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Assessment of the genetic diversity of a critically endangered species *Centaurea amaena* (Asteraceae)

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Abstract: *Centaurea amaena* is an endemic and endangered species listed as CR (critically endangered) 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 established gene flow (N_m) was 2.329, indicating a high migration rate between the populations. A 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% was 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 indicate that existing *C. amaena* populations should be conserved.

Keywords: *Centaurea*; endemic; genetic diversity; ISSR

INTRODUCTION

Turkey has quite a large geological and geomorphological diversity, and a wide variety of climate, topographic conditions and soil characteristics; it is located at the intersection of three different phytogeographical regions and is the center of differentiation of many genera and sections, thus having a quite rich flora [1]. *Centaurea* L. (Asteraceae) is an important and relatively large genus of Turkish flora. It is composed of approximately 743 species worldwide [2]. The genus is distributed in Southern and Central Europe, Anatolia, North Africa and the Caucasus. About 172 *Centaurea* species were identified in the flora of Turkey and the Eastern Aegean Islands [3]. Turkey is a center of diversity for *Centaurea*. Recently, with the latest additions, the number of *Centaurea* species has reached to 220 in Turkey [4-7]. Therefore, the endemism rate is approximately 60%. The *Centaurea amaena* Boiss. & Balansa included in the Sect. *Phalolepis* is a critically endangered endemic species and grows on the rocky slopes of Kayseri province.

Population genetics is a cornerstone of conservation biology [8]. The long-term persistence of a species depends on maintaining adequate genetic diversity within and between populations [9]. Detailed knowledge on the genetic structure of plant populations is required for conservation of available resources [10, 11].

Molecular markers; random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) are widely used to predict genetic variations at intraspecies and interspecies levels and to identify individual differences between populations. ISSR are usually dominant markers and allow more stringent amplification

[12,13]. ISSR markers have effectively been used in the fields of genetic diversity, phylogenetic analysis and evolutionary biology [14-18].

Genetic diversity levels have been reported for several endemic members of *Centaurea*, including *C. solstitialis* L. [19], *C. corymbosa* Pourr. [20], *Centaurea tenorei* Guss. ex Lacaita and *C. parlatoris* Heldr. [21], *C. cineraria* L. [22], *Femeniasia balearica* (J.J.Rodr.) Susanna [23], *C. horrida* Badarò [24], *C. nivea* (Bornm.) Wagenitz, [25], *Centaurea parlatoris* [26], *C. lycaonica* Boiss. & Heldr. [27], *C. stoebe* L. [28], *C. alba* L. [29], *C. tentudaica* (Rivas Goday) Rivas Goday & Rivas Mart. [30].

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 the critically endangered *C. amaena*.

MATERIALS AND METHODS

Plant sampling

The *C. amaena* is distributed in 2 populations in a small area between the Erciyes and Yılanlı mountains of Kayseri province. The two populations together cover an area of about 0.55 km^2 and the total number of individuals was determined as about 5672 (Supplementary Fig. S1). The distance between the 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 (Supplementary Table S1). 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 using the plant genomic DNA miniprep kit (Bio-Basic, Canada). DNA quantity and purity were assessed using Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA) and 1% agarose gel electrophoresis. DNA concentrations of all samples were diluted to 2 ng/μL, prepared for PCR and stored at -20°C. One individual from each population was used for the initial screening. 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 (Supplementary Table S2).

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 $MgCI_2$ (25 mM). The amplifications were performed using a thermal cycler (Bio-Rad, California, USA) that was programmed as follows: initial denaturation step at 94°C for 5 min; 35 cycles of 94°C for 45 s at a specific annealing temperature 1 min, 72°C for 1 min; final extension of 72°C for 7 min. The amplified PCR products were separated by 2% agarose gel electrophoresis in 1×TBE buffer at 80 V for 2 h and stained with ethidium bromide. 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, Taipei, Taiwan).

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) [31]. A dendrogram was generated based on Dice's coefficient matrix by the unweighted pair group method with arithmetic mean (UPGMA) to determine the genetic relationships among populations [32]. Principal component analysis (PCA) was also performed to evaluate the genetic relationships existing among the genotypes.

The POPGEN v.1.32 [33] was used to calculate genetic diversity parameters such as the observed number of alleles (A_o/N_a), the effective number of alleles (A_e/N_e), Nei's [34] gene diversity (H), Shannon's information index (SI), the percentage of polymorphic bands (PPB), total genetic diversity (H_T), genetic diversity within populations (H_s), the genetic differentiation coefficient (G_{st}) among populations and Nei's [35] genetic distance (D_N) between populations. In addition, the gene flow among the populations were calculated using the formula $N_m=0.5(1- G_{st})/G_{st}$ [36].

The genetic variation within and between populations was calculated using AMOVA [37] (Arlequin ver. 3.0 software) (University of Geneva, Geneva, Switzerland). The 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 [38].

RESULTS

A total of 50 ISSR primers was used in this study. From those, 13 primers producing polymorphic and reproducible products for the 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% (Supplementary Table S2).

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 (N_a) ranged from 1.637 to 1.686, while the effective number of alleles (N_e) 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 the species level, N_a, N_e, *H,* and *SI* were 1.784, 1.544, 0.306 and 0.447, respectively (Table 1). The P1 population displayed a higher level of variability (PPB 68.63 %) than the 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 about 17.6% of the total variation were between populations and that 82.4% of the variation were found within the populations. The mean gene flow (N_m) among *C. amaena* populations was 2.329, showing a high gene flow. The genetic distance (D_N) value and genetic identity between P1 and P2 populations were determined as 0.1563 and 0.8553, respectively.

Table 1. 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

N_a – observed number of alleles; N_e – effective number of alleles; H – Nei's genetic diversity; SI – Shannon's information index; PPB – percentage of polymorphic bands

UPGMA cluster analysis

The Dice similarity matrix was used to carry out cluster analysis by the 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. The 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. 1).

The relationships between populations were also assessed by 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. 2).

The results of AMOVA revealed that genetic variation within population was 75.10%, while the variance among populations was 24.89% (P<0.001). The 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

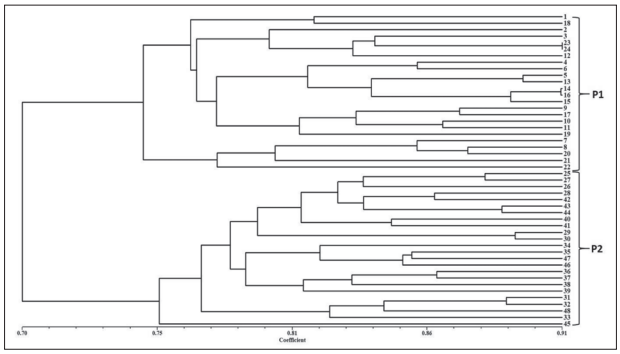

Fig. 1. UPGMA dendrogram of genetic relationships among 48 genotypes of *C. amaena* based on Dice's similarity coefficient.

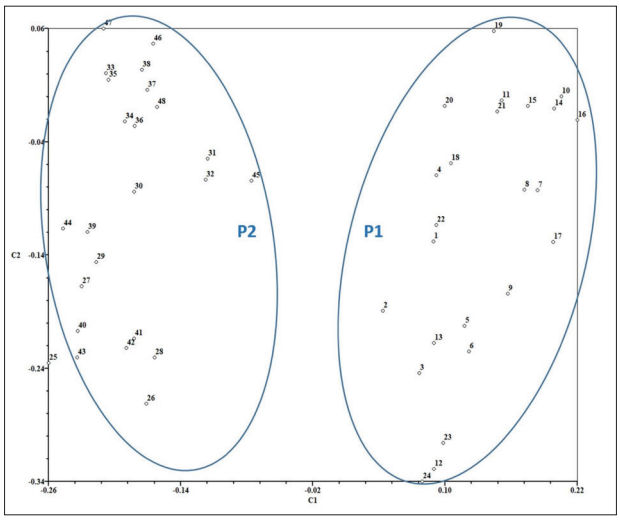

Fig. 2. Two-dimensional graph of *C. amaena* genotypes by obtained by PCA.

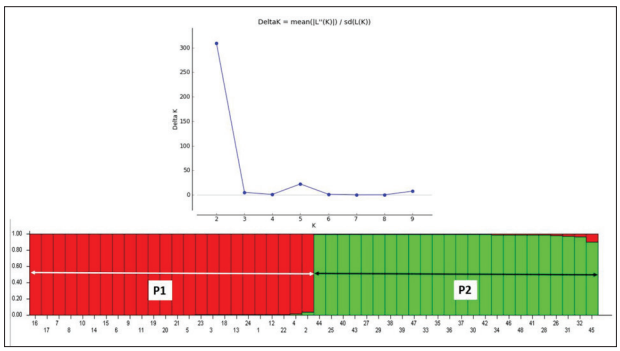

Fig. 3. Delta-K values and population structure analysis of *C. amaena*.

structure showed that *C. amaena* populations were best represented by two genetic groups (ΔK=2). Two populations of *C. amaena* were efficiently separated into two subgroups. There were 24 accessions in subpopulation P1 and 24 accessions in subpopulation P2 (Fig. 3).

DISCUSSION

The primary objective of conservation of genetic resources is to preserve the 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 the species' life-history characteristics, environmental impacts and demographic history [39,40]. Thus, conservation management plans often require knowledge of population dynamics and relative levels of genetic diversity within the genetic structure of the species [41]. The importance of genetic diversity in maintaining biodiversity and evolutionary processes, and in conservation biology of rare and endemic plant species, have been recognized by researchers for decades [8].

In this study, genetic variation was investigated within and among *C. amaena* populations using ISSR markers. In general, endemic plant species tend to maintain lower genetic diversity than widespread species [9]. 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 the 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) and *Lilium regale* (*PPB*: 97.3%, *h*: 0.198, *I*: 0.333) [17,25,27,42,43].

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 (AFLPs, SSRs, allozymes). The genetic diversity of *C. corymbosa* was investigated via microsatellites [44] and their heterozygosity (H_e) values were determined to be in the range of 0.36-0.62. By isozyme analysis of seven endemic *Centaurea* species, it was noted that heterozygosity values varied between 0.126 for *C. cineraria* subsp. *cineraria,* to 0.276 for C. *todari* [22]. A considerable amount of genetic variation was identified in endemic

Table 2. 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	-	[25]
C. lycaonica	narrow	0.201	-	[27]
C. horrida	narrow	-	0.123	[24]
C. parlatoris	narrow	-	0.176	[26]
C. tentudaica	narrow	-	0.023	[30]
***C. cineraria* group**	narrow	0.222	-	[22]
***C. cineraria* gr. -*C. jacea* gr.**	narrow	-	0.24-0.43	[28]
C. solstitialis	widespread	0.095	-	[19]
Femeniasia balearica	narrow	0.30	-	[23]

species *C. horrida* (He=0.603–0.854) using SSR markers [24]. In the narrow endemic species of *C. tentudaica*, high levels of genetic diversity were detected (P95=60.61, H_e=0.287) by allozyme analysis [30]. It was suggested [24] 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 variations at both the species and population levels. For instance, outcrossing taxa have the greatest diversity, while autogamous taxa have the lowest diversity [45]. It was observed [18] that the breeding system of *C. amaena* was facultative xenogamous, and this may be one of the reasons for its 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* [24], *C. nivea* [25] and *C. lycaonica* [27].

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 a moderate level of genetic differentiation among the populations. A wide variety of F_{ST} and G_{ST} values has been obtained from studies on *Centaurea* taxa and are summarized in Table 2. High genetic diversity and low population differentiation in endemic and rare plants have been attributed to several factors – insufficient time to reduce genetic diversity following isolation, population size reduction and significant gene flow [46,47].

Populations tend to diverge when gene flow has a low value, whereas when gene flow has a high value, populations tend to remain uniform [48]. The N_m value indicates whether genetic drift can produce substantial genetic variation between populations. If N_m is high (≥1), gene flow is strong enough to avoid significant differentiation caused by genetic drift [49]. The value of effective gene flow (N_m) of *C. amaena* was found as 2.329, which indicates it is sufficient to avoid population differentiation due to random genetic drift.

Herein, a total of 48 genotypes of *C. amaena* from 2 populations were examined for genetic diversity by using 13 ISSR primers. 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 the present analyses were reliable. In the similarity analysis using the UPGMA method based on the Dice similarity coefficient, the 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 [50].

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 a more useful technique for grouping individuals with a scatterplot presentation [51]. Like the phylogenetic tree, the results of PCA revealed that individuals of P1 and P2 were scattered from one another.

Structure analysis of *C. amaena* (ΔK=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 PCA 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 [14, 52].

For analysis of molecular variance, genotypes were classified according to 2 subpopulations as based on structure analysis. AMOVA 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%). The estimated F_{ST} value (F_{ST}=0.248, P<0.001) value was found to be close to the mean level of among-population differentiation in endemic and narrow species [53].

CONCLUSION

Centaurea amaena is an endangered species with a very limited distribution with only two populations in the 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 spoiled, 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 [54]. To this end, habitat conservation is the preferred strategy to preserve the genetic diversity of *C. amaena* in this region.

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Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Atasagun_8004_Data%20Report.pdf

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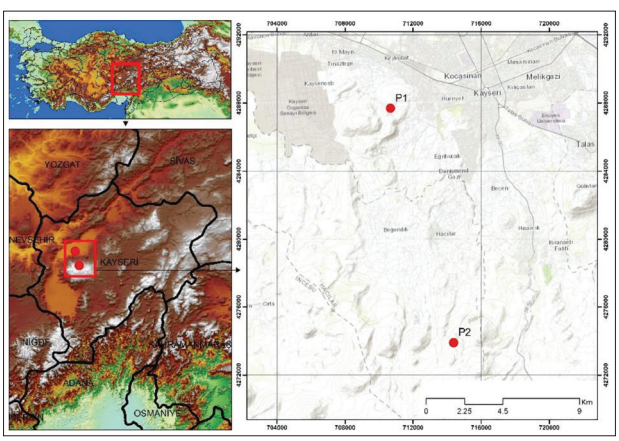
Supplementary material

Supplementary Table S1. 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



Supplementary Fig. S1. Geographical location of *Centaurea amaena* populations.

Supplementary Table S2. 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	No. 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