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A preliminary study on the genetic diversity of the critically endangered *Centaurea nivea* (Asteraceae)

Emel Sözen* & Burcu Özaydın

*Anadolu University, Science Faculty, Biology Department, Yunusemre Campus, Eskişehir 26470, Turkey (*corresponding author's e-mail: esozen@anadolu.edu.tr)*

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Centaurea nivea (Asteraceae) is endemic to Turkey and distributed only in the Eskişehir Province. It is under risk of extinction due to anthropogenic disturbance, so conservation strategies must be developed urgently. In this study, RAPD technique was used to estimate the level of genetic diversity of *C. nivea*. Twenty RAPD primers generated 234 bands, of which 215 were polymorphic (91.88%). A high level of genetic diversity was detected both at population (PPB = 72.90%, I = 0.3790, H_s = 0.2527) and at species level (PPB = 91.88%, I = 0.4510, H_t = 0.2963). A moderate level of genetic differentiation among populations was also revealed by Nei's gene diversity analysis (14.73%), Shannon's information measure (15.96%) and AMOVA (7.97%). Patterns of genetic variation among the populations of *C. nivea* may indicate that the closely located and fragmented populations of *C. nivea* were likely derived from a previously large population.

Key words: *Centaurea nivea*, conservation, genetic diversity, RAPD

Introduction

Centaurea nivea is a perennial, rhizomatous plant, belonging to the family Asteraceae. According to Wagenitz (1975) and herbarium records, *C. nivea* is known only from five populations, all of which are located in a narrow region of Eskişehir Province of western Anatolia, Turkey. The species has been classified as critically endangered according to the IUCN categories and listed in the Red Data Book of Turkish Plants (Ekim *et al*. 2000). Today, *C. nivea* populations are under risk of extinction due to rapid urbanization and should receive a priority in terms of conservation measures.

The maintenance of genetic diversity in rare or endangered species is a major goal of conservation programs, since long-time survival of species in a changing environment depends on the preservation of sufficient genetic variation within and among populations (Barrett & Kohn 1991). Understanding of the genetic variation within and among populations is therefore required for the establishment of efficient conservation strategies for endemic and endangered plant species (Hamrick & Godt 1996a, Lande 1999).

In recent years, a number of molecular markers including allozymes (Hamrick & Godt 1996a) and PCR-based DNA markers, such as

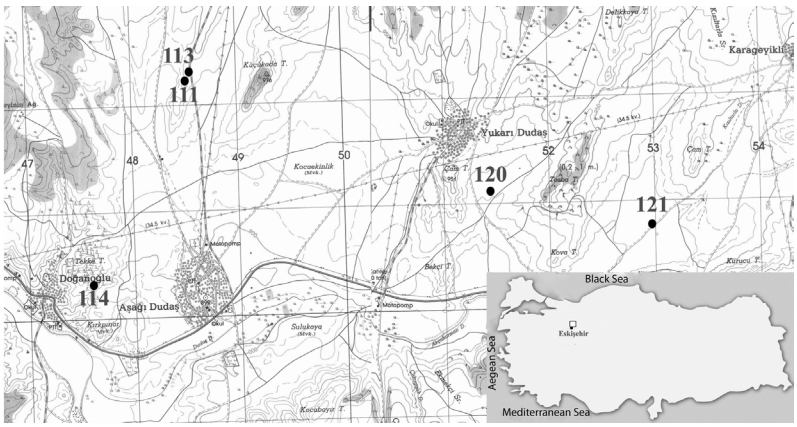

Fig. 1. Map showing the geographical locations of the five analyzed populations of *Centaurea nivea* in Turkey. For the meaning of the codes 111, 113, 114, 120 and 121, *see* Table 1.

random amplified polymorphic DNA (RAPD; Li & Jin 2006, Sun *et al*. 2006), inter simple sequence repeats (ISSR; Xie *et al*. 2005, Cao *et al*. 2006), simple sequence repeats (SSR; Rossetto *et al*. 2004) and amplified fragment length polymorphism (AFLP; Isagi *et al*. 2004, Vilatersana *et al*. 2007) have been widely used to study genetic variation and differentiation of endemic and endangered plant species. Among these, the RAPD markers (Williams *et al*. 1990) have been frequently employed for estimating the levels of genetic variation within and among plant populations due to their advantages such as random sampling of the whole genome, high levels of polymorphism, and easy performance (Nybom 2004). The RAPD technique requires small amounts of DNA, which is highly important when working with rare species (Artyukova *et al*. 2004). Some limitations of RAPDs, such as low reproducibility, have been largely overcome through improved, strictly applied laboratory techniques and scoring procedures (Nybom & Bartish 2000).

Genetic diversity levels have been estimated for several endemic members of *Centaurea*, including *C. corymbosa* (Freville *et al*. 2001), *C. tenorei* and *C. parlatoris* (Palermo *et al*. 2002), *C. cineraria* (Bancheva *et al*. 2006), *Femeniasia balearica* (formerly *C. balearica*, Vilatersana *et al*. 2007), and *C. horrida* (Mameli *et al*. 2008). To our knowledge, however, no information on the genetic variation in *C. nivea* was available. The aim of the present study was therefore to estimate the level of genetic variation and genetic differentiation in the *C. nivea* populations by using RAPD markers. The results presented here may be suggestive for developing conservation strategies for this critically endangered and endemic plant species.

Material and methods

Plant sampling

Centaurea nivea is a small, rhizomatous, perennial plant; its decumbent stem is 3–19 cm in height, with white tomentose leaves generally rhomboid or lanceolate in shape (Wagenitz 1975, Özaydın 2007). The plant flowers June to July. The flowers are bright-yellow with red and black stripes, and they are not fragrant. Like the other species of the genus, *C. nivea* is a diploid with 2n = 18 (Uysal 2006). It grows only in calcareous soils.

Centaurea nivea is known from only five natural populations. The geographic distribution of *C. nivea* is narrow, the species occurs only in a restricted region of the Eskişehir Province (Fig. 1). Healthy leaf tissues of 10 individual plants were randomly sampled from each population (Table 1). The distance between sampled plants was at least 10 m to decrease the possibility of sampling from the same clone. The leaf samples were placed in plastic bags, kept on ice during transport to the laboratory and stored at –20 °C until they were processed. Herbarium specimens were collected from each population, taxonomically characterized and deposited at the Herbarium of Anadolu University (ANES).

DNA extraction and RAPD-PCR amplification

Genomic DNA was isolated from 0.2–0.5 g of powdered leaf tissue by using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of DNA were determined by using Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA). DNA was diluted to 4 ng μl^{-1} and stored at 4 °C for RAPD amplification.

A set of 30 random 10-mer primers was purchased from Thermo Inc., Burlington, USA. After screening, 20 primers that amplified clear, reproducible banding patterns were chosen for further studies (Table 2). PCR amplifications were performed in 25 μl reaction mixture containing 10 ng of template DNA, 1X *Taq* polymerase buffer and 1 U of *Taq* polymerase (Fermentas, Maryland, USA), 2.5 mM $MgCl_2$, 1 mM dNTP, 1 μM primer. Amplifications were carried out in Progene Thermal Cycler (Techne Inc., Burlington, USA) that was programmed for initial denaturation step at 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and a final elongation at 72 °C for 7 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 μg ml^{-1}) and digitally photographed with a UVIpro gel documentation

Table 1. Populations of *Centaurea nivea* examined in the RAPD analysis.

Population code	Locality	Altitude (m a.s.l.)	Coordinates	Sample size
111	Eskişehir; Mihalıççık-Alpu 24 km	940	39°50´43.9´´N, 31°13´47.0´´E	10
113	Eskişehir; Aşağı Dudaş-Bahçekuyu	949	39°50´48.8´´N, 31°13´47.5´´E	10
114	Eskişehir; Doğanoğlu	920	39°51´15.4´´N, 31°14´02.0´´E	10
120	Eskişehir; Eskişehir-Mihalıççık 60 km	930–950	39°50´11.2´´N, 31°15´51.1´´E	10
121	Eskişehir; Mihalıççık-Eskişehir 20 km	955	39°49´58.5´´N, 31°16´54.4´´E	10

Table 2. Attributes of RAPD primers used for generating RAPD markers from 50 individuals of *Centaurea nivea* sampled from five populations.

Primer	Sequence 5´–3´	No. of bands scored	Number of polymorphic bands	Polymorphic bands (%)
P1	GTGACGCCGC	10	10	100
P2	CCGGACACGA	13	13	100
P3	GAGGCGGCGA	10	10	100
P4	AGCGTCGACT	13	11	84.6
P5	CTGCGACGGT	10	10	100
P6	GCGCGGCAGT	9	6	66.6
P7	TTGCGCCCGG	14	14	100
P8	AGACGCCGAC	15	13	86.6
P9	GGGAAGAGAG	11	11	100
P10	GGGTGTGGTT	9	8	88.8
P11	GGCCGATGAT	12	10	83.3
P12	AGTCGGGTGC	8	7	87.5
P13	ACCGGCTTGT	13	11	84.6
P14	CAGCACTGAC	13	13	100
P17	GTAGCACTCC	8	7	87.5
P19	CCACGGGAAG	16	16	100
P20	GTCTCTCAGG	6	5	83.3
P21	ACGGTGCCTG	17	14	82.3
P24	CGCCCTGGTC	17	17	100
ID10	GAGAGCCAAC	10	9	90
Total	20	234	215	91.88

system (UVItec, Cambridge, UK). Molecular weights of PCR products were estimated using a 1 kb DNA ladder (Fermentas). PCR reactions were repeated twice to ensure reproducibility. A negative control with no DNA template was also included in each of the PCR amplifications.

Data analysis

Since RAPD 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 mobility were assumed to be homologous. Staining intensity of bands was not considered as a difference. The data matrix was analyzed using software program POPGENE 1.32 (Yeh *et al*. 1999) as a dominant mode of inheritance in a diploid organism, assuming Hardy-Weinberg equilibrium. The population genetic parameters including the percentage of polymorphic loci (PPB), the effective number of alleles (n_e), observed number of alleles per locus (n_a), and Nei's (1973) gene diversity (h) were calculated (Table 3). At the species level, total heterozygosity (H_t), heterozygosity within population (H_s), coefficient of gene differentiation (G_{st}) and gene flow (N_m) were measured using Nei's (1973) gene diversity statistics. Nei's unbiased genetic similarity and genetic distance matrix (Nei 1978) was generated with POPGENE. Diversity levels were also calculated using Shannon's information measure (I, Lewontin 1972). I was calculated at two levels: the average diversity within populations (I_{pop}) and the total diversity (I_{sp}) with POPGENE. The proportion of diversity among populations was estimated as $(I_{sp} - I_{pop})/I_{sp}$.

An analysis of molecular variance (AMOVA) was applied to estimate the variance components, partitioning the variation within and among populations. Input data files for AMOVA ver. 1.55 (Excoffier *et al*. 1992) were created using AMOVA-PREP (Miller 1998). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations.

Results

Genetic diversity

Twenty primers produced 234 reliable bands in 50 individuals, corresponding to an average of 11.7 bands per primer. The size of the RAPD bands varied from 200 bp to 3500 bp but most were from 500 to 1500 bp. Among the 234 loci, 215 were polymorphic (91.88%) at the species level. The percentage of polymorphic bands (PPB) per population ranged from 70.94% to 75.21% with an average of 72.90% (Table 3). An example of the polymorphism detected with primer 4 is shown in Fig. 2. The mean number of alleles (n_a) was 1.729, while the effective number (n_e) was 1.430. Assuming Hardy-Weinberg equilibrium, the average gene

Table 3. Genetic variability within populations of *Centaurea nivea*, as revealed by the RAPD analysis. PPB, percentage of polymorphic loci; n_a = observed number of alleles; n_e = effective number of alleles; h = Nei's genetic diversity; I = Shannon's information index. For the meaning of the codes 111, 113, 114, 120 and 121, *see* Table 1.

Population code	Number of polymorphic bands	PPB (%)	n_a (SD)	n_e (SD)	h (SD)	I (SD)
111	173	73.93	1.739 (0.440)	1.438 (0.368)	0.256 (0.191)	0.384 (0.267)
113	168	71.79	1.718 (0.451)	1.428 (0.375)	0.249 (0.196)	0.372 (0.274)
114	166	70.94	1.709 (0.455)	1.411 (0.359)	0.244 (0.190)	0.367 (0.268)
120	176	75.21	1.752 (0.432)	1.437 (0.358)	0.258 (0.187)	0.389 (0.262)
121	170	72.65	1.726 (0.446)	1.437 (0.372)	0.254 (0.193)	0.381 (0.271)
Average	170.6	72.90	1.729 (0.017)	1.430 (0.011)	0.252 (0.006)	0.379 (0.009)
Species	215	91.88	1.919 (0.273)	1.492 (0.324)	0.296 (0.158)	0.451 (0.209)

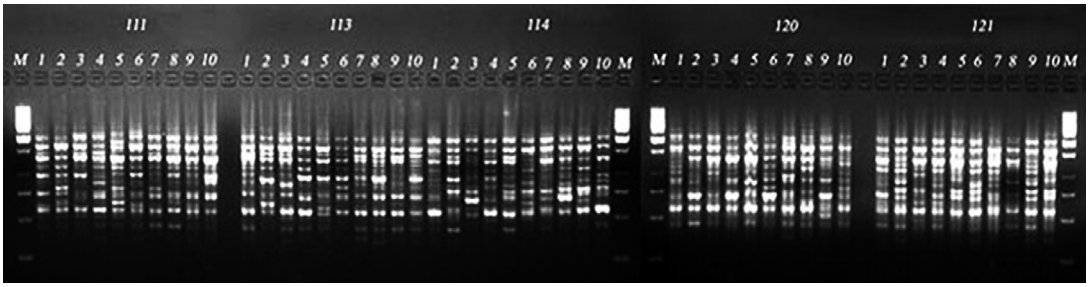

Fig. 2. Electrophoresis of RAPD PCR products amplified with primer 4 for the five populations of *Centaurea nivea*. Abbreviations as in Fig. 1 (*see* Table 1). Lane M is 1kb DNA ladder (Fermentas).

Table 4. Analysis of molecular variance (AMOVA) within and among *Centaurea nivea* populations. df = degree of freedom; SSD = sums of squares; MSD = mean square deviations.

Source of variation	df	SSD	MSD	Variance component	Total variance (%)	P^a
Among populations	4	262.6	65.65	3.04	7.97	< 0.001
Within populations	45	1582.8	35.17	35.17	92.03	< 0.001

[a] *P* values are the probabilities of having more extreme variance component than the observed values by chance alone. Significance tests after 1000 permutations.

diversity was 0.252 (H_s) within populations and 0.296 (H_t) at the species level. The Shannon's information indices ranged from 0.367 to 0.389 with an average of 0.379 at the population level (I_{pop}) and 0.451 at the species level (I_{sp}). Among the five populations, population 120 exhibited the highest level of variability (PPB = 75.21%, h = 0.258, I_{pop} = 0.389, respectively), whereas population 114 exhibited lowest level of variability (PPB = 70.94%, h = 0.244, I_{pop} = 0.367, respectively; *see* Table 3). No specific bands were detected in either population.

Population genetic structure

There is a moderate genetic differentiation among the populations of *C. nivea*. The coefficient of genetic differentiation between populations (G_{st}, as estimated by the partitioning of the total gene diversity) was 0.147. This finding is in broad agreement with the results of the Shannon's diversity index and AMOVA (Table 4) which suggested that 15.96% and 7.97% of the total variation was partitioned among the populations, respectively. The level of gene flow (N_m) between populations was estimated to be 2.894 individuals per generation, indicating that there is a high migration rate. Genetic identities between populations varied from 0.927 to 0.964 with an average ± SD of 0.943 ± 0.011. The highest identity (0.964) was found between populations 120 and 121, while the lowest (0.927) between populations 111 and 121 (Table 5).

Discussion

Centaurea nivea is a critically endangered endemic species belonging to the *Cheirolepis* section of *Centaurea* genus (Ekim *et al*. 2000). It survives only in few closely located populations in western Anatolia, Eskişehir region and is in danger of extinction due to human disturbances and urbanization. As compared with microsatellite and allozyme markers, RAPDs have

Table 5. Nei's unbiased genetic distance among the populations of *Centaurea nivea*.

Population	111	113	114	120
113	0.0429	–		
114	0.0630	0.0544	–	
120	0.0642	0.0643	0.0532	–
121	0.0754	0.0736	0.0516	0.0366

some limitations such as low reproducibility and dominant allelic expression (Lee *et al*. 2002). However, these markers have been widely used in plant population studies because they detect variation in the entire genome, they produce higher levels of polymorphisms than allozymes and their analysis can be performed easily and faster than microsatellites (Nybom & Bartish 2000, Nybom 2004). In this study, therefore, the genetic variation within and among populations of *C*. *nivea* was investigated by using RAPD markers. This is the first attempt at estimating the genetic variability of this species towards its conservation.

Generally, species with small geographic ranges tend to maintain less genetic diversity than geographically widespread species (Hamrick & Godt 1989). Based on such a consideration, a low diversity level was expected in *C*. *nivea*, however, the observed genetic diversity was very high both at the population (P = 72.90%, h = 0.252, I = 0.379) and species (P = 91.88%, h = 0.296, I = 0.451) levels. These results may indicate that *C*. *nivea* has no history of sufficiently severe or long-lasting population bottlenecks to cause loss of genetic diversity. Thus the size of geographic distribution is not a good predictor of genetic diversity for *C*. *nivea*.

Using RAPD or ISSR markers, a high level of genetic diversity have also been reported for many other rare or narrow endemic plant species such as *Leucopogon obtectus* (P = 90%; Zawko *et al*. 2001), *Oxytropis chankaensis* (P = 81.9%; Artyukova *et al*. 2004), *Sinocalycanthus chinensis* (P = 68.84; Li & Jin 2006), *Hippophae rhamnoides* subsp. *sinensis* (P = 88.79%; Sun *et al*. 2006) and *Swertia przewalskii* (P = 94%; Zang *et al*. 2007). A high level of variability could play an important role in the local adaptation of such species.

Although it is difficult to make direct comparisons when different marker systems (e.g. allozymes, AFLP and SSRs) were used, it appears that the genetic diversity level of *C*. *nivea* is similar to that of other endemic *Centaurea* species. For example, in *C*. *tenorei*, the amount of genetic diversity was assessed by means of allozymes (Palermo *et al*. 2002). The percentage of polymorphic loci varied from 33.3% (*C*. *tenorei* subsp. *tenorei* and subsp. *montaltensis*) to 83.3% (*C*. *tenorei* subsp. *maritima* and *C*. *parlatoris*). The lowest expected heterozygosity (H_e) was observed in *C*. *tenorei* subsp. *tenorei* (0.08), while the highest was detected in *C*. *parlatoris* (0.34). Another allozyme analysis of seven endemic *Centaurea* species of Sicily yielded heterozygosity values ranging from 0.126 for *C*. *cineraria* subsp. *cineraria* to 0.276 for *C*. *todari* (Bancheva *et al*. 2006). In another rare species, *Femeniasia balearica* (Asteraceae), quite high levels of genetic variation were found by using AFLP markers (Vilatersana *et al*. 2007). Freville *et al*. (2001) studied the genetic diversity of *C*. *corymbosa* by means of microsatellites and found heterozygosity (H_e) values in the range of 0.36–0.62. By using the same microsatellite markers a considerable amount of genetic variation was found (average H_e = 0.603–0.854) in the endangered species *C*. *horrida* (Mameli *et al*. 2008). It was suggested that high genetic diversity values observed in all these *Centaurea* species may have played a role in allowing them to survive in a harsh and highly stressing environment (Mameli *et al*. 2008).

Species biology, life strategies and especially reproduction systems of plant species play a major role in determining the level of genetic variation (Hamrick & Godt 1989). For example, outcrossing species commonly have higher levels of genetic diversity and lower differentiation between populations (Hamrick & Godt 1996b). Although little is known about the breeding system of *C*. *nivea*, our preliminary field observations indicated that this plant reproduces both sexually and asexually. Pollination is likely achieved by small insects and winds. We have observed a low level of seed set and seed viability (average of three viable seeds per capitulum). Under laboratory conditions seed germination rates of *C*. *nivea* were moderate (56% max.), but how many seeds germinate successfully in their natural habitats is unknown. However, it was reported that the successful establishment of one or a few seedlings should be enough to maintain high levels of genetic variation in plant populations (Watkinson & Powell 1993). On the other hand, there are several studies proposing that asexual reproduction may promote the maintenance of unexpectedly high genetic diversity in plants (Schnabel & Hamrick 1990, Houston & Houston 1994, Mitton &

Grant 1996). Therefore, it is possible to speculate that sexual reproduction was more common in the recent history of this species and subsequent clonal growth has maintained a significant part of its genetic diversity. Further studies on the breeding system and pollination biology would be helpful for a better understanding of the genetic variation in *C. nivea*.

The majority of the genetic variation in *C. nivea* was found within rather than among populations as estimated by Nei's gene diversity statistics (85.27%), Shannon's information measure (84.04%) and AMOVA (92.03%). Such results were reported previously in several studies of rare and endangered species including *Leucopogon obtectus* (Zawko *et al*. 2001), *Nouelia insignis* (Luan *et al*. 2006) and *Hippophae rhamnoides* (Sun *et al*. 2006). High diversity and low population partitioning in rare plants have been attributed to a number of factors (Zawko *et al*. 2001), including insufficient time for genetic diversity to be reduced following a natural reduction in population size and isolation, adaptation of genetic system to small population conditions, recent fragmentation (human disturbance) of a once continuous genetic system, and extensive gene flow (Maguire & Sedgley 1997). In *C. nivea*, the effective gene flow was estimated as $N_m = 2.89$, which indicates sufficient genetic exchange among populations contributing towards preventing the populations' differentiation. A high level of genetic diversity and low level of genetic differentiation in *C. nivea* may indicate that the closely located and fragmented populations have likely been derived from a previously large population.

Centaurea nivea has a very restricted distribution in Eskişehir region, with only five populations. If the present habitat is damaged, the species will inevitably be confronted with extinction. Although many plant species are being rescued by *ex situ* methods, and reintroductions, the single most important way to conserve a plant species is through the protection of the habitat in which it lives (Li *et al*. 2002). Therefore, the preferred strategy for preserving genetic variation of *C. nivea* in this region should be to protect its habitats. On the other hand, the seed and germplasm collections in botanical gardens should also be of practical value for the conservation of genetic variation in *C. nivea*, in such cases sampling intensively from one or two populations should be enough to preserve most of the genetic variability.

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