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Genetic diversity and molecular characterization of natural *Pancratium maritimum* L. populations by DNA markers

Ceren ELİBOL[1], Behiye Banu BİLGEN[2,*]

[1]Department of Agricultural Biotechnology, Faculty of Agriculture, Eskişehir Osmangazi University, Eskişehir, Turkey
[2]Department of Agricultural Biotechnology, Faculty of Agriculture, Namık Kemal University, Tekirdağ, TURKEY

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Abstract: Bulbous plants play an important role in Turkey's biodiversity due to their great potential use in various industries. Sea daffodils (*Pancratium maritimum*), one aspect of Turkey's biological richness, represent an important bulbous plant that generally spreads on sand dunes and can be seen only on the Mediterranean coast and on its certain beaches in our country. In this study, the genetic structure and genetic diversity of four natural *P. maritimum* populations have been determined by RAPD and nrSSR primers. Eight RAPD and four nrSSR loci were analyzed. All RAPD and nrSSR loci, except SSR-20, were found to be polymorphic. Genetic diversity parameters such as mean number of alleles for each nrSSR loci (N_a = 3.313), effective allele number (N_e = 2.190), Shannon's information index (I = 0.728), observed heterozygosity (H_o = 0.449) and expected heterozygosity (H_e = 0.396) were calculated. A rather high proportion of the genetic diversity (81% for nrSSR, 72% for RAPD) was due to within-population variation and the remaining part was due to variation between populations. According to the acquired UPGMA phenogram for RAPD and nrSSR data, the İğneada and Çamlıkoy populations, which are geographically close, are also genetically the most similar populations. The STRUCTURE analysis results supported the constructed UPGMA phenogram for the studied sea daffodil populations. The results of this study include important information about the genetic structure of the studied populations.

Key words: Genetic conservation, genetic diversity, nrSSR, *Pancratium maritimum*, RAPD, sea daffodil

1. Introduction

Turkey is considered as one of the most important gene centers in the world due to its varied geography, topography, climatic diversity, geology, and ecology. Moreover, being a transitional zone between Europe and Asia, located in the Euro-Siberian, Mediterranean, and Irano-Turanian phytogeographical regions, there is a rich floristic structure in our country (Şekercioğlu et al., 2011). Turkey is located in both Mediterranean and Near Eastern gene centers and this contributes significantly to its genetic diversity. The presence of plant genetic resources increases the importance of biological diversity (Demir, 2013). The gene centers ensure very important gene resources for the future sustainability of many plant species. Unfortunately, the important plant genetic resources are seriously threatened throughout the world by overpopulation, urbanization, tourism, pollution, industry, overgrazing, habitat loss, diseases, climate change, and poor legislation (FAO, 2010; Ogwu et al., 2014).

Plants having underground perennation organs (such as bulbs, corms, tubers, or rhizomes) are called geophytes. They have an important contribution to Turkey's biodiversity because approximately 600 geophytes naturally grow in our country (Koyuncu and Alp, 2014; Gümüş, 2015). Amaryllidaceae, with approximately 1100 species belonging to 85 genera, is a family of bulbous plants. In Turkey this family is represented by 33 taxa and 28 species belonging to 5 genera except the ornamental ones coming from abroad to Turkey (Güner et al., 2000; Gümüş, 2015). *Pancratium* L. is a genus of Amaryllidaceae with 21 species in the world. *Pancratium maritimum* (sea daffodils) is the only species of the genus *Pancratium* that grows naturally in Turkey. Sea daffodils grow mainly on the coastal sands of the Mediterranean, Black, and Caspian seas, and also the European part of Atlantic Ocean (De Castro et al., 2012). In Turkey, *P. maritimum* grows naturally on sandy beaches of the Thrace region, İstanbul, Bolu, Bartın, Sinop, Samsun, Giresun, Trabzon, Antalya, Mersin, İzmir, and Adana (Davis, 1984; Yaltırık and Efe, 1996; Gümüş, 2015). Briefly, *P. maritimum* is a bulbous species capable of vegetative reproduction and its seeds are dispersed by water and wind due to their specialized

* Correspondence: bbilgen@nku.edu.tr

structure (Zahreddine et al., 2004; De Castro et al., 2016). The haploid chromosome number of the species is n = 11 (De Castro et al., 2012). *P. maritimum* populations in their original habitats in Turkey are threatened by extinction like other populations on the coasts of the Mediterranean and Black seas (Gümüş, 2015). *P. maritimum* has ornamental, medicinal, pharmacological, and ecological properties, and as a consequence certain urgent conservation and management strategies are needed in order to prevent the decrease of populations of these plants (Ioset et al., 2001). The excess use of flowers for ornamental purposes, urbanization, usage of sandy beaches for tourism, sunbathing, and sand dune erosion seriously threaten the species and cause a very significant decrease of its populations (Nikopoulos et al., 2008; Schlacher et al., 2008; Demir et al., 2010; Ciccarelli, 2015; De Castro et al., 2016).

Several studies about the chemical composition (Georgiev et al., 2011; El-Hadidy et al., 2012; Sanaa et al., 2013, 2014), pharmaceutical usage (Hetta and Shafei, 2013; Ibrahim et al., 2013), germination and seedling production (Balestri and Cinelli, 2004), in vitro micropropagation (Bogdanova et al., 2009; Nikopoulos and Alexopoulos, 2008), and genetic diversity with morphological and molecular markers of *P. maritimum* have been conducted (Zahreddine et al., 2004; Grassi et al., 2005; Sanaa and Fadhel, 2010; De Castro et al., 2012; El-Hadidy et al., 2012; Di Maio and De Castro, 2013; Sanaa et al., 2014; Giovino et al., 2015; Perrone et al., 2015; Sanaa et al., 2015; De Castro et al., 2016). Based on the reviewed literature, only one previous study about genetic characterization with RAPD and SRAP primers has been carried out with regard to the two populations of sea daffodils from Turkey (Hocagil et al., 2010).

A sustainable development strategy aims at the protection and utilization of natural sources for many years instead of preventing their usage (Ogwu et al., 2014). For this reason, the determination of genetic structure with molecular approaches is also needed in order to protect the great important genetic resources of the *P. maritimum*, especially for both economic and biodiversity aspects and to provide appropriate production strategies. The determination of the genetic diversity, which is one of the most important components of biological diversity, is important for healthy ecosystems and sustainability. In order to prevent the reduction of the natural populations of *P. maritimum*, conservation methods with both traditional and biotechnological approaches and production activities should be carried out, and also should be supported by molecular studies. SSRs have been the most widely used markers for the study of the genetic structure of plants because they are codominant, highly informative, polymorphic, neutral, and reproducible (Vieira et al., 2016). The main purposes of this study were as follows: 1) to determine the genetic structure of the studied populations by comparing data from RAPD and nrSSR analysis; 2) to estimate the genetic diversity parameters; and 3) to provide basic information for management strategies and genetic resource conservation programs of *Pancratium maritimum* by studying genetic intra- and interpopulation variation.

2. Materials and methods

2.1. Plant materials

Fresh leaves of at least 20 individuals from each of the four natural *P. maritimum* populations were collected in August 2014 and June 2015 without destroying the selected populations during sampling. The studied populations were from İğneada Floodplain Forests National Park (Kırklareli), Çamlıkoy National Park (Tekirdağ), Pamucak Coast (İzmir), and Belek Coast (Antalya) (Figure 1). In order to avoid the sampling of genetically same individuals due to vegetative reproduction, sampled individuals were at least 10 m in distance apart from each other. Fresh leaves were stored at –80 °C until DNA isolation.

2.2. DNA isolation

For the DNA isolation, the leaves belonging to individuals of each population were used. Each sample was ground with a ball mill (Retsch MM400). Total genomic DNA was isolated by using the i-genomic Plant DNA Extraction Mini Kit (Intron Biotechnology) following the instruction manual. The DNA in samples was quantified with the Qubit 2.0 Fluorometer and also controlled by electrophoresis on 1% agarose gels with RedSafe Nucleic Acid Staining Solution in 1X TBE buffer at 80 V constant for 30 min and visualized under UV light (gel imaging system, Vilber Lourmat Quantum ST5). The extracted DNA samples were diluted to 10 ng/µL for PCR analysis and stored at –20 °C for further use.

2.3. RAPD analysis

Eight RAPD (OPA-01, OPA-02, OPB-10, OPB-12, OPN-06, OPN-12, OPV-08, and OPV-18) primers were selected for analysis (Table 1). Different DNA template (10 ng, 20 ng, 30 ng), primer (5 pmol, 10 pmol, 15 pmol), $MgCl_2$ concentrations (1.5 mM, 2 mM, 2.5 mM, 3 mM), and Taq DNA polymerase concentrations (1 U, 1.5 U, 2 U) were analyzed in order to determine the optimum conditions for RAPD analysis and reproducibility. The final PCR reactions were performed in volumes of 10 µL containing 1X reaction buffer, 20 ng of DNA, 10 pmol primer, 3 mM $MgCl_2$, 0.3 mM dNTP, and 2 U of Taq DNA polymerase. The PCR profile for RAPD primers consisted of 3 min of denaturing at 94 °C, followed by 45 cycles of 1 min of denaturing at 94 °C, 1 min of annealing at 37 °C, and 1 min of extension at 72 °C, with a final extension of 10 min at 72 °C. PCR products were visualized on 1.7% agarose gels using a 100-bp DNA ladder to determine the size of RAPD bands during data collection. All the samples

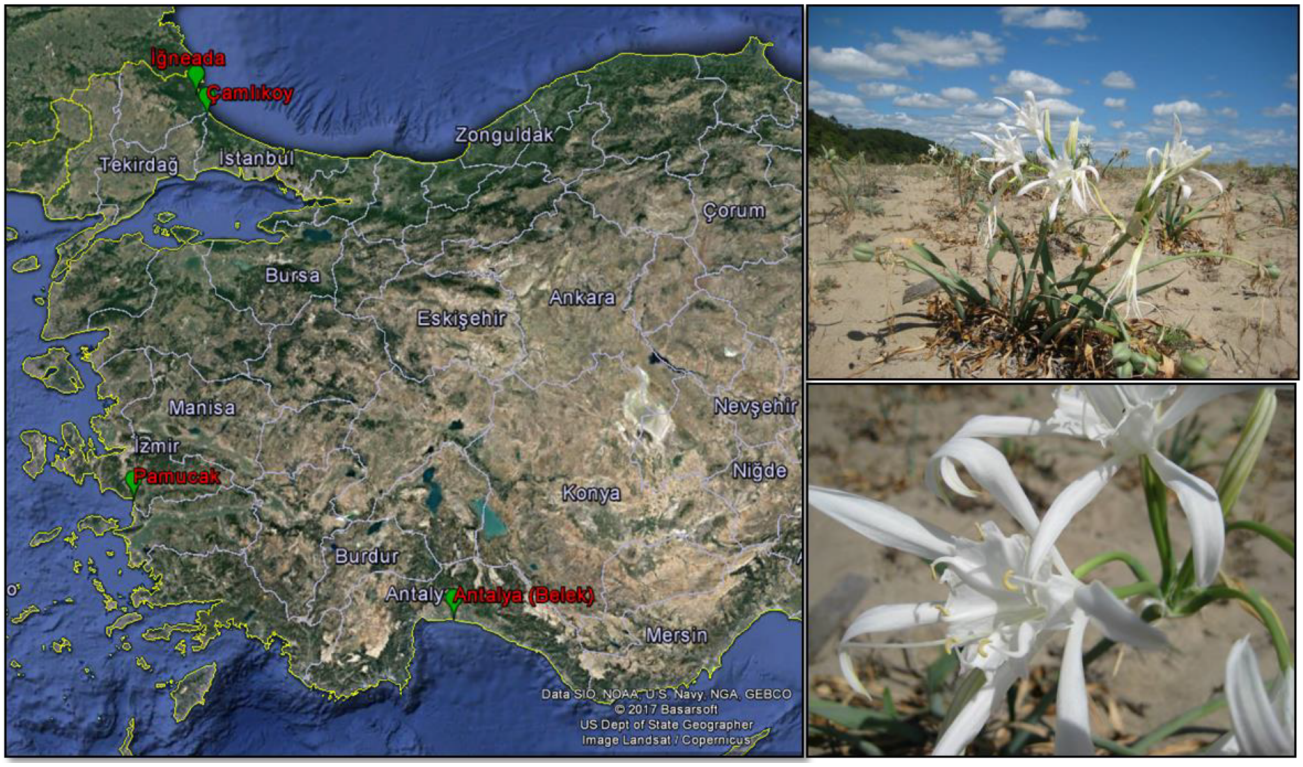

Figure 1. Geographic locations of 4 studied *P. maritimum* populations and pictures of some individuals analyzed (Google Earth ©2016).

Table 1. Characteristics of the RAPD and nrSSR markers used to analyze the genetic diversity of *P. maritimum* populations.

Locus	Primer sequences (5′-3′)	Tm (°C)	Observed allele size range (bp)	Observed number of loci/alleles
RAPD				Loci
OPA-01	CAGGCCCTTG	37	230–1940	11
OPA-02	TGCCGAGCTG		295–1690	10
OPB-10	CTGCTGGGAC		250–1500	6
OPB-12	CCTTGACGCA		315–1380	9
OPN-06	GAGACGCACA		270–1400	15
OPN-12	CACAGACACC		490–2350	6
OPV-08	GGACGGCGTT		380–1360	7
OPV-18	TGGTGGCGTT		160–1500	10
nrSSRs				Alleles
SSR15	F: 5’-PET-GATATCCTCAAACGCC-3’ R: CGTCTTCCCCTTCTCTGG	54	189–193	3
SSR20	F: 5’-NED-GACTATTGGGCCATATTGGG-3’ R: CCTGAATACACTCGCAATCC	56	131	1
SSR27	F: 5’-VIC-ATGGAGGTTTATGAGATGGC-3’ R: CATATCTCTCTCCTCCACC	56	196–205	5
SSR38	F: 5’-FAM-TGACGAGGATGAAGCTCC-3’ R: ACCTGTTTGACCCCTCAC	54	115–135	11

were analyzed twice (some of them three times) for eight primers to assure the reproducibility of the PCR product.

2.4. nrSSR analysis

Four nrSSR (SSR-15, SSR-20, SSR-27, and SSR-38) loci from 21 developed nuclear microsatellite markers (nrSSR) were chosen for this study (Di Maio and De Castro, 2013). The characteristics of the used primers are shown in Table 1. Forward primers were 5′ end-labeled with fluorochromes (PET, NED, VIC, and FAM). The PCR amplifications were performed as described by Di Maio and De Castro (2013). The DNA amplifications by PCR were carried out using the Applied Biosystems Veriti Thermal Cycler and Applied Biosystems ProFlex PCR System Thermal Cycler. Amplified allele fragments were separated using a 3500 Genetic Analyzer (Applied Biosystems, Life Technologies, UK) and their sizes were determined with reference to the GeneScan 500 LIZ size standard using GeneMapper software version 5.0 (Applied Biosystems).

2.5. Data analysis

The size of PCR products on gels for each RAPD primer was determined using Vision-Capt Software (Vilber Lourmat). PCR products were scored as discrete binary states (present/absent). For the analysis of RAPD data, genetic diversity parameters such as polymorphic loci (P), allele frequencies, allelic richness, effective number of alleles, expected heterozygosity, and Shannon's information index were calculated using POPGENE software version 1.31 (Yeh et al., 1999) and GenAlEx Version 6.3 (Peakall and Smouse, 2006). For the analysis of nrSSR data, allelic and genotypic frequencies, effective numbers of alleles (N_e), Shannon's information index (I), observed and expected heterozygosity (H_o and H_e, respectively), fixation index (F), and polymorphic information contents (PIC) were calculated using POPGENE software version 1.31 (Yeh et al., 1999) and GenAlEx Version 6.3 (Peakall and Smouse, 2006).

The analysis of molecular variance (AMOVA) method was applied using ARLEQUIN Version 3.11 (Excoffier et al., 2005) to determine the genetic variation within and among populations and the significance of AMOVA results was evaluated with 1000 permutations of the acquired data. Genetic relationships among populations were estimated using Nei's unbiased genetic distance measure (Nei, 1987). Cluster analysis, based on Nei's genetic distance matrix, was performed by using the unweighted pair group method (UPGMA). Bayesian model-based cluster analysis was also performed for nrSSR data using STRUCTURE 2.3.4 software in order to identify gene pools of studied populations (Pritchard et al., 2000). In order to obtain the appropriate K value from the data, based on the work of Evanno et al. (2005), STRUCTURE HARVESTER software was used (Earl and vonHoldt, 2012).

3. Results

Eight RAPD primers initially revealed 78 polymorphic bands among 84 samples belonging to four populations. Some of the bands had very poor staining; these bands were discarded from the final dataset after analyzed three times. Therefore, 74 polymorphic bands with 2 alleles (present/absent) were used in the statistical analysis. Polymorphisms of RAPD loci for the Antalya, Pamucak, İğneada, and Çamlıkoy populations were 62%, 76%, 81%, and 87%, respectively. The scored loci numbers ranged from 6 (OPB-10 and OPN-12) to 15 (OPN-06). Observed allele size ranged from 160 to 2350 when all RAPD primers evaluated (Table 1). The estimated mean number of observed alleles (N_a) was 1.645. The highest number of effective alleles (N_e) was observed in the Pamucak population (1.503), whereas the lowest N_e was found in Çamlıkoy population (1.357). When all populations were evaluated, overall mean N_e was 1.434 (Table 2). Overall mean Shannon's information index (I) and expected heterozygosity (H_e) were estimated as 0.385 and 0.255, respectively. H_e was lowest in the Çamlıkoy population (0.224) and highest in the Pamucak population (0.285) (Table 2).

Using four nrSSRs (SSR-15, SSR-20, SSR-27, and SSR-38), 20 alleles with a mean of 3.313 alleles per population and locus were identified. Considering the four studied sea daffodil populations, SSR-38 has the highest number of alleles (11 alleles) and the locus SSR-20 has the lowest number of alleles (one allele) (Table 1). Genetic diversity estimations by four nrSSR loci in the four studied populations are presented in Table 3. In relation to how informative the selected SSR markers were, 3 of them (75%) were polymorphic, except SSR-20, showing a moderate overall PIC value of 0.345. SSR-38 was a highly informative marker with a PIC value of 0.714 (PIC > 0.50). SSR-15 and SSR-27 were moderately informative markers with 0.367 and 0.300 PIC values, respectively (0.25 < PIC < 0.50).

Based on nrSSR analysis, the mean number of alleles per locus (N_a) ranged from 3.00 (İğneada and Çamlıkoy) to 4.00 (Antalya). The overall mean number of effective alleles per locus (N_e) was 2.19 (varied from 1.860 to 2.405). The highest number of private alleles (four) was determined in the Antalya population and the lowest number of private alleles (one) was determined in the Pamucak population (Table 3). There were no private alleles in the İğneada or Çamlıkoy populations. The Antalya population has one private allele (193 bp) for SSR-15, two private alleles (200 and 202 bp) for SSR-27, and one private allele (135 bp) for SSR-38. The Pamucak population has only 1 private allele (204 bp) for SSR-27. Overall mean Shannon's information index (I) was estimated as 0.728, the highest in the Antalya population (0.779) and lowest in the İğneada population

Table 2. Genetic diversity estimated using eight RAPD loci in *P. maritimum* populations.

Population	N*	N_a*	N_e*	I*	H_e*
Antalya	20	1.486 (±0.084)	1.401 (±0.045)	0.341 (±0.034)	0.231 (±0.024)
Pamucak	20	1.608 (±0.086)	1.503 (±0.045)	0.420 (±0.032)	0.285 (±0.023)
İğneada	22	1.811 (±0.060)	1.474 (±0.041)	0.425 (±0.028)	0.281 (±0.021)
Çamlıkoy	22	1.676 (±0.082)	1.357 (±0.037)	0.352 (±0.027)	0.224 (±0.020)
Overall mean	21	1.645 (±0.040)	1.434 (±0.021)	0.385 (±0.015)	0.255 (±0.011)

*N = Sample size, N_a = mean number of alleles per locus, N_e = effective number of alleles, I = Shannon's information index, H_e = expected heterozygosity (Nei, 1987), ± standard errors in parentheses.

Table 3. Genetic diversity estimated using four nrSSR loci in *P. maritimum* populations.

Population	N*	P_{PL}*	N_a*	N_e*	S*	I*	H_o*	H_e*	F*
Antalya	20	75	4.00 (±1.732)	2.405 (±0.998)	4	0.779 (±0.402)	0.363 (±0.220)	0.384 (±0.175)	0.236 (±0.244)
Pamucak	20	75	3.25 (±1.315)	2.196 (±0.866)	1	0.699 (±0.356)	0.388 (±0.194)	0.363 (±0.165)	–0.007 (±0.109)
İğneada	20	75	3.00 (±1.354)	1.860 (±0.311)	0	0.666 (±0.264)	0.500 (±0.205)	0.397 (±0.134)	–0.280 (±0.312)
Çamlıkoy	20	75	3.00 (±1.354)	2.298 (±0.686)	0	0.767 (±0.349)	0.546 (±0.228)	0.438 (±0.159)	–0.269 (±0.330)
Overall mean	20	75	3.313 (±0.656)	2.190 (±0.344)	1.25 (±0.73)	0.728 (±0.155)	0.449 (±0.097)	0.396 (±0.072)	–0.080 (±0.125)

*N = Mean sample size, P_{PL} = polymorphic loci (%), N_a = mean number of alleles per locus, N_e = effective number of alleles, S = total number of private alleles observed, I = Shannon's information index, H_o = observed heterozygosity, H_e = expected heterozygosity (Nei 1987), F = fixation index, ± standard errors in parentheses.

(0.666). Expected heterozygosity (H_e) and observed heterozygosity (H_o) were 0.396 and 0.449 on average, respectively (Table 3). The H_o among all of the loci was 0.599; H_e was 0.528. The observed heterozygosity was higher than the expected value, and this caused a negative mean inbreeding coefficient (F_{IS} = –0.111 ± 0204) (Table 4). The F_{IS} value across all of the loci ranged from –0.528 to 0.285 and the F_{ST} values ranged from 0.036 to 0.149 (Table 4).

Nei's (1987) genetic distance coefficient ranged from 0.034 (İğneada-Çamlıkoy) to 0.272 (Çamlıkoy-Antalya) among all possible population pairs for RAPD data. Thus, a high proportion (72%) of genetic variation was due to the differences within populations according to AMOVA analysis (Table 5). AMOVA results for nrSSRs show that 81% of the diversity was due to intrapopulation variation. Moreover, genetic variation among populations accounted for 19% of the total variation (Table 5).

UPGMA trees for both RAPD and nrSSRs according to Nei's genetic distance are shown in Figure 2. The phenograms for both RAPD and nrSSR data were divided into two clusters, where cluster I grouped two northern populations of sea daffodil from Turkey (İğneada and Çamlıkoy) and cluster II grouped the other two sea daffodil populations from the southern and southwestern part of Turkey (Pamucak and Antalya). A STRUCTURE

Table 4. Genetic parameters for each of the polymorphic SSR loci used in the analysis of *P. maritimum* populations.

Locus	H_o	H_e	F_{IS}	F_{ST}
SSR15	0.725	0.474	–0.528	0.036
SSR27	0.263	0.367	0.285	0.149
SSR38	0.808	0.741	–0.091	0.107
Mean (±SE)	0.599 (±0.091)	0.528 (±0.139)	–0.111 (±0.204)	0.097 (±0.029)

Table 5. The analysis of molecular variance (AMOVA) at RAPD and nrSSR loci.

Source of variation	df*	Sum of squares	Variance components	Percentage of variation
RAPD				
Among populations	3	266.824	3.774	28%
Within populations	80	780.295	9.754	72%
nrSSR				
Among populations	3	28.678	0.392	19%
Within populations	77	125.100	1.625	81%

*df = Degrees of freedom.

analysis performed with the given groups using nrSSR data also showed two main genetic clusters for K = 2, as in the UPGMA tree (Figure 3). In Figure 3, each column represents an individual belonging to a population; each color (red and green) denotes a population cluster. The STRUCTURE analysis indicated the existence of two major gene pools, one of them represented by the İğneada and Çamlıkoy populations and the other represented by the Antalya and Pamucak populations.

4. Discussion

Genetic diversity has significant value to enable a population's existence in the future while environmental conditions change. Consequently, the maintenance of high genetic diversity in plant populations and the conservation of these populations are the most important issues for genetic resource management studies. In this study, four nrSSR loci and eight RAPD primers were analyzed. All nrSSR loci, except SSR-20, were found to be polymorphic (75%). A total of 20 alleles for nrSSR loci and 74 polymorphic bands (loci) for RAPD analysis were determined. Zahreddine et al. (2004) studied the genetic structure of *P. maritimum* populations in Lebanon with 10 RAPD markers. They reported that 110 out of 118 bands were polymorphic (93%) across all primers. Grassi et al. (2005) used AFLP analysis in order to study gene flow and genetic diversity of 10 *P. maritimum* populations in the Northern Tyrrhenian Sea. In their study, the polymorphism level was estimated to be low both within populations and among them as 3.56%. In the work of Sanaa and Fadhel (2010), 14 mainland and 5 island *P. maritimum* populations were investigated to determine the genetic structure via 7 isozyme loci, and 12 out of 18 loci were found to be polymorphic. Di Maio and De Castro (2013) developed 21 SSR markers for sea daffodil by using 48 samples gathered from Israel, Italy, and Spain. They reported that 19 out of 21 SSR loci were polymorphic. In our study, we selected 4 nrSSR loci from the study of Di Maio and De Castro (2013) according to the detected number of alleles and polymorphism levels as well as PIC values. In their study, they reported that 4 nrSSR loci (SSR-15, SSR-20, SSR-27, and SSR-38) were polymorphic, having allele numbers higher than four and PIC values around 0.500 or above. In our study, PIC values for SSR-15 (0.367) and SSR-27 (0.303) were lower than the reported values of Di Maio and De Castro (2013). This result might be caused by the geographic differences of the studied populations and the differences of analyzed numbers of individuals in these two studies; the level of self-pollination in the studied populations could be another reason. The PIC value for SSR-38 is compatible between our study (0.714) and Di Maio and De Castro's study (0.752). De Castro et al.

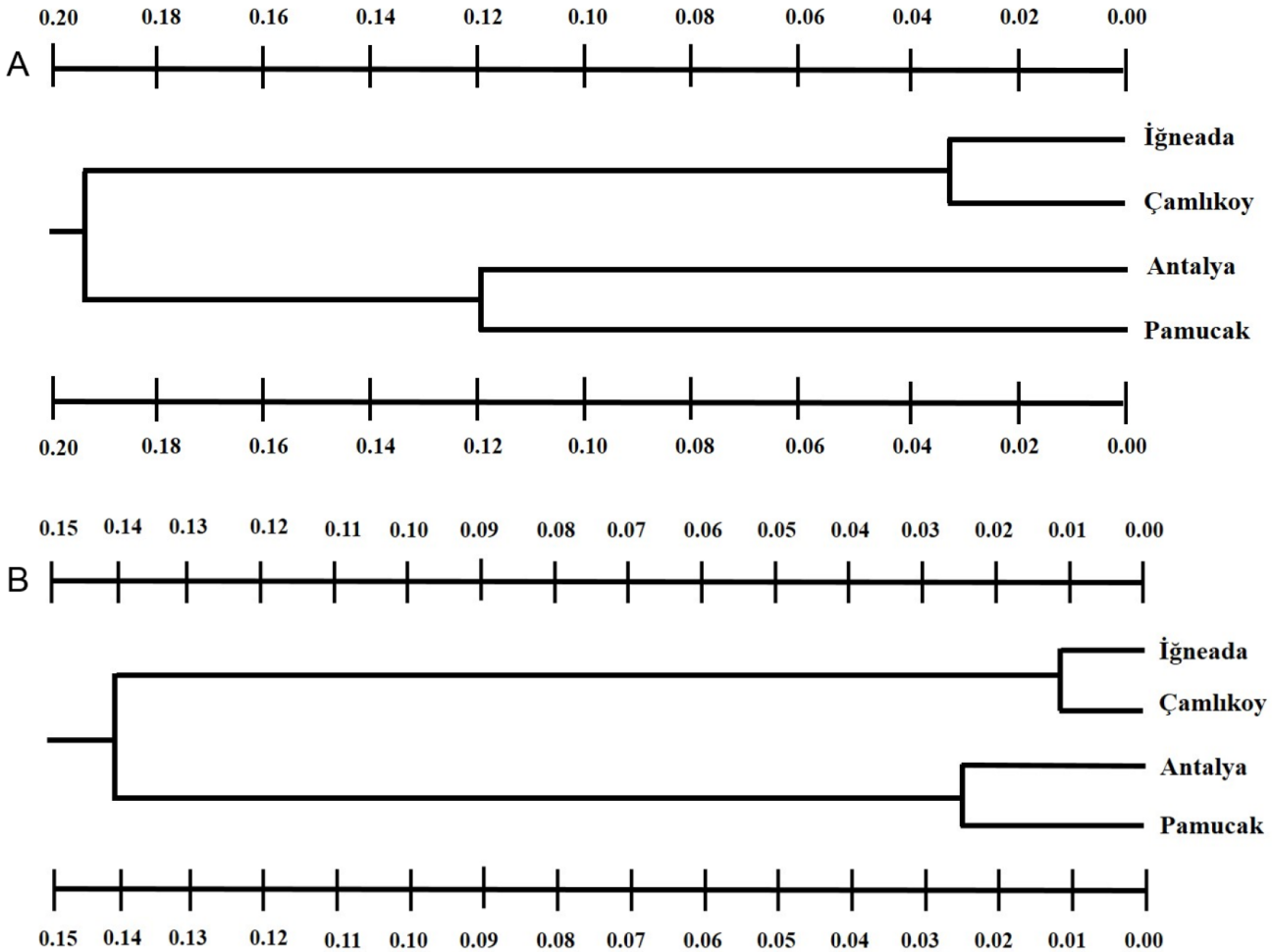

Figure 2. Phenogram constructed using Nei's (1987) genetic distance values for four studied *P. maritimum* populations for RAPD (A) and nrSSR (B).

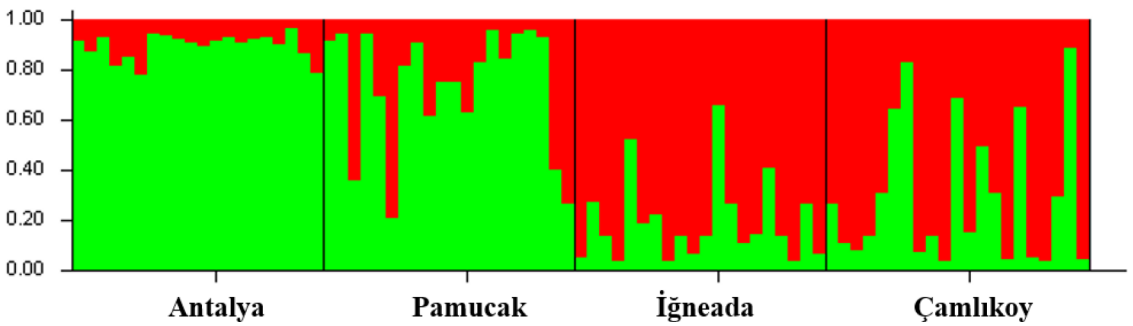

Figure 3. Bayesian structure analysis of the studied *P. maritimum* populations using STRUCTURE 2.3.4 software (K = 2).

(2016) carried out another comprehensive study involving 48 populations with six nrSSR loci (3 of them were the same as in our study) and they reported 17 to 27 alleles per loci. The high allele number per locus resulted from the high number of samples (867 individuals) and also from the differences in the gene pool of geographically different populations.

The genetic diversity level within populations is one of the main parameters in evolutionary and gene conservation studies. In order to maintain the health of

the individuals that constitute populations and to increase their potential to cope with selection and environmental changes, high levels of genetic diversity should be seen in populations. The basic measure of the genetic diversity at a locus is the allelic richness and also the private allelic richness. Thus, the private allele numbers as well as allelic richness were estimated for each population in this study. According to our results, we determined 4 private alleles in the Antalya population and 1 private allele in the Pamucak population (Table 3). We recommend high priority for these two populations in genetic resource conservation and management studies. De Castro et al. (2016) reported 42 private alleles in 17 studied populations, with 1 private allele for the Turkish (Karasu-Sakarya) population. They also reported 6 private alleles for populations from Israel and Croatia and 5 private alleles for Rhodes and two different populations from Italy. The numbers of individual analyzed in these populations were different from each other (De Castro et al., 2016).

Nei's (1987) overall mean observed heterozygosity (H_o = 0.449) was relatively higher than the mean expected heterozygosity (H_e = 0.396) in the studied populations for SSR data. RAPD data analysis also resulted in a low level of overall mean expected heterozygosity (H_e = 0.255). In general, if the observed heterozygosity is higher than the expected heterozygosity, there might be a breaking of genetic isolation. The breaking of genetic isolation might affect the level of heterozygosity for the İğneada and Çamlıkoy populations. Additionally, the low level of heterozygosity might be due to the small population size, relatively narrow genetic base, and population bottlenecks. The studied populations have small size and their genetic base might be narrow, and this might cause the low level of heterozygosity. The low level of observed genetic diversity might be seen due to habitat fragmentation and genetic drift, because their flowers are overcollected and sometimes people collect their bulbs; therefore, the population size tends to be decreasing. Smaller population size might lead to random loss of genetic variability due to genetic drift. Another important point that we found was a statistically significant relationship between geographic distances of population pairs and their N_m values ($r = -0.945$, $P \leq 0.05$, $df = 4$). As geographic distance between population pairs increased, the level of gene flow decreased. Di Maio and De Castro (2013) also reported higher observed heterozygosity in some SSR loci (such as SSR-19, SSR-21, SSR-22, and SSR-28) in their three studied populations. In the work of De Castro et al. (2016), the mean observed heterozygosity and mean expected heterozygosity were calculated as 0.60 and 0.54, respectively. The mean inbreeding coefficient (F_{IS}) of the studied sea daffodil populations for each locus was calculated as -0.008 ± 0.125 in this study. This means that the studied populations show infrequent inbreeding. De Castro et al. (2016) reported the mean F_{IS} value as -0.09 ± 0.03 in their 48 studied populations for each locus. An F_{IS} value below 0.1 might be caused by the low level of inbreeding among populations. Our F_{IS} results were compatible with those of De Castro et al. (2016). The low level of inbreeding in the analyzed *P. maritimum* populations might be related to the reproductive strategy of individuals such as vegetative reproduction, out-crossing, or in-crossing pollination. The breeding system of *P. maritimum* exhibits variation among populations according to their ecological conditions (Medrano et al., 1999).

According to AMOVA results for nrSSRs and RAPDs, 19% and 28% of the total genetic diversity might respectively be assigned to interpopulation differences and the remaining part to variation among individuals within populations. Zahreddine et al. (2004) reported that 53% of the variation in Lebanese populations based on 10 RAPD primers was within populations, whereas 47% was among populations. De Castro et al. (2016) also utilized AMOVA and they indicated that 23% of the genetic variation was due to among-population variations as well. Gene flow is one of the most significant factors that affect intra- and interpopulation genetic variation. Gene flow being disrupted by habitat fragmentation may cause a decrease in genetic diversity. This promotes genetic differentiation between populations. Strong selection pressures might also cause a loss of genetic variation. If any decrease in genetic variation is noticed, in situ and ex situ conservation studies should be planned immediately. According to the UPGMA phenogram based on both RAPD and nrSSR, geographically close populations constituted the same cluster (Figure 2). The genetic distance values obtained from RAPD and SSR analysis supported each other. According to STRUCTURE analysis, populations in cluster I were from the Black Sea, while in cluster II there were two Mediterranean populations. The genetic diversity level also was higher in the studied Black Sea populations than the Mediterranean populations. The relatively higher level of differentiation observed in the studied populations may indicate habitat fragmentation and this might have detrimental effects on gene flow. There might be strong selection pressure on the studied Mediterranean populations causing reduction in genetic diversity, loss of some alleles, and genetic drift. De Castro et al. (2016) reported that two genetic pools of 48 studied populations presented similar frequency, and the analyzed Turkish population (Karasu-Sakarya) had a higher similarity to an Italian population than to eastern and western peripheral populations.

Pancratium maritimum, with its wide range of dispersal area in Mediterranean countries, is threatened by many factors, especially tourism, urbanization, and excessive

collection from the natural habitat. For example, the decrease in natural sea daffodil populations was reported in Spanish and Tunisian populations due to the same reasons (Sanaa and Fadhel, 2010). According to Zahreddine et al. (2004), *P. maritimum* populations on the coasts of France seriously decreased due to urbanization and they are under conservation programs. Crete is another region where the sea daffodils are evaluated as threatened. Zahreddine et al. (2004) reported Lebanese *P. maritimum* populations as vulnerable (VU) according to the regional IUCN Red List due to habitat fragmentation and loss. In Turkey, it has been considered as rare and endangered (EN) in the Red Data Book of Turkish Plants due to the existing habitat destruction in natural populations (Ekim et al., 2000), but it has not been evaluated yet by the IUCN and this species should be evaluated as soon as possible; otherwise, important genetic resources could be lost.

In conclusion, natural *P. maritimum* populations are threatened seriously by factors such as random collection from the habitat, inadequate education of local people, pollution, tourism, and urbanization (Nikopoulos et al., 2008; Schlacher et al., 2008; Demir et al., 2010; Gümüş, 2015). Habitat loss and fragmentation have the potential to affect genetic variation, so these factors need to be considered for conservation and management studies. Precautions should be taken and genetic resource conservation studies should be planned by authorities. Our results related to the studied four populations reveal important statistical data about the genetic structure of these populations. It is important to undertake genetic characterization studies for the remaining/unstudied populations in Turkey. New studies to be carried out using effective molecular markers such as SSRs, including all populations in our country, will play an important role in the identification of strategies for protection of gene resources. The results of our study are also very useful for appropriate management strategies for both in situ and ex situ conservation. Propagation with tissue culture techniques would also be beneficial for conservation programs of selected populations and ex situ conservation studies can be obtained by collecting enough seeds/bulbs from all populations of Turkey and by cultivating them in suitable habitats.

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