Assessment of Genetic Diversity of a Critically Endangered Species, Centaurea amaena (Asteraceae)

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Research Article

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Abstract

Centaurea amaena is an endemic and endangered species and listed as CR in Turkey. ISSR markers were used to detect the level of genetic diversity in two natural populations of *C. amaena.* A total of 50 ISSR primers were used and 13 primers producing polymorphic and reproducible products were selected. These primers yielded 102 amplified discernible loci, of which 80 (78%) were polymorphic. A high level of genetic diversity was detected both at population and species levels; the effective number of alleles (*Ne*) was 1.544, the observed number of alleles (*Na*) was 1.784, the Nei's genetic diversity (*H*) was 0.306, and Shannon's information index was 0.447. The determined gene flow (Nm) was 2.329, indicating a high migration rate between the populations. Moderate level of genetic differentiation (G_{ST}: 0.176) was also observed. Analysis of molecular variance (AMOVA) revealed that 24.89% of the total genetic diversity resided among populations, while 75.10% within the populations. Cluster analysis showed that samples from the same locality clustered together and there was no cross-clustering between the samples. The patterns of genetic variation indicates that existing *C. amaena* populations should be conserved.

Introduction

Turkey has quite a large geological and geomorphological diversity, a wide variety of climate, topographic conditions and soil characteristics, is located at intersection of three different phytogeographical regions and is the center of differentiation of many genera and sections, thus has quite a rich flora (Erik and Tarıkahya 2004). *Centaurea* L. (Asteraceae) is an important and relatively large genus of Turkish flora. It is composed of approximately 743 species worldwide (POWO 2022). The genus is distributed especially in Southern and Central Europa, Anatolia, North Africa and the Caucasus. About 172 *Centaurea* species were identified in the flora of Turkey and the Eastern Aegean Islands (Wagenitz 1975). Turkey is a center of diversity for *Centaurea*. Recently, with the latest additions, number of *Centaurea* species has reached to 220 in Turkey (Hamzaoğlu and Koç 2020; Duman et al. 2021; Ozbek 2021; Şirin et al. 2022). Therefore, the endemism rate is approximately 60%. The *Centaurea amaena* Boiss. & Balansa Balansa included in the Sect. *Phalolepis* is a critically endangered endemic species and grows on rocky slopes of Kayseri province.

Population genetics is a cornerstone of conservation biology (Laikre 2010). Long-term persistence of a species depends on maintaining adequate genetic diversity within and between the populations (Ellstrand and Elam 1993). Detailed knowledge on genetic structure of plant populations is required for conservation of available resources (Ottewell et al. 2016; Chung et al. 2020).

Molecular markers (RAPD, ISSR, SSR, AFLP and RFLP) are widely used to predict genetic variations at intraspecies and interspecies levels and to identify individual differences between populations. The inter simple sequence repeats (ISSR) are usually dominant markers and allow more stringent amplification (Wolfe et al. 1998; Hilooğlu and Sözen 2017). ISSR markers have effectively been used in the fields of genetic diversity, phylogenetic analysis and evolutionary biology (Petrova et al. 2017; Sozen et al. 2017; Atasagun et al. 2018; Sevindik et al. 2020; Sevindik and Efe 2021).

Genetic diversity levels have been reported for several endemic members of *Centaurea*, including *C. solstitialis* (Sun 1997), *C. corymbosa* (Freville et al. 2001a), *Centaurea tenorei* and *C. parlatoris* (Palermo et al. 2002), C. *cineraria* (Bancheva et al. 2006), *Femeniasia balearica* (Vilatersana et al. 2007), C. *horrida* (Mameli et al. 2008), C. *nivea* (Sözen and Özaydin 2009), *Centaurea parlatoris* (Bancheva et al. 2011), C. *lycaonica* (Uysal et al. 2012), C. *stoebe* (Geraci A et al. 2012), C. *alba* (Requena Ordóñez 2017), *C. tentudaica* (Moreyra et al. 2021).

This study was conducted to investigate the level of genetic diversity within and between two *C. amaena* populations with the use of ISSR markers. Prospective outcomes are expected to provide essential information for establishing effective conservation strategies for critically endangered *C. amaena*.

Material And Methods

Plant Sampling

The *C. amaena* is distributed with 2 populations in a small area between Erciyes and Yılanlı Mountains of Kayseri province. Two populations together cover an area of approximately 0.55 km^2 and the total number of individuals was determined as approximately 5672 (Fig. 1). The distance between two populations is about 15 km. For this study, 24 individuals of *C. amaena* were randomly sampled from each population in the natural distribution area of the species in 2019 (Fig. 1, Table 1). Fresh leaves were placed in plastic bags, kept on ice during transport to the laboratory and stored at -20 °C until DNA isolation.

DNA extraction and ISSR-PCR amplification

Total DNA was extracted from young leaves via plant genomic DNA miniprep kit (Bio-Basic, Canada). The DNA quantity and purity were assessed using Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA) and 1% agarose gel electrophoresis. Then, DNA concentrations of all samples were diluted to 2 ng/µl, prepared for PCR and stored at -20°C. A total of 50 ISSR primers (University of British Columbia, Canada) were screened for PCR amplification. After screening, 13 primers that produced clear and reproducible polymorphic fragments were chosen for ISSR analyses (Table 2).

The ISSR-PCR reactions were conducted in a total reaction volume of 20 µL containing 2 µL of template DNA (2 ng/µL), 2 µL PCR Buffer (10X), 0.2 µL Taq DNA polymerase, 0.5 µL of each primer (10 pmol/µL), 1 µL dNTPs (10 mM), and 3 µL $MgCl_2$ (25 mM). The amplifications were performed using a thermal cycler (Biorad, California, USA) that was programmed as follows: an initial denaturation step at 94 °C for 5 min; 35 annealing cycles of 94°C for 45 s, at a specific annealing temperature 1 min, 72 °C for 1 min; a final extension of 72 °C for 7 min. The amplified PCR products were separated in 2% agarose gel electrophoresis with ethidium bromide in 1 x TBE buffer at 80 V for 2 h. The electrophoresis results were visualized and recorded with a gel imaging system. Molecular weights of the amplified products were estimated using a 100 bp DNA ladder (Geneaid).

Data analysis

The amplification products were scored as: present (1) or absent (0) in each individual. Only clear and distinct products were used in statistical analysis. The data were analyzed using the Numerical Taxonomy Multivariate Analysis System (NTSYSpc version 2.1) (Rohlf 2000). A dendrogram was generated based on Dice's coefficient matrix by the unweighted pair group method arithmetic average (UPGMA) to determine genetic relationships among populations (Dice 1945). A Principal component analysis (PCA) was also performed to evaluate the genetic relationships existing among the genotypes.

The POPGEN v.1.32 (Yeh, Yang & Boyle, 1999) was used to calculate genetic diversity parameters such as: the observed number of alleles (Ao/Na), effective number of alleles (Ae/Ne), Nei's (1973) gene diversity (H), Shannon's information index (SI), the percentage of polymorphic bands (PPB), total genetic diversity (HT), genetic diversity within populations (Hs), the genetic differentiation coefficient (Gst) among populations and Nei's (1978) genetic distance (DN) between populations. In addition, the gene flow among the populations were calculated using the formula Nm = 0.5 (1-GST)/GST (McDermott and McDonald 1993).

The genetic variation within and between populations was calculated using AMOVA (Excoffier et al. 2005) (Arlequin ver. 3.0 software) (Univ. of Geneva, Geneva, Switzerland). Significance levels of the variance components were determined using permutations with 1000 replicates. A structure test (STRUCTURE 2.3.3 statistical software) was used to group individuals from different populations (Pritchard et al. 2000).

Results

A total of 50 ISSR primers was used in this study. From those, 13 primers producing polymorphic and reproducible products for estimation of genetic diversity in endemic *C. amaena* were selected. A total of 102 bands with fragment lengths ranging from 300 and 2700 bp were obtained with an average of 7.84 bands per primer, 80 of these bands were polymorphic. The polymorphism ratio per primer ranged from 50 to 100% with an average of 78% (Table 2).

The percentage of polymorphic bands (PPB) per population ranged from 63.73% (P2) to 68.63% (P1) with an average of 66.18 % ± 3.4, while at the species level, this value was 78.43%. The mean observed number of alleles (*Na*) ranged from 1.637 to 1.686, while the effective number of alleles (*Ne*) varied between 1.425 - 1.448. The Nei's gene diversity values (*H*) ranged from 0.245 to 0.259 with an average of 0.252, and the Shannon's information index (*SI*) varied between 0.361 - 0.383 with an average of 0.372. At species level, *Na, Ne, H* and *SI* were 1.784, 1.544, 0.306 and 0.447, respectively (Table 3). P1 population displayed a higher level of variability (PPB 68.63 %) than P2 population (PPB 63.73 %).

According to Nei's gene diversity statistic, the total genetic diversity was determined as (H_t)= 0.306±0.03. Of these, 0.252 was composed of within-population genetic diversity (H_s) and 0.054 among population genetic diversity (D_{ST}). The mean genetic differentiation coefficient (G_{ST}) between populations was found

to be 0.176, indicating that approximately 17.6% of the total variation between populations and that 82.4% of the variation was found within the populations. The mean gene flow (N_m) among *C. amaena* populations was found as 2.329, showing that high gene flow. The genetic distance (D_N) value and genetic identity between P1 and P2 populations was determined as 0.1563 and 0.8553, respectively.

UPGMA cluster analysis

The Dice similarity matrix was used to carry out cluster analysis by UPGMA method. The correlation between the similarity matrix and the dendrograms was determined by the Mantel test of matrix correspondence. The correlation coefficient of the Mantel test revealed a significant correlation between geographical and genetic distance (Correlation coefficient r =0.7821). Similarity coefficient values varied between 0.70 - 0.91 with an average value of 0.81. A clear division of 48 samples was provided with the UPGMA clustering map. Notably, it was evident that samples from the same populations clustered together. Present dendrogram had two distinct clusters. The first major group contained all genotypes belonging to the Perikartın (P1) population, while the second major group contained all genotypes including the Yılanlı population (P2). Major groups generally showed that samples were consistent with their regional sources (Fig. 2).

The relationships between populations were also assessed through Principal component analysis (PCA). Two genetically distinct clusters were determined in terms of genetic diversity among the genotypes. The first two principal coordinates (P1 and P2) accounted for 7.64% and 26.29% of the total variation, respectively (Fig. 3).

The results of AMOVA revealed that genetic variation within population was 75.10 %, while the variance among populations was 24.89% (P<0.001). Estimated F_{ST} value was determined as 0.248.

Population genetic structure was also evaluated with the Bayesian clustering approach implemented in STRUCTURE v.2.3.4. Bayesian cluster analysis of genetic structure revelaed that *C. amaena* populations were the best represented by two genetic groups (K=2). Two populations of *C. amaena* were efficiently separated into two sub-groups. There were 24 accessions in sub-population P1 and 24 accessions in sub-population P2 (Fig. 4).

Discussion

The primary objective of conservation of genetic resources is to preserve the broad-based genetic diversity within each of the species that has a known or potential value to ensure their availability to current and future generations. The genetic diversity of a plant is structured at different spatial scales (for example, geographic areas, populations, between neighboring individuals) and largely designated by species' life-history characteristics, environmental impacts and demographic history (Engelhardt et al. 2014; Penas et al. 2016). Thus, conservation management plans often require knowledge of population dynamics, relative levels of genetic diversity within genetic structure of the species (Perez-Collazos et al. 2008). The importance of genetic diversity in maintaining biodiversity and evolutionary processes and in

conservation biology studies of rare and endemic plant species has been recognized by researchers for decades (Laikre 2010).

In this study, genetic variation was investigated within and among *C. amaena* populations using ISSR markers. In general, endemic plant species tend to maintain low genetic diversity than widespread species (Ellstrand and Elam 1993). Contrarily, the genetic diversity of *C. amaena* was high at both population *(P* = 66.18 %, *h* = 0.252, *I* = 0 .372) and species *(P* = 78.43%, *h* = 0.306, *I*= 0.447) levels. These results may indicate that *C. amaena* did not have a history of severe or prolonged population bottlenecks sufficient to cause loss of genetic diversity. Similarly, many endemic species with high genetic diversity have been reported. For instance; *Centaurea nivea (P*= 91.88%, *h* = 0.296, *I*= 0.451), *C. lycaonica* (P:90.62%, h:0.2706, I:0.4148), *Verbascum alyssifolium (P*= 99.74%, *h* = 0.2651, *I*= 0.4206), *Teucrium leucophyllum (P*= 99.31%, *h* = 0.263, *I*= 0.418), *Lilium regale* (*PPB*: 97.3%, *H*: 0.198, *I*: 0.333) (Sözen and Özaydin 2009; Uysal et al. 2012; Wu et al. 2015; Hilooglu and Sozen 2017; Sozen et al. 2017).

The level of genetic diversity of *C. amaena* appears to be similar to that of the other endemic *Centaurea species*, although direct comparison is difficult when using different marker systems (eg AFLP, SSRs, allozyme). Freville et al. (2001b) investigated the genetic diversity of *C. corymbosa* via microsatellites and determined heterozygosity (He) values in the range of 0.36-0.62. By isozyme analysis of seven endemic *Centaurea* species, it was determined that heterozygosity values varied between 0.126 for C. *cineraria* subsp. *cineraria* to 0.276 for C. *todari* (Bancheva et al. 2006). A considerable amount of genetic variation was identified in endemic species *Centaurea horrida* (He=0.603–0.854) by using SSR markers (Mameli et al. 2008). In narrow endemic species of *Centaurea tentudaica*, quite high levels of genetic diversity were detected (P95 = 60.61, He = 0.287) by allozyme analysis (Moreyra et al. 2021). Mameli et al. (2008) suggested that high values of genetic diversity observed in these *Centaurea* species might have played a role in their survival in a challenging and stressful environment.

Reproductive biology of a species plays an important role in determining genetic variation at both the species and population levels. For instance, outcrossing taxa have the greatest diversity, while autogamous taxa have the lowest diversity (Hamrick and Godt 1996). Atasagun et al. (2018) determined that the breeding system of *C. amaena* was facultative xenogamous. This may be one of the reasons for high level of genetic diversity.

The greatest amount of genetic diversity in C. *amaena* was found within the population rather than among populations as estimated by Nei's gene diversity (82.35%), Shannon's information index (83.66%), and AMOVA (75.10%). Similar results have been previously reported in various studies of the following endangered species; *C. horrida* (Mameli et al. 2008), *C. nivea* (Sözen and Özaydin 2009), *C. lycaonica* (Uysal et al. 2012).

G_{ST} values above 0.30 indicate a high level of genetic differentiation, while G_{ST} values between 0.05-0.15 indicate a low level of genetic differentiation between populations. In *C. amaena*, the G_{ST} value was

determined as 0.176, indicating moderate level of genetic differentiation among the populations. A wide variety of F_{ST} and G_{ST} values have been obtained from studies on *Centaurea* taxa (Table 4). High genetic diversity and low population differentiation in endemic and rare plants have been attributed to a number of factors; insufficient time to reduce genetic diversity following isolation, population size reduction and significant gene flow (Maguire and Sedgley 1997; Zawko et al. 2001).

Populations tend to diverge when gene flow has a low value, whereas when gene flow has a high value, populations tend to remain uniform (Geraci Anna et al. 2012). The Nm value indicates whether genetic drift can produce substantial genetic variation between populations. If Nm is high (≥1), gene flow is strong enough to avoid significant differentiation caused by genetic drift (Slatkin and Barton 1989). The value of effective gene flow (*Nm*) of *C. amaena* was found as 2.329, which indicates sufficient to avoid population differentiation due to random genetic drift.

A total of 48 genotypes of *C. amaena* from 2 populations were examined for genetic diversity by using the 10 ISSR primers in this study. The cophenetic correlation coefficient (r) among the populations was determined as 0.7821 using the normalized Mantel. This value shows that the dendrogram represented the similarity matrix very well and present analyzes were reliable. In the similarity analysis using the UPGMA method based on Dice similarity coefficient, two populations were obviously differentiated. In the UPGMA tree, two main clusters were observed. The first cluster was composed of P1 population individuals, whereas the second cluster had only P2 population individuals. It was observed that individuals belonging to each population were grouped together. It has been stated that this tree topology may be affected by the genetic structure of populations, which may be associated with genomic forces such as mutations, deletions and insertions (Filiz et al. 2014).

PCA analysis of *C. amaena* revealed the cumulative sum of the first two eigen values as 26.29%. Once the first two or three principal axes were able to explain 25% or more of the total variation, PCA may be more useful technique for grouping individuals with a scatterplot presentation (Mohammadi and Prasanna 2003). Similar to the phylogenetic tree, the results of PCA revealed that individuals of P1 and P2 were scattered from one another.

The Structure analysis of *C. amaena* (*K*=2) genotypes revealed that each population represented an independent unit, as all individuals were clustered according to their population status. This pattern was also supported by UPGMA and PCoA analysis, in which the genotypes clustered similarly. It also shows that the populations had a simple pedigree and that the genetic exchange between each pair of populations was low. Similar results were also observed in studies with endemic plant species with small and isolated populations (Petrova et al. 2017; Wang et al. 2020).

For analysis of variance, genotypes were classified according to 2 sub-populations from Structure analysis. The AMOVA analysis revealed the total variation among the populations as 24.89% and total variation within the populations as 75.10%. The variation rate within the populations was found to be

significantly high (75.10%). Estimated F_{ST} value (Fst = 0.248, p < 0.001) value was found to be close to the mean level of among-population differentiation in endemic and narrow species (Nybom 2004).

Conclusion

Centaurea amaena is an endangered species with a very limited distribution with only two populations in Kayseri region. It was determined that the main factor threatening the species was anthropogenic related (including construction, tourism, habitat fragmentation). If the existing habitats are continuously damaged, the species will inevitably be confronted with extinction. It is important to understand patterns of genetic variability to develop efficient conservation strategies for endangered plants. Increasing population size and genetic diversity are among the main objectives of the many conservation and management programs (Frankham et al. 2002). To this end, habitat conservation is the preferred strategy to preserve the genetic diversity of *C. amaena* in this region.

Declarations

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Competing interests B. Atasagun declares no conflicts of interest.

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Tables

Table 1. Sampling details of the *C. amaena* populations

Populations	Latidute/ Longitude	Altitude (m)	Population Size	Sample Size	Sample Number
P1 (Yılanlı)	38º 42' 42" N 035º 25' 22" E	1223	1347	24	1-24
P2 (Perikartın)	38º 35' 12" N 035º 27' 40" E	2246	4325	24	25-48

Table 2. Detailed features of the ISSR markers used in PCR amplification of *C. amaena*

Primers	Sequence of primers (5’ - 3’)	Annealing Temperature (ºC)	No. of bands scored	Number of polymorphic bands	Polymorphism ratio %
UBC 805	TATATATATATATATAC	31	9	8	88.9
UBC 808	AGAGAGAGAGAGAGAGC	48.8	6	4	66.7
UBC 809	AGAGAGAGAGAGAGAGG	48.2	6	3	50
UBC 811	GAGAGAGAGAGAGAGAC	47	6	6	100
UBC 812	GAGAGGAGAGAGAGAA	45	8	7	87.5
UBC 814	CTCTCTCTCTCTCTCTA	45	7	7	100
UBC 816	CACACACACACACACAT	45	9	7	77.8
UBC 840	GAGAGAGAGAGAGAGACT	47.4	10	10	100
UBC 855	ACACACACACACACACCT	53.1	8	6	75
UBC 868	GAAGAAGAAGAAGAAGAA	43.2	6	3	50
ISSR 1	ACACACACACACACACG	54	7	5	71.4
ISSR 43	GTGTGTGTGTGTGTGTYA	51.4	10	6	60
ISSR 47	AGAGAGAGAGAGAGAGY	47.9	10	8	80
Total	**13**		**102**	**80**	**78**

Y: C/T

Table 3. Genetic structure of *C. amaena* populations based on ISSR data

Populations	N_a	N_e	H	SI	PPB (%)
P1	1.686±0.43	1.448±0.37	0.259±0.19	0.383±0.28	68.63
P2	1.637±0.48	1.425±0.38	0.245±0.20	0.361±0.29	63.73
Average	1.662±0.04	1.437±0.01	0.252±0.01	0.372±0.02	66.18
Species	1.784±0.41	1.544±0.37	0.306±0.19	0.447±0.27	78.43

Na, observed number of alleles; Ne, effective number of alleles; H, Nei's genetic diversity; SI, Shannon's information index; PPB, percentage of polymorphic bands

Table 4 Mean G_{ST} and F_{ST} values of some *Centaurea* taxa from previous studies

Species	Distribution	G_{ST}	F_{ST}	References
C. nivea	narrow	0.147	-	(Sözen and Özaydin, 2009)
C. lycaonica	narrow	0.201	-	(Uysal *et al.,* 2012)
C. horrida	narrow	-	0.123	(Mameli *et al.,* 2008)
C. parlatoris	narrow	-	0.176	(Bancheva *et al.,* 2011)
C. tentudaica	narrow	-	0.023	(Moreyra *et al.,* 2021)
***C.cineraria* group**	narrow	0.222	-	(Bancheva *et al,.* 2006)
***C. cineraria* gr. -*C.jacea* gr.**	narrow	-	0.24-0.43	(Geraci A *et al*., 2012)
C. solstitialis	widespread	0.095	-	(Sun, 1997)
Femeniasia balearica	narrow	0.30	-	(Vilatersana *et al*., 2007)

Figures

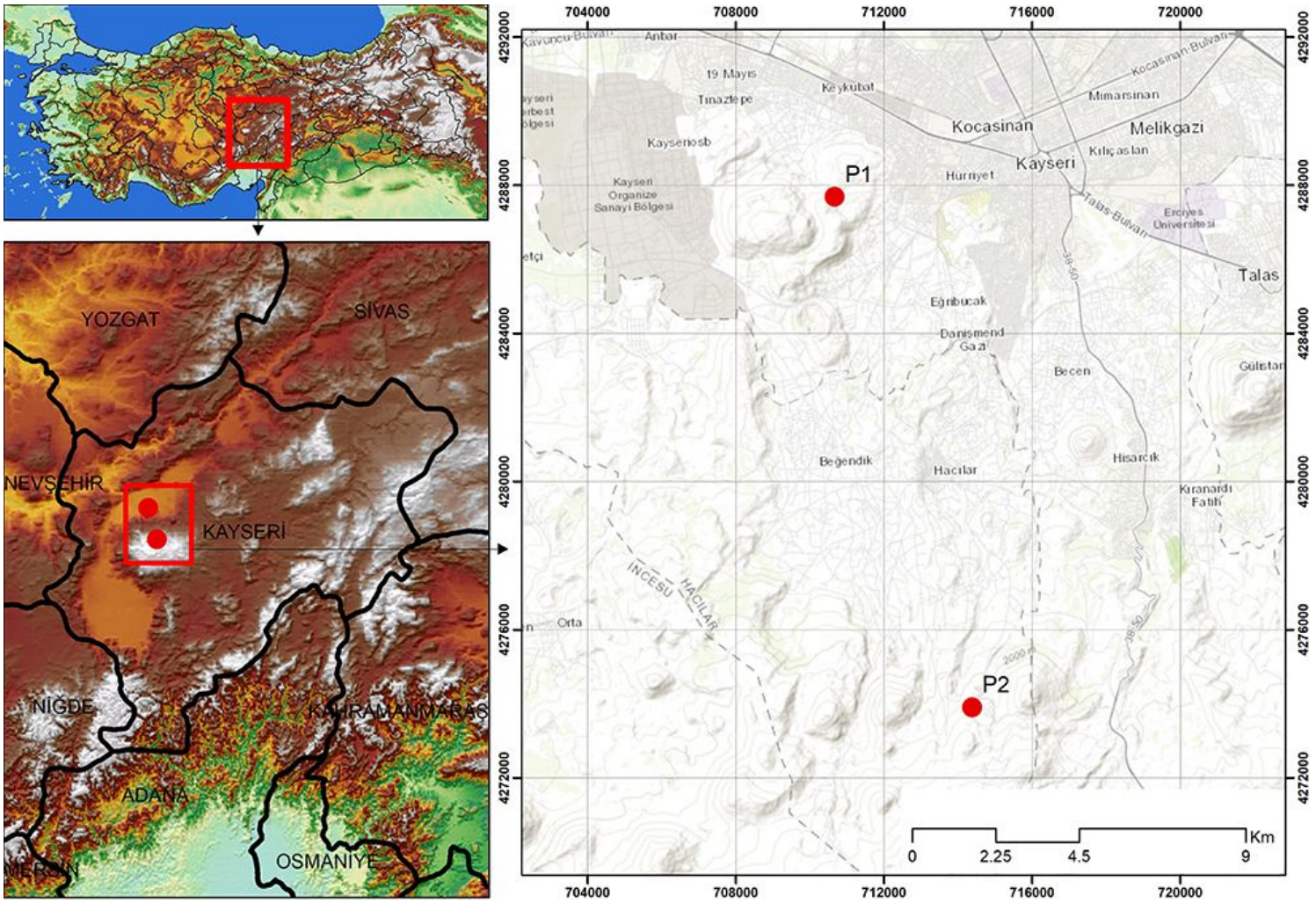

Figure 1

Geographical location of *Centaurea amaena* populations

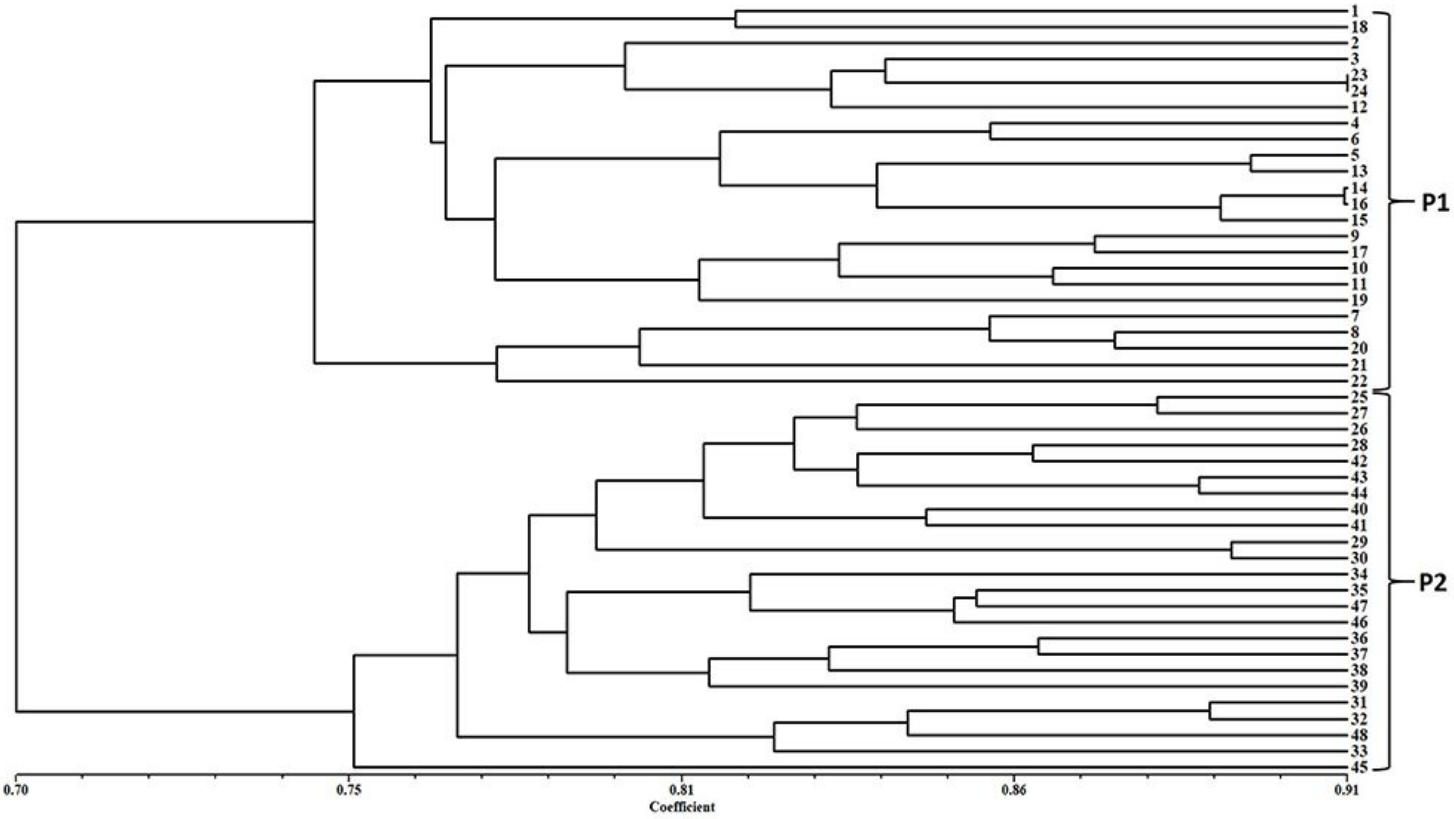

Figure 2

UPGMA dendrogram of genetic relationships among 48 genotypes of *C. amaena* based on Dice's similarity coefficient

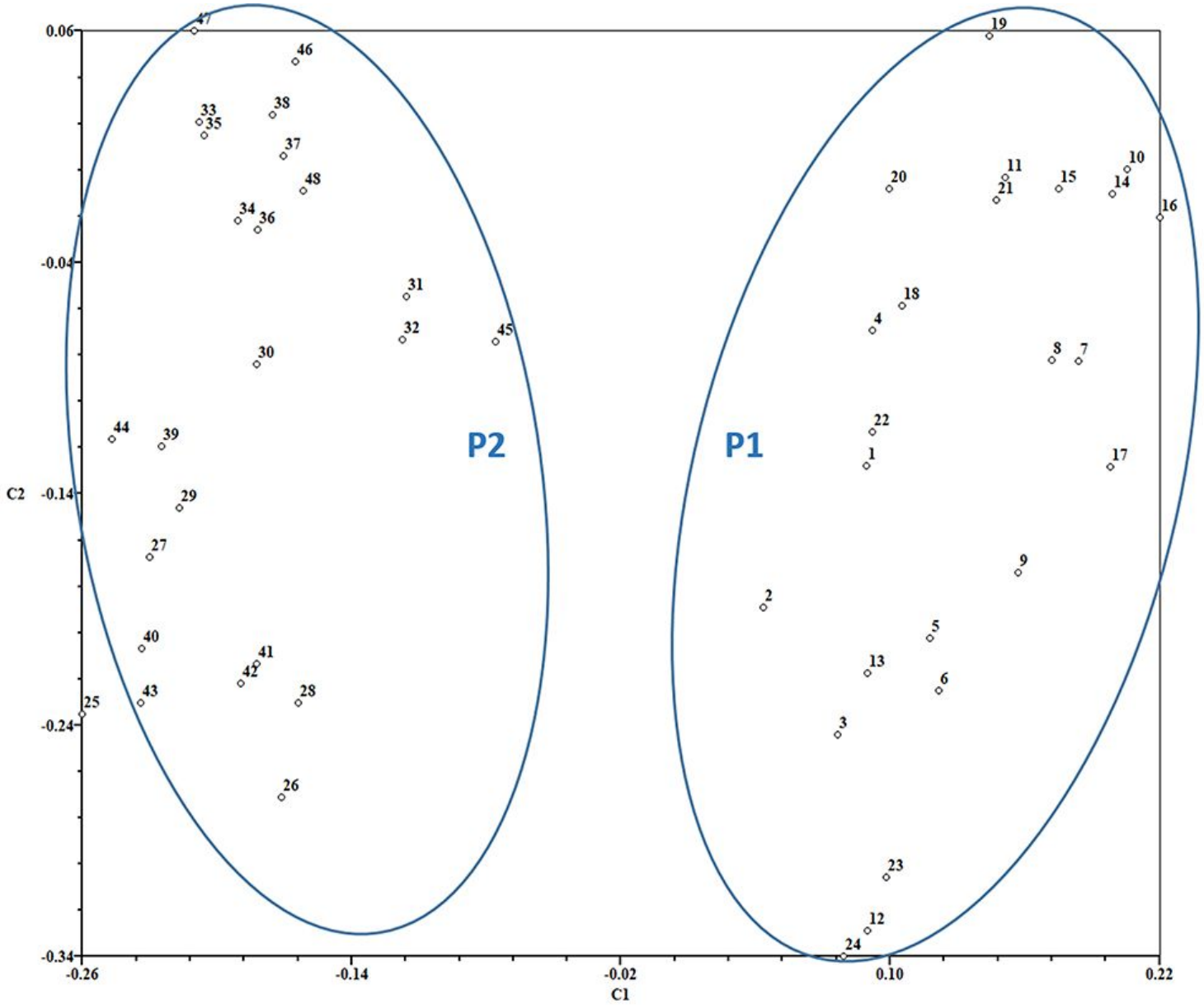

Figure 3

Two-dimensional graph of *C. amaena* genotypes by PCA

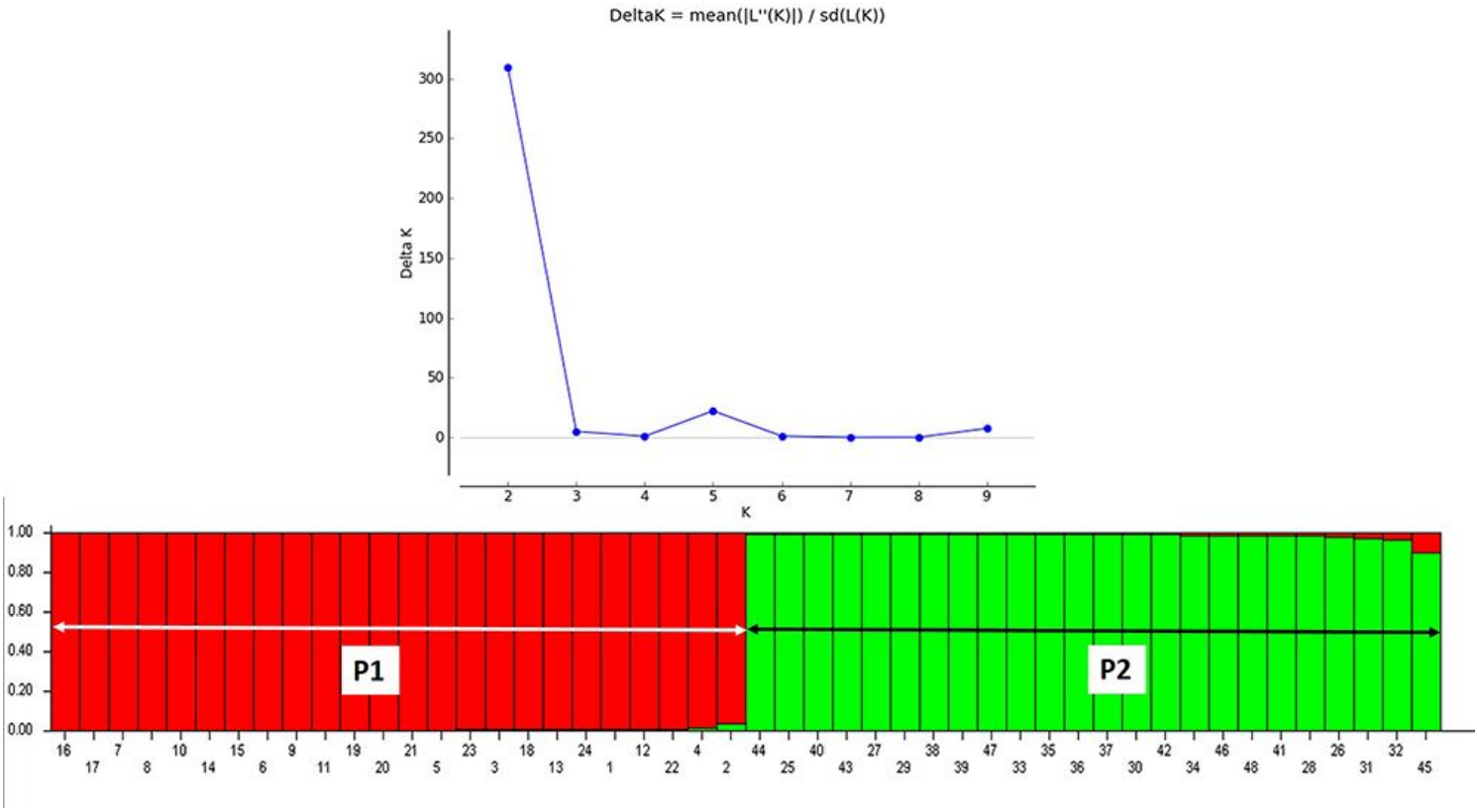

Figure 4

Delta-K values and Population structure analysis of *C. amaena*