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Comparative RAPD analysis and pollen structure studies of *Bellis perennis* L.

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Abstract: Random amplified polymorphic DNA (RAPD) is one of the easiest and most commonly used molecular techniques for genetic variability analysis. In the present study, the genetic diversity of analysis of *Bellis perennis* L. collected in 4 Turkish geographical locations was investigated by RAPD-PCR analysis. In addition to RAPD, pollen analysis of the 4 plant populations was also conducted. Out of 40 RAPD primers tested, 12 exhibited distinct banding patterns showing 45% to 79% polymorphism. Plants from these 4 localities were almost the same morphologically. Considerable differences were observed in the RAPD profile and pollen shape of plants from the Rize/Dereköy location. The results of this study clearly show that there are significant variations in the field collected populations of *B. perennis* from different geographic and climatic locations in Turkey. Environmental conditions may help us to determine the genomic structures of species.

Key words: *Bellis perennis*, RAPD-PCR, pollen

Bellis perennis L. türünün RAPD ve polen yapılarının karşılaştırılmalı analizi

Özet: Genetik çeşitliliği ortaya çıkarmak için kullanılan Rastgele Çoğaltılmış Polimorfik DNA (RAPD) yöntemi çok yaygın kullanılan ve basit bir yöntemdir. Bu çalışmada Türkiye'nin dört farklı lokasyonundan toplanmış *Bellis perennis* L. türü RAPD-PZR yöntemi ile analiz edilmiştir. RAPD yöntemine ilave olarak dört populasyonun polen analizleri de yapılmıştır. Kullanılan 40 RAPD primerinden 12 primer polimorfik bulunmuş ve polimorfizm oranı % 45 ila % 79 arasında değişmiştir. Dört lokaliteden toplanan bitki örneklerinin morfolojik olarak hemen hemen birbirinin aynı olduğu belirlenmiştir. Fakat Rize/Dereköy bölgesinden toplanan örneklerin RAPD ve polen analizleri sonucu diğer örneklerden farklı olduğu gözlenmiştir. Bu çalışmalardan farklı yörelerden ve farklı iklim şartlarının olduğu bölgeden toplanmış *Bellis perennis* türleri arasında önemli bir varyasyon olduğu belirlenmiştir. Çevresel faktörler genomik yapının belirlenmesinde etkili olabilir.

Anahtar sözcükler: *Bellis perennis*, RAPD-PZR, polen

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Introduction

The *Bellis* L. group has been included in the subtribe *Bellidinae* (tribe Astereae) along with another 117 genera representing more than 3000 species (Bremer, 1994). *Bellis perennis* L. is a perennial herbaceous plant that is native to almost the whole of Europe, Turkey, Cyprus, Syria, and Azerbaijan (Avato & Tava, 1995). The local names of *B. perennis* in Turkey are koyungözü, koyun çiçeği, and çayır papatyası (Baytop, 1994, 1999). *B. perennis* is variable in terms of the size of the capitulum and coloration of ligules. It has red, red purple, pink, or white flowers. It has been used in folk medicine in the treatment of rheumatism and as an expectorant (Wray et al., 1991; Hansel, 1992). It has also been employed as a vulnerary and against ecchymoses in veterinary medicine (Avato & Tava, 1995). Yazıcıoğlu and Tuzlacı (1995) reported that *B. perennis* was used to relieve stomach ache in Trabzon, Turkey, and there is also a report on the antiulcerogenic effect of *B. perennis*. Furthermore, it has been shown that the plant possesses antifungal activity (Desevedavy, 1989). Selected strains of this species are cultivated in Europe for decoration (Davis, 1995).

Morphological traits for the classification of plants are used for determining diversity and relationships among plants species. However, these traits are not sufficient because of environmental influences on them. It is only in recent years that the usefulness of molecular markers has been investigated as a means of characterising and discriminating different species more precisely (Benharrat et al., 2002). Random amplified polymorphic DNA (RAPD) analysis is often used to discriminate between closely related species or to detect variability within species (Williams et al., 1993; Benharrat et al., 2002; Awasthi et al., 2004; Aksoy et al., 2007; Akcicek et al., 2005; Açık et al., 2009).

The present study was carried out to screen RAPD primers and perform pollen analysis to show the genetic variability in 4 Turkish *B. perennis* populations representing 4 geographical locations in Turkey.

Materials and methods

Plant materials

Four populations of *B. perennis* L. were analysed in this study. The collection sites were Rize/Sivrikaya, Rize/Dereköy, Rize/Köhçer, and Antalya/Alanya. Thirty samples for each location were used (35 for Rize/Dereköy). Voucher specimens are kept at the Herbarium of Gazi University GAZI, Ankara, Turkey.

Pollen Analysis

For scanning electron microscopy (SEM), pollen grains were dried and gold-coated. All counts and measurements were based on 10 grains per sampling site. The general terminology follows Nilsson and Praglowski (1992), Vezey et al. (1992), and Pınar and Adıgüzel (1998). SEM observations were performed using a JEOL type electron microscope.

DNA extraction

First 100 mg of frozen leaves was ground in liquid nitrogen using a mortar. DNA was extracted according to the 2× CTAB method modified by Steward and Porter (1995) with the addition of 2% PVP to 2% CTAB. The powder was suspended in 1 mL of extraction buffer (of 2% PVP to 2% CTAB) and incubated at 65 °C for 90 min. Phenol:chloroform: isoamyl alcohol (24:24:1) was added to remove polysaccharide contaminants 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 isoproponol, and the mixture allowed to sit at room temperature for 40 min. Following centrifugation at 5000 rpm for 3 min, it was washed twice with 76% ethanol. The pellets were dissolved in TE buffer.

RAPD-PCR and gel electrophoresis

The PCR amplifications were performed as described by Williams et al. (1990). A set of 20 oligonucleotides were used. Amplification and reaction conditions were described in Aksoy et al. (2007). Amplification reactions were performed using the protocol reported by Williams et al. (1990). The amplification reactions were set in 100 µL of final volume of reaction mixture containing 1 unit of Taq polymerase, 10 mL of 10× Taq DNA polymerase buffer, 10 pmol primer, 25 ng of genomic DNA, and 1 mM $MgCl_2$. DNA amplification was performed in a Biometra thermocycler (T Personel). The thermocycler was programmed for 45 cycles, each of which had the following temperature profile: 30 s at 96 °C, 30 s at 35 °C, and 30 s at 72 °C. Then 15 µL of amplification products were loaded in a 2% agarose

gel that was run for 4 h at 90 mV in 1× TAE buffer. A 100 base-pair DNA ladder was used as molecular weight marker. The gel was stained with ethidium bromide and photographed under UV light (Maniatis et al., 1989).

Data analysis

In the study of overall genetic variation, fragments that were readable and reproducible were used. Bands were scored as either present (1) or absent (0) for all species studied. The genetic distance and the polymorphism among the population were calculated using the POPGENE program (Nei, 1972). A cluster analysis technique of the unweighted pair-group method of arithmetic averages (UPGMA) was used to construct the phylogenetic tree.

Results and discussion

Forty RAPD primers were screened in order to select those that produce reproducible and polymorphic bands for the study of *Bellis* populations from Rize/Dereköy, Rize/Sivrikaya, Rize/Köhçer, and Antalya/Alanya. Out of the 40 primers tested, 12 revealed distinct band profiles. The 12 primers that produced amplification are listed in Table 1. The amplification products ranged from 100 bp to 3 kb in size. The size of amplified product ranged from 100 to 3000 bp.

For the statistical analysis, the genetic distances between the 4 populations were calculated (Table 2). The dendrogram was constructed using UPGMA cluster analysis (Figure 1). The average distance between the populations ranged from 53% to 79%. The phylogenetic tree was generated with the use of the POPGENE computer program. The dendrogram shows that the differences between the population of Rize/Dereköy and the populations of Rize/Sivrikaya, Rize/ Köhçer, and Antalya/Alanya were 69%, 79%, and 76%, respectively. The differences between the population of Rize/Sivrikaya and the populations of Rize/Köhçer and Antalya/Alanya were 53% and 76%, respectively. The genetic distance between the *B. perennis* population from Antalya/Alanya and the population from Rize/Köhçer was 66%. Hence the positioning of populations on the tree branch clearly indicates the presence of genetic diversity among the populations. However, interestingly the Rize/Dereköy population, which is in the same geographic areas as the other 2 populations from Rize, showed an identity separate from that of the other 2 Rize populations.

Table 1. Random oligonucleotide primers used for RAPD analysis.

Primer name	Primer sequence
OPA04	AAGTCCGCTC
A2	TGCCGAGCTG
OPR 03	ACACAGAGGGT
B7	GGTGACGCAG
OPB 10	CTGCTGGGAC
OPW 10	TCGCATCCCT
OPC 02	GGTCTACACC
OPA07	GAAACGGGTG
OPU 16	CTGCGCTGGA
OPR03	ACACAGAGGGT
M13	GAGGGTGGCGGTTCT
OPA 17	GACCGCTTGT

Table 2. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

pop ID	Rize/ Sivrikaya	Rize/ Köhçer	Rize/ Dereköy	Antalya/ Alanya
Rize/Sivrikaya	**	58	50	46
Rize/Köhçer	53	**	45	51
Rize/Dereköy	69	79	**	46
Antalya/Alanya	76	66	76	**

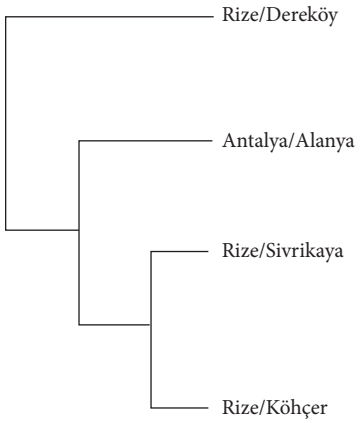

Figure 1. UPGMA dendrogram based on RAPD data showing relationships among the 4 populations of *B. perennis*.

The pollen grains are echinate-perforate, rarely echinate-rugulate-perforate (only Rize-Dereköy), tricolporate, isopolar, radially symmetrical and spheroidal, rarely oblate (only Antalya-Alanya). The sizes of pollen grains are 20-22 × 20-25 mm. The length of the spine ranges between 2.5 and 4 mm. Spines with numerous perforations at the base, each of which measures much less than 1 mm. mm^2 perforations are 2-8 (Table 3) (Figure 2a-h). SEM observations show that the most different pollen belonged to the Rize/Dereköy population.

In summary, genetic variability using RAPD markers and pollen analysis revealed clear genetic variations in *B. perennis* with reference to their geographical locations in Turkey. Morphological studies have shown considerable variations in the *Bellis* populations, which grow almost all over Turkey. RAPD primers OPA17, OPA04, A2, OPR03, B7, OPB10, OPW10, OPC02, OPA07, OPU16, OPR03, and M13 revealed a high level of genetic diversity in the 4 populations of *B. perennis*, enabling these complicated species to be clearly differentiated. This study also demonstrated that RAPD marker can be supported by pollen analysis for the *B. perennis* populations. Phylogenetic analysis of *B. perennis* populations from various parts of Turkey revealed a phylogeographic pattern in their distribution (except the Rize/Dereköy population), as it is clear from the results that all 4 geographical populations were demonstrated in 4 clusters. Cluster I contained the Rize/Köhçer and Rize/Sarıkaya populations and cluster II the Antalya/Alanya population, whereas in cluster III the Rize/Dereköy is represented as a separate identity. The separation of the Antalya/Alanya population can be explained by its geographic distance from the other populations. The Rize/Dereköy population, which is near the other 2 Rize populations, is interestingly different from those 2 populations despite the geographical conditions at the collection sites for the 3 populations from Rize, being more similar to each other than those of the Antalya/Alanya population.

In conclusion, on the basis of our results it is clear that there are significant variations in the field collected populations of *B. perennis* from different geographic and climatic locations in Turkey. This shows that environmental stresses such as different climatic conditions generate adaptive genomic structures, but polymorphism can appear even in the same climatic conditions in the same species. Since this plant is extensively used medicinally, it is important for its efficient use and conservation to detect genetic diversity. The genetic diversity observed by RAPD marker needs to be further supported by using other molecular markers such as microsatellite, ISSR (Alam et al., 2009), and mitochondrial DNA. In addition to molecular analysis, pollen analysis appears to be useful to observe differences within species.

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Table 3. Pollen measurements of *B. perennis* populations.

Localities	P (µm)	E (µm)	Pollen type	Ornamentation	Spine length (µm)	$µm^2$ perforations	Aperture type
Antalya/Alanya	20 µm	25 µm	Oblate	Echinate-Perforate	3-4 µm	7-8	Tricolporate (operculate)
Rize/Dereköy	22 µm	23 µm	Spheroidal	Echinate-Rugulate-Perforate	2.5-3 µm	4-5	Tricolporate (operculate)
Rize/Köhçer	20 µm	20 µm	Spheroidal	Echinate-Perforate	3-4 µm	2-3	Tricolporate (operculate)
Rize/Sivrikaya	21 µm	22 µm	Spheroidal	Echinate-Perforate	3-4 µm	4-5	Tricolporate (operculate)

P: Polar axis, E: Equatorial axis

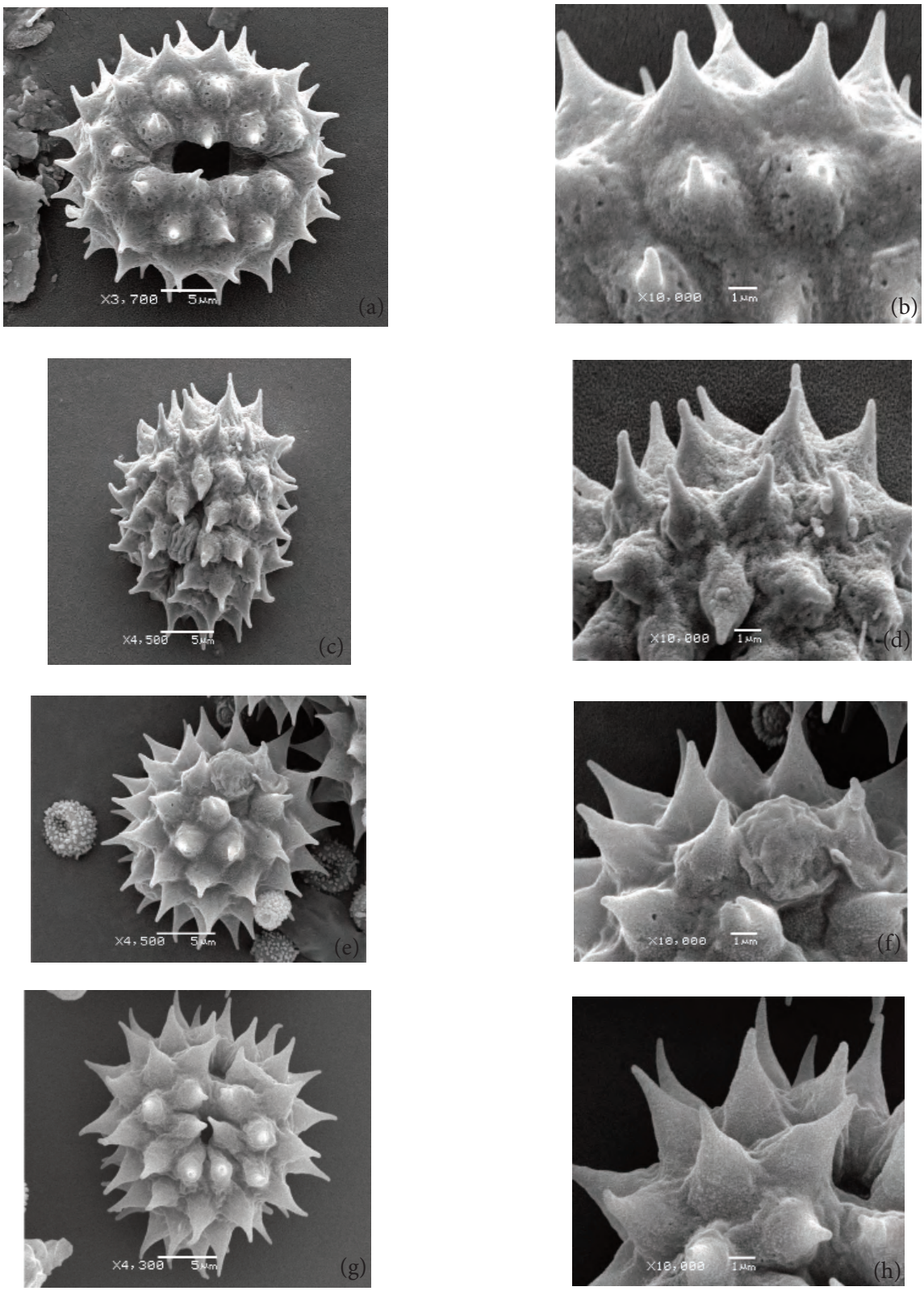

Figure 2. SEM photos of the 4 *B. perennis* populations.

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