereria V (2007). Demography, genetic and morphological variability of the endangered *Sophronitis sincorana* in the Chapada Diamantina Brazil. Plant Syst. Evol. 267 :129-146.

Clark MS (1997). Extraction of whole genomic DNA by the CTAB method, Plant molecular biology, a laboratory manual, Springer 5-8.

Davis PH, Mill RR, Tan K (1988). Flora of Turkey and the East Aegean Islands, Edinburgh at the University Press, Edinburgh. 10: 166-169.

Demiralp H, Çelik S, Köksel H (2000). Effects of oxidizing agents and defatting on the electrophoretic patterns of flour proteins during dough mixing. Eur. Food Res. Technol. 211: 322-325.

Ekim T, Koyuncu M, Vural M, Duman H, Aytaç Z, Adıgüzel N (2000). Türkiye Bitkileri Kırmızı Kitabı (Red Data Book of Turkish Plants). Barışcan Ofset, Ankara.

Eren Ö (2007). The genus *Rhaponticoides* Vaill. (Asteraceae) in Turkey: a new species and first key. Plant Syst. Evol. 267: 13-23.

Excoffier LGL, Schneider S (2005). Arlequin Version 3.0: An Integrated software package for population genetics data analysis. Evol. Bioinform. Online 1: 47-50.

Greuter W (2003). The Euro-Med treatment of Carduae (Compositae): Generic concepts and required names. Willdenowia 33: 49-61.

Güner A, Özhatay N, Ekim T, Başer KHC (2000). Flora of Turkey and the East Aegean Islands (Supplement 2). Edinburgh.

Hellwig FH (2004). Centaureinae (Asteraceae) in the Mediterranean - history of ecogeographical radiation. Plant Syst. Evol. 246: 137-162.

http://www.dnr.state.md.us/wildlife/espaa.asp

http://www.epa.qld.gov.au/nature_conservation/endangered_plants

Kaundun S, Park Y (2002). Genetic Structure of Six Korean Tea Populations as Revealed by RAPD-PCR Markers. Plant Genet. Resour. 42: 594-601.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277: 680-684.

Maniatis T, Fristsh EF, Sambrook J (1989). In Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, Chapter 1: 25-29.
Nei M (1972). Genetic distance between populations. Am. Nat. 949: 283-292.
Sangwan NS, Yadav U, Sangwan RS (2003). Genetic diversity among elite varieties of the aromatic grasses, Cymbopogon martini. Euphytica. 130: 117-130.
Saraswati R, Matoh T (1993). Identification of *Sesbonia* species from electrophoretic patterns of seed proteins. Trop. Agric. (Trinidad). 70: 282-285.
Sharma TR, Jana S (2002). Random amplified polymorphic DNA (RAPD) variation in *Fagopyrum tataricum* Gaertn. accessions from China and the Himalayan region. Euphytica 127: 327-333.
Steward CN, Porter DM (1995). RAPD profiling in biological conservation - an application to estimating clonal variation in rare and endangered Iliamna in Virginia. Biol. Conserv. 2:135-142.
Wagenitz G (1975). *Centaurea* L. In: Davis PH (Ed) Flora of Turkey and the East Aegean Islands, Edinburgh at the University Press, Edinburgh. 5: 465-585.
Wagenitz G (1986). *Centaurea* in South-West Asia: Patterns of distribution and biodiversity. Proc. Roy. Soc. Edinburgh 89B: 11-21.