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Genetic variation and population differentiation of *Dorystaechas hastata* endemic to Turkey: Implications for conservation management

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Abstract: *Dorystaechas hastata* is a relict endemic medicinal and aromatic plant under risk of extinction. Assessment of genetic diversity and association between geographic distribution and genetic variation could benefit to the conservation efforts. The objectives of this study were to assess genetic diversity and population differentiation of the species in its natural habitat. The genetic structure of 56 accessions sampled from 15 populations encompassing whole natural distribution of the species were analyzed with both sequence-related amplified polymorphism (SRAP) and inter-primer binding site (iPBS) molecular markers. The 146 polymorphic markers were generated by 13 SRAP and 11 iPBS primers. The similarity of the accessions ranged from 53% to 91%, with a mean similarity value of 72%. The populations formed two main groups. Principle component analyses (PCoA) and cluster analysis explained 64.9% and 9.4% of the variations with the first and second eigen vectors. The overall genetic diversity of *D. hastata* was relatively high at the species level, while it is relatively low at the population level. The pairwise allelic differences between populations (F_{ST}), an indication of population differentiation, ranged from 0.15 (Tünektepe -Hacısekiler) to 0.76 (Altınyaka - Sivridağ). The AMOVA results indicated that 51% and 49% of the total variation resided among and within populations, respectively. Geographic distance and/or isolation seem to have strong effect on the genetic differentiation among populations. Results indicate that a representative core collection can be established without significant compromise on genetic diversity. Having the highest genetic diversity Tahtalı, Güllük (Termessos), Beldibi, and Beycik populations must be conserved immediately in nature and might be used to initiate domestication/breeding programs.

Key words: Medicinal and aromatic plants, sequence-related amplified polymorphism (SRAP), inter-primer binding site (iPBS), polymorphic, cluster analyses

1. Introduction

Dorystaechas Boiss. & Heldr. ex Benth., commonly known as "Çalba", is a monotypic and morphologically isolated genus of the Lamiaceae family (Hedge, 1982). Considered as single species, *D. hastata* Boiss. & Heldr. ex Benth. is a relict endemic species (Celep and Dirmenci, 2017) with a narrow distribution range in Antalya, Turkey and defined as "Vulnerable" in IUCN Red List Categories (Ekim et al., 2000). The distribution of the species is restricted to Konyaaltı, Kemer, Korkuteli and Kumluca districts of western Antalya, Turkey, from sea level up to 2000 m altitude (Hedge, 1982). The species is a woody shrub with unique aesthetic appearance, possessing a considerable morphological variation for plant height (14–185 cm), plant diameter (40–620 cm), flower spike length (3–26 cm), and leaf size (Selim et al., 2021). *D. hastata* has been used in medical and perfumery industry due mainly to presence of intense volatile, aromatic oil and antioxidant contents (Meriçli and Meriçli, 1986; Venturella et al., 1988; Öztürk, 1990; Uluben et al., 2004; Karagözler et al., 2008; Erkan et al., 2011; Ozcan et al., 2016). It is also consumed as herbal tea by local inhabitants (Ozcan et al., 2016) against common cold and as a culinary herb (Meriçli and Meriçli, 1986; Erkan et al., 2011). *D. hastata* blooms between March and July, and flowers are borne on upright attractive flower spikes with a great potential to be used as an ornamental plant. The species is under risk of extinction due mainly to uncontrolled mass collection for its pharmacological properties. Immediate domestication of this species as part of ex-situ conservation is suggested (Öztürk, 1990; Selim and Sever Mutlu, 2016), and in vitro germination and propagation was reported (Erdağ et al., 2010). Cultivation or domestication of this species has yet to be initiated. Moreover, information is lacking regarding

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genetic diversity, population genetic structure and gene flow or population fragmentation of this endangered species.

Genetic diversity is a mechanism that allows adaptation of plant populations to changing environmental conditions (Hughes et al., 2008; Helm et al., 2009). Variations in dispersion patterns of species, accumulation of mutations, changes in population size, landscape structure and habitat quality, genetic drift and inbreeding are historical and evolutionary processes that affect genetic diversity in populations. Interruption of gene flow between isolated and fragmented habitats decreases genetic variation (Spielman et al., 2004; Ouborg et al., 2006). These effects have been shown to be more dramatic in populations of endemic species (Frankham, 1997; Hamner et al., 2012). Torres-Diaz et al. (2007) stated that, in natural populations, geographical range of endemic species were good determiners of their genetic diversity, which is expected to be low in endemic species due to narrow geographical distribution (Ledig and Conkle, 1983; Karron et al., 1988; Wolf et al., 2000; Doğan et al., 2016) even though there are studies reporting otherwise (Premoli et al., 2001; Zhang et al., 2009).While the gene flow in animal populations is related to the ability of individuals' movement, in plant populations, it is up to 90%, related to pollen dispersal and seed propagation (Petit et al., 2005). Molecular markers are one of the reliable and time-saving methods for determining gene flow, genetic variation, and differentiation between populations. Different DNA based molecular markers are used to identify genetic variations in plant populations (Agarwal et al., 2008). The DNA based sequence-related amplified polymorphism (SRAP) and inter-primer binding site (iPBS) markers have also been used to determine genetic diversity in many plant species (Borna et al., 2017; Zahumenická et al., 2018). Using molecular marker data, allelic richness, heterozygosity, polymorphism rate, genetic diversity index, genetic differentiation coefficient, and genetic distances are calculated to assess the level of genetic variation in populations (Yang et al., 2015; Doğan et al., 2016). Allelic richness is commonly used as a measure of genetic diversity. It could be integrated to studies by molecular markers (Van Zonneveld et al., 2012; Vinceti et al., 2013; Szczecińska et al., 2016). Population differentiation expressed as allelic richness occurs due to isolation, drift, founder effects, and local selection (Jolivet and Bernasconi, 2007). This knowledge in turn makes it possible to develop more effective and reliable recommendations on protection strategies of endangered species (Jeong et al., 2012).

Although several genetic studies on other members of Lamiaceae family were reported (Sheidai et al., 2016; Tabaripour et al., 2018), this is the first report for *D. hastata's* intra-species genetic variation. Assessment of populations is crucial for development of conservation strategies, selection, and cultivation of candidate accessions to initiate domestication/breeding programs. Therefore, this study was conducted to determine the genetic variation and population differentiation of *D. hastata* using populations encompassing its whole natural distribution of the species in its natural habitat and specify the populations that must be conserved immediately in nature.

2. Materials and methods

2.1. Plant materials

The natural populations of *D. hastata* reside within the study area, in Kemer-Kumluca-Korkuteli-Konyaaltı districts of Antalya, located in Eastern Mediterranean region, southwest of Turkey. The 59 accessions were derived from 15 different populations encompassing whole natural distribution of the species ranging from 4 m to 1862 m from the sea level (Table 1, Figure S1). Depending on the size of populations, 3 to 5 individuals, at least 100 m apart with distinguishing flower characteristics and/or plant architecture were sampled at each location (Figure S2). Field work was conducted between March 2016 and August 2017 (Table 1).

2.2. DNA isolation and analysis of molecular markers

Young leaves (15–20 g.) were harvested from each accession and placed into zip-lock type sealable plastic bag with silica gel and transported to molecular plant genetics laboratory of Department of Agricultural Biotechnology, Akdeniz University, Antalya, Turkey. Samples were taken between March and August, covering flowering stage of the species. Total genomic DNA was extracted from fresh leaf tissue according to cetyl trimethylammonium bromide (CTAB) DNA extraction procedure (Doyle and Doyle, 1990). Quality of extracted DNA was examined by running on 1 % agarose gel.

2.2.1. Sequence-related amplified polymorphism (SRAP) assay

The 208 SRAP primer combinations, 13 forward (Me1 to Me13) and 16 reverse primers (Em1 to Em16), were used to screen two bulk DNA, each prepared from sampling one individual accession of a given population. The thirteen SRAP primer combinations that yielded highest scorable markers were determined (Table 2).

2.2.2. inter-primer binding site (iPBS) assay

A total of 47 iPBS primers were tested using the bulk DNA, each consisting of 20 different accessions. The iPBS primers yielding the best amplification with high number of markers were chosen (Table 3). The PCR amplification conditions were similar to Kalendar et al. (2011). The amplified iPBS fragments were separated and visualized with the same procedure as for SRAP.

Table 1. Sampling sites and locational characteristics for fifteen *D. hastata* populations in Antalya, Turkey.

Population name code	Population code	Locality code	Latitude (N) Longitude (E)		Altitude
Altınyaka	A	A-1	16°60′55″	40°55′696″	+1137
		A-2	26°53′89″	40°54′633″	+1125
		A-3	26°53′45″	40°54′648″	+1106
Alakır	AL	AL-1	25°65′24″	40°57′796″	+1202
		AL-2	25°63′25″	40°57′401″	+1205
		AL-3	25°22′66″	40°53′718″	+1151
		AL-4	24°91′92″	40°48′308″	+1059
Sivridağ	S	S-1	27°01′43″	40°83′868″	+1255
		S-2	27°02′79″	40°83′669″	+1250
		S-3	27°07′64″	40°83′530″	+1204
Söğütcuması	SO	SO-1	26°42′75″	40°67′287″	+1465
		SO-2	26°46′86″	40°66′214″	+1399
		SO-3	26°47′18″	40°66′202″	+1400
Hisarçandır	H	H-1	27°43′33″	40°72′232″	+964
		H-2	27°44′88″	40°72′155″	+979
		H-3	27°43′63″	40°72′306″	+937
		H-4	27°43′27″	40°72′363″	+906
		H-5	27°43′82″	40°72′372″	+904
Üçoluk	UC	UC-1	26°99′33″	40°58′499″	+1087
		UC-2	26°99′58″	40°55′952″	+1053
		UC-3	26°97′20″	40°57′623″	+1081
		UC-4	26°96′46″	40°58′214″	+1083
Tahtalı	T	T-1	27°10′37″	40°58′478″	+1094
		T-2	27°10′36″	40°58′519″	+1102
		T-3	27°08′47″	40°58′665″	+1105
		T-4	27°08′32″	40°58′686″	+1113
Gölcük	GL	GL-1	27°43′86″	40°96′097″	+1009
		GL-2	27°44′24″	40°96′102″	+1008
		GL-3	27°22′60″	40°96′317″	+971
		GL-4	27°42′76″	40°96′316″	+974
Beycik	BY	BY-1	26°98′99″	40°42′820″	+743
		BY -2	26°85′64″	40°43′212″	+1023
		BY -3	26°85′58″	40°43′181″	+1009
		BY -4	26°87′29″	40°43′101″	+ 956
Tünektepe	TN	TN-1	28°18′18″	40°78′383″	+40
		TN-2	28°10′11″	40°77′482″	+96
		TN-3	28°04′77″	40°76′911″	+90
Beldibi	BL	BL-1	28°10′88″	40°69′170″	+16
		BL-2	28°03′29″	40°63′036″	+61
		BL-3	28°02′61″	40°69′115″	+7
		BL-4	28°06′98″	40°69′157″	+4

Table 1. (Continued).

		BL-5	28°09′74″	40°69′305″	+46
Feslikan	F	F-1	36°49′12″	30°23′830″	+1862
		F-2	36°49′11″	30°23′832″	+1853
		F-3	36°49′09″	30°23′810″	+1853
		F-4	36°49′06″	30°23′782″	+1853
Kesmeboğazı	K	K-1	27°51′73″	40°53′623″	+158
		K-2	27°56′25″	40°53′662″	+104
		K-3	27°59′04″	40°53′514″	+109
Hacısekiler	HC	HC-1	28°25′20″	40°79′757″	+231
		HC-2	28°24′67″	40°79′789″	+233
		HC-3	28°24′29″	40°79′817″	+235
Güllük (Termessos)	GU	GU-1	27°43′86″	40°96′097″	+1009
		GU-2	27°44′24″	40°96′102″	+1008
		GU-3	27°22′60″	40°96′317″	+971
		GU-4	27°42′76″	40°96′316″	+974

2.3. Data analysis

To construct a binary matrix, clear and highly reproducible marker bands were taken into consideration and visually scored as present (1) or absent (0). The PCoA and cluster analyses based on Pearson genetic similarity coefficient were performed using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 software package (Exeter Software, Setauket, New York, , NY, USA) (Rohlf, 2000) to reveal the genetic relationships among populations. Eigen values were calculated based on SQRT (LAMBDA) parameters for PCoA. The Unweighted Pair Group Method with Arithmetic Average (UPGMA) in the SHAN module was used to build the dendrogram revealing the genetic relationships among 15 populations of *D. hastata*. Mantel test (Mantel, 1967) was performed for estimating correlation between SRAP and iPBs similarity matrices using NTSYS-pc v2.1 software (Rohlf, 2000).

Genetic diversity parameters including number of polymorphic loci (NP), percentage of polymorphic loci (PPL), observed number of alleles per locus (N_a), effective number of alleles per locus (N_e), Nei's gene diversity (h) (Nei, 1978) and Shannon's information index (I) were determined in POPGENE version 1.32 (Yeh et al., 1997). The Nei genetic distance (Weising, 2005) was determined among the populations and was used for the grouping of the accessions in population level. Genetic differentiation of the populations was studied by AMOVA with 1000 permutations and pairwise F_{ST} were performed in ARLEQUIN software 3.1 (Excoffier et al., 2005). Phylogenetic trees and dendrograms were conducted using Genetic Distance (D) (Huson and Bryant, 2006) package program.

3. Results and discussion

The genetic diversity of populations was determined by SRAP and iPBS molecular markers. Because SRAP specifically targets the functional gene regions and iPBS the retrotransposons, the combination of the two marker systems is expected to shed a better light for genomic differences and hence, evolutionary history of the species.

The 13 of the 208 SRAP primer combinations produced the highest scorable marker bands. The screening of 56 accessions yielded 165 SRAP markers, 158 of which were polymorphic (Figure S3a). The average percentage of the polymorphic bands was 99.8% and ranged from 87.75 to 100%, indicating a high genetic diversity among individuals. The total number of the amplified bands per primer varied from 2 to 25 with an average of 12.6 bands (Table 4). The size of amplified products ranged from 50 to 1450 bp. The Em7-Me7 combination was the most polymorphic with 24 bands. Another member of Lamiaceae, *Thymus daenensis* Celak, was screened with fourteen SRAP primer combinations to estimate the genetic diversity of 79 accessions (Talebi et al., 2015). The 14 SRAP primer combinations amplified 240 bands with an 82.5% polymorphism. In a similar study, Aghaei et al. (2017) used SRAP markers to detect genetic diversity of five *Salvia* species (*S. virgata* Jacq., *S. nemorosa* L., *S. officinalis* L., *S. cereal* L. and *S. sclarea* L.) of Lamiaceae family where fourteen primer combinations amplified 265 fragments for 54 accessions with a 96% polymorphism rate. Chen et al. (2013) also studied genetic diversity in basil (*Ocimum* spp), another member of Lamiaceae, using 10 SRAP marker combinations and 37 accessions representing four species of (*Ocimum basilicum* L., *O. americanum* L., *O.*

Table 2. The 13 SRAP primer combinations showing high levels of polymorphism.

Primer Combination	Sequence (5'–3')
Em 1 -Me 9	GACTGCGTACGAATTAAT TGAGTCCAAACCGGACG
Em 1 - Me 11	GACTGCGTACGAATTAAT TGAGTCCAAACCGGAAC
Em 2 - Me 7	GACTGCGTACGAATTTGC TGAGTCCAAACCGGACG
Em 3 - Me 7	GACTGCGTACGAATTGAC TGAGTCCAAACCGGACG
Em 4 - Me 7	GACTGCGTACGAATTTGA TGAGTCCAAACCGGACG
Em 5 - Me 3	GACTGCGTACGAATTAAC TGAGTCCAAACCGGAAT
Em 7 - Me 7	GACTGCGTACGAATTCAA TGAGTCCAAACCGGACG
Em 8 - Me 2	GACTGCGTACGAATTCAC TGAGTCCAAACCGGAGC
Em 9 - Me 5	GACTGCGTACGAATTCAG TGAGTCCAAACCGGAAG
Em 9 - Me 11	GACTGCGTACGAATTCAG TGAGTCCAAACCGGAAC
Em 10 - Me 12	GACTGCGTACGAATTCAT TGAGTCCAAACCGGAGA
Em 11 - Me 11	GACTGCGTACGAATTCTA TGAGTCCAAACCGGAAC
Em 13 - Me 3	GACTGCGTACGAATTCTG TGAGTCCAAACCGGAAT

gratissimum Forssk. and *O. tenuiflorum* L.), amplifying 741 bands with an average polymorphic ratio of 93%.

The 47 iPBS primers tested, the 11 were selected that yielded the best amplification and reproducible bands. In total, 192 iPBS markers were obtained with a 97.9% polymorphism ratio (Figure S3b) that ranged from 86.6 to 100%. The number of the amplified bands per primer varied from 12 to 27 with an average of 17.5 bands. The size of amplified products ranged from 200 to 2500 bp. The primer '2076' produced the highest number of marker bands (27). Similarly, the iPBS markers were successfully used to assess the genetic diversity of *Leonurus cardiaca* L., another Lamiaceae species (Borna et al., 2017). Seven iPBS primers produced 191 bands, ranging from 180 to 4000 bp in size, indicating that the iPBS marker system can reveal genomic diversity in *L. cardiaca*. Strid (1987) reported the

Table 3. The selected iPBS primers, their nucleotide sequence, and optimum annealing temperatures.

Primer	Sequence (5'-3')	Annealing temperatures
2076	GCTCCGATGCCA	51 °C
2375	TCGCATCAACCA	50 °C
2383	GCATGGCCTCCA	46 °C
2387	GCGCAATACCCA	46 °C
2277	GGCGATGATACCA	45 °C
2217	ACTTGGATGTCGATACCA	46 °C
2230	TCTAGGCGTCTGATACCA	51 °C
2232	AGAGAGGCTCGGATACCA	56 °C
2237	CCCCTACCTGGCGTGCCA	46 °C
2239	ACCTAGGCTCGGATGCCA	58 °C
2251	GAACAGGCGATGATACCA	56 °C

chromosome number of *D. hastata* as 2n = 20. The 357 markers were generated (Table 4) by 13 SRAP and 11 iPBS primers presented 35.7 markers per haploid chromosome, a 2.8 cM marker density considering each chromosome as 100 cM in length.

Genetic variation is an important criterion for plant populations in the process of coping with and adapting to environmental changes (Koohdar et al., 2016). The genetic diversity of endemic species is expected to be low because of their narrow geographical distribution (Hamrick et al., 1979; Ledig and Conkle, 1983; Karron et al., 1988; Wolf et al., 2000; Doğan et al., 2016). However, results from present study confirmed exactly the opposite with a high degree of polymorphism on species basis (96.9%). Similar results were reported in other endemic species. For example, polymorphism rates within the species *Saussurea chabyoungsanica* Im (Jeong et al., 2012), *Ottelia acumianata* (Gaghep.) Dandy (Zhang et al., 2009) and *Uechtritzia armena* Freyn & Sint. (Doğan et al., 2016) were 95.2%, 79.4 %, and 96.2%, respectively.

3.1. Cluster analysis and PCoA

An UPGMA clustering of genetic similarity among the accessions is presented in Figure 1. According to dendrogram calculated using similarity matrix generated by combined SRAP and iPBS markers, the similarity ratio of the accessions ranged from 53% to 91%, with a mean similarity value of 72%. The lowest intra-population genetic variation was found within the Altınyaka population (10%). While two main groups are evident, statistical cut line points eight subgroups. All accessions of Altınyaka, Alakır, Tünektepe, Hacısekiler populations and two accessions from each of Tahtalı and Güllük (Termessos) formed the first main group. All accessions

Table 4. List of the selected SRAP and iPBS primers and the degree of polymorphism.

Primer	Total bands (n)	Polimorphic bands (np)	Polimorphism ratio (%)
Em 9 Me 5	12	12	100
Em 11 Me 11	8	7	87.8
Em 1 Me 11	11	11	100
Em 13 Me 3	5	5	100
Em 1 Me 9	21	21	100
Em 4 Me 7	11	10	90.9
Em 8 Me 2	14	13	92.9
Em 5 Me 3	12	11	91.7
Em 9 Me 11	15	15	100
Em 10 Me 12	2	2	100
Em 2 Me 7	16	15	93.8
Em 3 Me 7	13	12	92.3
Em 7 Me 7	25	24	96
2076	27	27	100
2375	20	20	100
2383	12	11	91.7
2387	15	13	86.7
2277	18	17	94.4
2217	20	20	100
2230	13	13	100
2232	14	14	100
2237	21	21	100
2239	12	12	100
2251	20	20	100
Polimorphism	357	346	96.9

of Hisarçandır, Söğütcuması Sivridağ, Üçoluk, Beldibi, Feslikan, Beycik, Kesmeboğazı, Gölcük populations and only two accessions of Tahtalı and Güllük (Termessos) populations formed the other main group. The Tahtalı and Güllük (Termessos) populations have shared accessions in both main groups. The results indicate that the species basically diverged into two loosely associated subgroups. The Altınyaka population (A2-A3) showed the lowest and the Tahtalı and Güllük (Termessos) populations resided the highest within population genetic diversity, housing individuals belonging to both main groups.

The PCoA is one of the most useful statistical tools for understanding the genetic relationship among and within populations (Johnson, 1998) allowing the identification of the geographical origin of the respective populations (Erbano et al., 2015). The PCoA analysis of the genetic data based on SRAP and iPBS molecular markers was performed using the correlation matrix values and a spatial distribution graph of the accessions is presented in Figure 2. It revealed that the first, second, and the first three components comprised about 64.9%, 9.4% and 76.7 % of the total genetic variation, respectively. The *Cycas siamensis* Miq. is also a narrowly distributed, rare species, and, using 210 polymorphic AFLP markers, the first two eigen vectors explained only 20% of the variation (Yang et al., 2015). The number of eigen vectors that shed light on genetic diversity between accessions reveals that there are very high genetic differences in *D. hastata*. All accessions of Tünektepe, Alakır, Altınyaka, and Hacısekiler loosely grouped together (Figure 2, top left). Tünektepe – Hacısekiler are geographically in proximity, similar to Alakır – Altınyaka locations (Figure S1). PCoA results agree with cluster analyses where two main groups are formed, and Tahtalı and Güllük (Termessos) populations tended to separate from the others probably because they have accessions belonging to both groups. The Mantel test performed between the genetic distance matrices obtained from SRAP and iPBs produced significant regression value between the two ($r^2 = 0.882$, $p < 0.001$), indicating that distance matrixes obtained from the two marker systems are highly correlated.

3.2. Population structure and genetic diversity of natural populations

Based on the marker estimates, the genetic diversity parameters per population level and the total population level were shown in Table 5. Observed number of alleles per locus (Na), ranged from 1.17 to 1.56 among populations, with an average value of 1.37 and 1.97 at the population and species level, respectively. The effective number of alleles per locus (Ne) ranged from 1.12 to 1.36 among the populations, with an average value of 1.26 and 1.57 at the population and species level, respectively. The genetic diversity of *Uechtritzia armena,* another endemic species with a narrow geographic distribution, Na and Ne ranged between 1.64 and 1.69, and 1.23 and 1.26, respectively (Doğan et al., 2016). *Ottelia acumianata* is an endemic species to China and Zhang et al. (2009) reported that the Na and Ne varied between 1.84–1.89, and 1.75–1.76, respectively. Results showed that *D. hastata* has a much wider variation as indicated by Na and Ne values. Nei's gene diversity (h) ranged from 0.07 to 0.2, with 0.15 and 0.33 average at the population and species level, respectively. Doğan et al. (2016) reported that Nei's gene diversity index in *Uechtritzia armena* was 0.17 and 0.19 at the population and species level, respectively. Shannon's index (I) varied from 0.10 to 0.31 with an average of 0.22 and 0.5 at the population and species level, respectively. Similarly, a relatively high Shannon's index value 0.39 and 0.48 within populations and species level, respectively

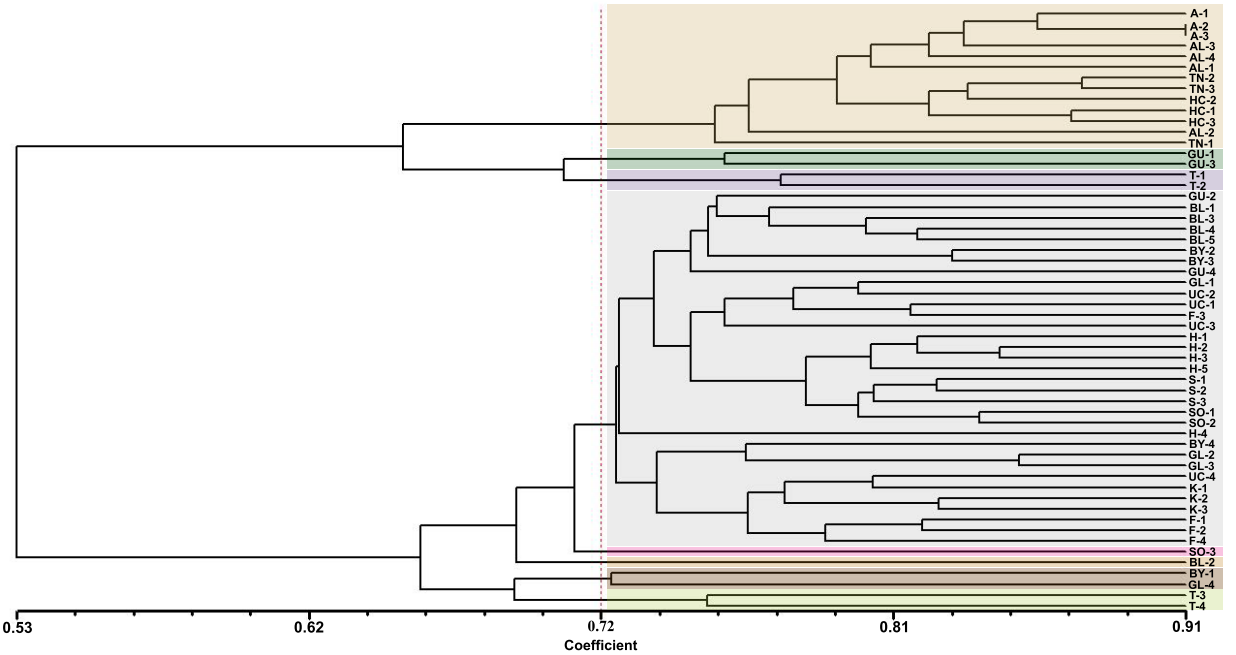

Figure 1. A UPGMA clustering of genetic (Jaccard) similarity among individuals and populations (Locality codes: HC: Hacısekiler, S: Sivridağ, F: Feslikan, TN: Tünektepe, H: Hisarçandır, UC: Üçoluk, BL: Beldibi, K: Kesmeboğazı, BY: Beycik, T: Tahtalı, GL: Gölcük, A: Altınyaka, SO: Söğütcuması, AL: Alakır, GU: Güllük).

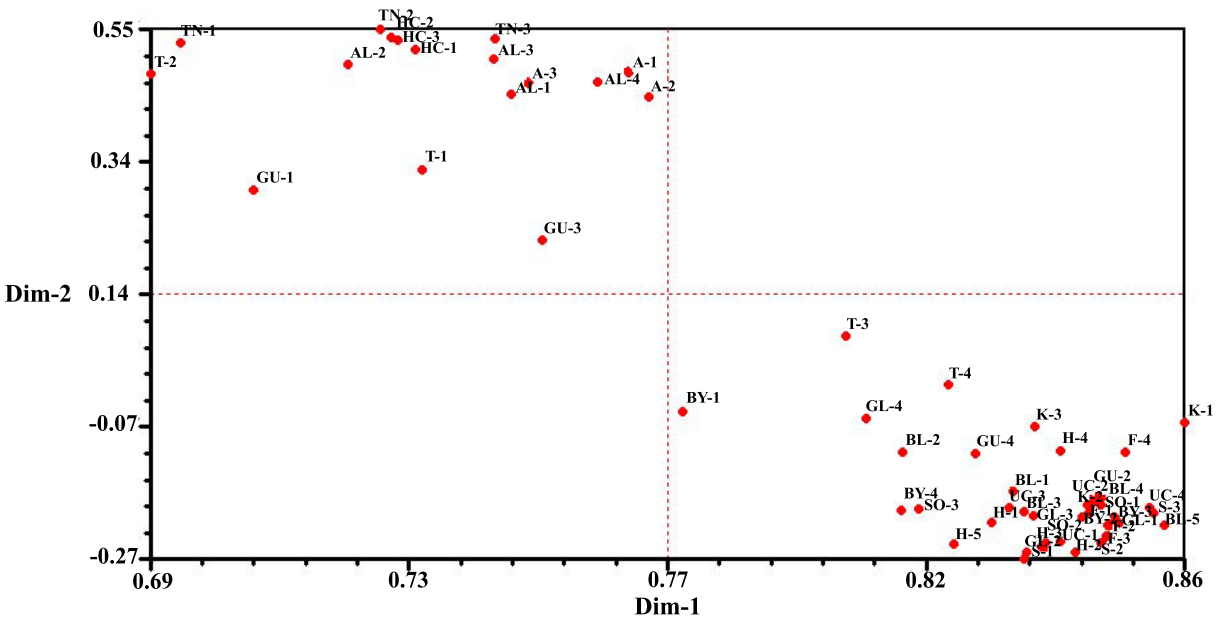

Figure 2. A PCoA analysis of the genetic data based on SRAP and iPBS molecular markers. Dim-1 and Dim-2 indicate %64.9 and %9.4 of the variation. (Population codes: HC: Hacısekiler, S: Sivridağ, F: Feslikan, TN: Tünektepe, H: Hisarçandır, UC: Üçoluk, BL: Beldibi, K: Kesmeboğazı, BY: Beycik, T: Tahtalı, GL: Gölcük, A: Altınyaka, SO: Söğütcuması, AL: Alakır, GU: Güllük).

was reported for another member of Lamiaceae species; *Eremostachys superba* Royle ex Benth. distributed in Indian Himalayas (Verma et al., 2007). Number of polymorphic loci (NP) ranged from 37 to 185, with an average of 111.2 and 346 at the population and species level, respectively. The percentage of polymorphic loci (PPL) of *D. hastata*

Table 5. Observed number of alleles per locus (Na), effective number of alleles per locus (Ne), Nei's gene diversity (h), and Shannon's information index (I) with standard deviations, number of polymorphic loci (NP), percentage of polymorphic loci (PPL) at both individual population level and the overall population level.

Populations	Na	Ne	h	I	NP	PPL(%)
Altınyaka	1.17 ± 0.37	1.12 ± 0.28	0.06 ± 0.16	0.09 ± 0.22	37	10.4
Gölcük	1.43 ± 0.49	1.28 ± 0.38	0.16 ± 0.20	0.23 ± 0.29	139	38.9
Beldibi	1.49 ± 0.50	1.34 ± 0.41	0.19 ± 0.21	0.27 ± 0.30	161	45.1
Beycik	1.46 ± 0.50	1.31 ± 0.40	0.17 ± 0.21	0.26 ± 0.30	150	42.0
Güllük (Termessos)	1.56 ± 0.50	1.36 ± 0.38	0.21 ± 0.20	0.31 ± 0.29	185	51.8
Üçoluk	1.39 ± 0.50	1.28 ± 0.40	0.15 ± 0.21	0.23 ± 0.30	130	36.4
Kesmeboğazı	1.28 ± 0.45	1.21 ± 0.36	0.11 ± 0.19	0.17 ± 0.28	92	25.8
Feslikan	1.35 ± 0.48	1.23 ± 0.36	0.13 ± 0.20	0.20 ± 0.28	117	32.8
Hisarçandır	1.40 ± 0.49	1.27 ± 0.38	0.15 ± 0.20	0.22 ± 0.29	133	37.3
Tahtalı	1.47 ± 0.50	1.31 ± 0.38	0.18 ± 0.20	0.26 ± 0.29	152	42.6
Sivridağ	1.27 ± 0.45	1.19 ± 0.35	0.11 ± 0.19	0.16 ± 0.30	90	25.2
Söğütcuması	1.27 ± 0.44	1.19 ± 0.34	0.11 ± 0.19	0.16 ± 0.27	88	24.6
Tünektepe	1.27 ± 0.44	1.19 ± 0.35	0.11 ± 0.19	0.16 ± 0.27	59	16.5
Hacısekiler	1.24 ± 0.43	1.19 ± 0.36	0.10 ± 0.19	0.15 ± 0.27	53	14.9
Alakır	1.36 ± 0.48	1.25 ± 0.38	0.14 ± 0.20	0.21 ± 0.29	82	28.0
All populations average	1.37 ± 0.12	1.26 ± 0.07	0.14 ± 0.04	0.21 ± 0.06	111.2	28.4
Average values on overall allele frequencies (Species)	**1.96 ± 0.17**	**1.56 ± 0.32**	**0.33 ± 0.15**	**0.49 ± 0.2**	**346**	**96.9%**

ranged from 10.4% to 51.8% at the population and species level, respectively. The PPL of *Lavandula multifida* L., another member of Lamiaceae distributed in Tunisia, was 13.8% at species and 73.2% at population level (Hnia and Mohamed, 2011).

It is important to determine the genetic diversity within and among populations in order to develop conservation and breeding programs of a species (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Borna et al., 2017). Determining the populations with the highest conservation value via molecular markers are effective from a long-term perspective to make better conservation plans and decisions (Szczecińska et al., 2016). Considering the genetic diversity of *D. hastata* natural populations with commonly used parameters as allelic richness (Van Zonneveld et al., 2012; Vinceti et al., 2013; Szczecińska et al., 2016) and population differentiations (Jolivet and Bernasconi, 2007), the highest levels of diversity occurred in Güllük (Termessos) population followed by Beldibi, Tahtalı and Beycik populations, and while the lowest level of genetic diversity present within Altınyaka population (Table 5). While the overall genetic diversity of *D. hastata* is relatively high at the species level, it is relatively low at the population level. Although *D. hastata* has a narrow distribution area and shows a relict endemic character, it has a large genetic diversity as confirmed by Nei and Shannon gene diversity indexes or AMOVA analyses. In general, species with narrow distribution tend to have lower genetic diversity than species with large distribution area (Hamrick et al., 1979), where former are generally more exposed to the effects of genetic drift, regression and low gene flow compared to common species. Although reports exist supporting this hypothesis (Ledig and Conkle, 1983; Linhart and Premoli, 1993; Wolf et al., 2000), endemic species with a narrow distribution area possessing high genetic variation were also reported (Karron et al., 1988; Gonzalez-Astorga and Nunez-Farfan, 2001; Jeong et al., 2012; Wang and Yan, 2013; Doğan et al., 2016).

3.3. Genetic differentiation of the populations

The AMOVA showed that 51% of the total variation resides among populations, the remaining 49% was due to differences among the individuals within populations (Table 6). Nearly similar results were reported in *Salvia fruticosa*, a close relative in Lamiaceae family (Mader et al., 2010). Specifically, species with narrow geographic distribution are more likely to be exposed to the effects of genetic drift, spontaneous and low rate of gene flow than common species (Ledig and Conkle, 1983; Linhart and Premoli, 1993; Wolf et al. 2000). On the contrary, genetic variation within and among populations of *D.*

Table 6. Analysis of Molecular Variance (AMOVA) for genetic diversity in 15 populations of *D. hastata*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	14	1250.6	10.6	51
Within populations	97	989.1	10.2	49
Total	111	2239.7	20.8	
Species F_{ST}=0.51				

hastata is rather high. Similar conclusions were made concerning the population structure of other rare and endemic species such as *Erythronium propullans* A.Gray (Pleasants and Wendel, 1989), *Astragalus linifolius* Osterh. and *A. osterhoutii* M.E.Jones (Karron et al., 1988), *Brongniartia vazquezii* Dorado (Gonzalez-Astorga and Nunez-Farfan, 2001), *Opisthopappus longilobus* C.Schih and *O. taihangensis* (Ling) C.Schih (Asteraceae) (Wang and Yan, 2013), *Saussurea chabyoungsanica* Im (Jeong et al., 2012), *Petunia secreta* Stehmann & Semir (Turchetto et al., 2016), *Leonurus cardiaca* (Borna et al., 2017), and *Ottelia acuminata* (Zhang et al., 2009).

Pairwise combination F_{ST} values among populations revealed the genetic distances of the populations based on molecular markers (Table 7). The F_{ST} values, an indication of population differentiation to estimate pair wise allelic differences between populations, ranged from 0.15 (Tünektepe -Hacısekiler) to 0.76 (Altınyaka - Sivridağ) among populations of *D. hastata*. The geographic proximity and/or altitude/mountain range seemed to have strong effect on F_{ST} values, indicating level of gene flow (Table 7, Figure S1). The *F*st based grouping concurs with that of PCoA and cluster analysis. The relationship between geographic proximity and genetic similarity were further confirmed on the populations of Tünektepe - Hacısekiler, and Alakır - Altınyaka. In general, geographic proximity is expected to be the determinant in genetic relationship (Wu et al., 2015), which was the base of isolation by distance theory (Schaal, 1974; Loeschcke, 1987). However, pairwise differentiation of some populations unexpectedly low without regard to the geographical distances such as Feslikan-Kesmeboğazı and Hisarçandır-Sivridağ populations (Table 7, Figure S1). The lack of strong association between geographic distance and genetic variation may be attributed to lack of geographic isolation and reproductive strategy of the species. Reproductive biology has a special importance in rare and endemic species due to restricted population/ distribution sizes (Jorge et al., 2015). The strategy used for reproduction determines the quality and quantity of the offspring and, consequently, the movement of genes in time and space (Barrett, 2003; Barrett, 2010). There is no report on reproductive biology of this species. *Salvia sclareoides* Brot., a member of same family, is a facultative xenogamous species producing offspring with outcross and self-pollination. *Salvia sclarea* another species of the Lamiaceae family is pollinated by bees and several taxa of long-tongued insects (Şenol et al., 2017). The lack of strong correlation between genetic diversity and geographic distance may indicate outcrossing properties of *D. hastata*, which is thought to have a large distribution before the glacial period with a very restricted current distribution in Antalya. According to genetic diversity, the Güllük (Termessos) population is unique, consisted with the results of heterozygosity and diversity indexes. Collaborating the genetic variation, Güllük population possessed the highest and Altınyaka the lowest morphological variations as well (Selim et al., 2021). The Güllük (Termessos) and Tahtalı populations have the highest genetic diversity, hinting that they survived from the glacial period. The other populations tend to show the founder's effect.

Although the genetic diversity of endemic species is expected to be low due to their narrow geographical distribution, the high polymorphism value of *D. hastata* is associated with the species' relict character. The main distribution area of *D. hastata*, east of Tahtalı and Bereket Mountains, has deep valleys that descend to the sea and are closed to the north, so it was protected from the freezing cold of the glacial ages and was probably never glaciated (Davis, 1970). Thus, some Tethys flora elements of the tertiary period were able to survive there. Depending on the climatic conditions, it is thought that the biogeographic shrinkage in the distribution area of *D. hastata* brings along the richness of genetic material stuck in the limited area where the species is distributed today. *Globularia davisiana* O.Schwarz, *Eryngium thorifolium* Boiss. & *Echinops onopordum* P.H.Davis can be cited as an example of other local paleoendemic plant species with similar relict character within the distribution area of the species.

Considering the relict character of the species, four remarkable canyon areas stand out with their sheltered characters with narrow and fast topographic passages. These regions, which can be interpreted as biogeographical "shelter" locations of the species, are Mecine, Beldibi, Kemer, and Yarıkpınar Canyons from north to south, respectively. The population with the highest value in terms of genetic diversity of the species is also the population of Güllük (PPL 51.52%) located in the upper part of Mecine Canyon, located in the northernmost part. This situation is probably associated with the accumulation of genetic material during the glacial period in the drift of the species to the south due to adverse conditions from the north. The UPGMA and PCoA analysis support the view that the

Table 7. Matrix of F_{ST} values for each pairwise combination of *D. hastata* populations.

	A*	GU	BL	BY	GL	UC	K	F	H	T	S	SO	TN	HC	AL
A	0,000														
GU	0.462	0,000													
BL	0.648	0.289	0,000												
BY	0.714	0.288	0.307	0,000											
GL	0.714	0.381	0.343	0.327	0,000										
UC	0.666	0.334	0.327	0.321	0.268	0,000									
K	0.700	0.305	0.389	0.422	0.432	0.437	0,000								
F	0.737	0.345	0.425	0.371	0.440	0.366	0.358	0,000							
H	0.672	0.356	0.401	0.401	0.464	0.393	0.489	0.327	0,000						
T	0.447	0.211	0.485	0.472	0.469	0.443	0.456	0.491	0.469	0,000					
S	0.755	0.301	0.392	0.411	0.429	0.395	0.564	0.475	0.234	0.479	0,000				
SO	0.732	0.341	0.372	0.455	0.465	0.410	0.540	0.492	0.334	0.500	0.291	0,000			
TN	0.345	0.459	0.660	0.659	0.678	0.652	0.661	0.698	0.678	0.379	0.722	0.718	0,000		
HC	0.462	0.452	0.656	0.668	0.679	0.659	0.683	0.724	0.679	0.363	0.728	0.715	0.150	0,000	
AL	0.268	0.451	0.635	0.639	0.655	0.623	0.640	0.675	0.649	0.361	0.683	0.665	0.234	0.335	0,000

*Population codes: A: Altınyaka, GU: Güllük, BL: Beldibi, BY: Beycik, GL: Gölcük, UC: Üçoluk, K: Kesmeboğazı, F: Feslikan, H: Hisarçandır, T: Tahtalı, S: Sivridağ, SO: Soğütcuması, TN: Tünektepe, HC: Hacısekiler, AL: Alakır. F_{ST} is the proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance. High F_{ST} implies a considerable degree of differentiation among populations.

Güllük population is one of the two populations subject to the regional origin of the species. Similarly, populations housing high genetic diversity have the role of shelter, where Beldibi population (PPL 45.10%) is in the close vicinity of Beldibi Canyon, Tahtalı population (42.58%) in the upper part of Kemer Canyon and Beycik population (42.02%) in the upper part of the Yarıkpınar Canyon, the southernmost distribution of the species.

3.4. Conservation status and threat factors

As a relict endemic medicinal herb under risk of extinction due to human overexploitation, *D. hastata* deserves conservation consideration. Although, the Termessos (Güllük Dağı) and Olimpos (Beydağları Sahil) national parks, respectively inhabit Güllük and Tünektepe, Beldibi, Göynük, parts of Kemer ve Beycik populations, the rest of them (Sivridağ, Feslikan, Hisarçandır, Söğütcuması, Tahtalı, Uçoluk, Altınyaka and Alakır) is not within the National Parks' conservation area (Figure S1). Besides, there are Düzlerçamı, Sivridağ and partly Sarıkaya wildlife development areas within the distribution regions of the species; unfortunately these areas are also open to collecting raw material. Antalya Regional Directorate of Forestry (ARDF) issues annual quota permits to collect for industrial use. However, grazing pressure and uncontrolled mass collection by locals both within and outside conservation areas create an existential threat for the species. It is used and sold for tea and herbal remedy in the region. The Beldibi, Göynük, and Beycik populations in particular are under tourism or construction pressure. Construction for housing in Beycik and dam in Alakır poses specific threats to the populations in question. We witnessed loss of populations within a year; hence, the natural populations are progressively decreasing. It is strongly recommended that Species Action Plan should be prepared by the Ministry of Agriculture and Forestry to protect the species. Further field studies and monitoring should be carried out to protect the populations having unique genetic and morphologic characteristics.

Although the species has not been domesticated yet, the methods for in vitro germination and propagation (Erdağ et al., 2010) may aid to establish a rapid and mass production capacity, which in turn would alleviate the pressure on natural populations. In light of the study, genotypes for aromatic and medicinal characteristics should be determined and propagated in field, greenhouse, or tissue culture, and the ARDF should cease issuing quote for collection from natural habitat. It is recommended that a seed and plant germplasm bank that would represent the populations should be established immediately. Then, genotypes with highest diversity/value should be propagated and replanted in their respective habitats to avoid populations' extinction. For instance, the most

morphologically diverse genotypes resided in Güllük and Üçoluk, and valuable variations for attractive flower types for ornamental use existed in Beycik populations (Selim et al., 2021).

Threat category of the species has been identified as Vulnerable "VU" in the Turkish Red Data Book (Ekim et al., 2000). The species is re-evaluated according to Guidelines for Using the IUCN Red List Categories and Criteria, Version 14 (IUCN, 2019). The distribution area within the shortest continuous imaginary boundaries that can be drawn up to the Mediterranean coastline including all points, between the populations of Güllük, Feslikan, Hacısekiler, Tünektepe, Beldibi, Göynük, Beycik and Alakır, which are the outermost locations, is calculated as 1053 km^2 (Extend of occurrence-EOO). The Area of Occupancy (AOO) value with its widespread character is considered to be more than 500 km^2. During the course of study, several thousand individuals, estimated to be over 10000, have been observed. In addition, although there are deep valley regions subject to topographic isolation, the distribution area of the species is considered as a single population, continuing uninterrupted. According to the data of ARDF, the species is collected with a quota between 30%–70%. As long as the threat factors stated in this study (i.e. excessive gathering) continue, a rapid decline in the number of adult individuals is expected. Thus, the IUCN Red List Category of the species is identified as Vulnerable (VU B1b(i)+2b(ii,iii)). However, considering the size of the threat factors and the quantitative analyzes to be made on the populations of the species, it may also be possible to re-evaluate the species as Endangered (EN) in the near future.

4. Conclusion

Interest in utilization of medicinal and aromatic plants as pharmaceuticals, herbal remedies, flavorings, perfumes and cosmetics, and other natural products has increased substantially (De Vrient et al., 2017). Turkish flora has many undomesticated, traditionally used medicinal and aromatic plants; hence pressure is mounting on natural populations in the region including *D. hastata*. The species shows a relict endemic character and is in the vulnerable category according to the IUCN Red List. It was well established fact that fragmentation, isolation, and habitat loss have even more negative effect on relict and endemic species that are historically rare (Cruzan, 2001; Aguilar et al., 2008; De Vrient et al., 2017). This is the first comprehensive report on *D. hastata* accessions sampled from whole natural distribution of the species in Antalya, Turkey. The overall genetic diversity of *D. hastata* is relatively high, ranging from 53% to 91%, with a mean similarity value of 72%. We found that 51 and 49% of the total variation resided among and within populations, respectively. Populations tended to form two main groups, supported by the PCoA and cluster. The lack of strong relationship between genetic and geographic distance among the populations was evident. *D. hastata* populations differed for allelic richness. The highest levels of diversity occurred in Güllük (Termessos) population followed by Beldibi, Tahtalı, and Beycik. These populations might be considered with the highest conservation value in developing conservation plans for *D. hastata*. The variation present in *D. hastata* accessions may contribute to its cultivation, breeding, and conservation programs, as well as our understanding evolution of this valuable relict endemic medicinal plant. The species is thought to have a large distribution before the glacial period and currently has a very restricted distribution in Antalya. Nevertheless, Güllük (Termessos) and Tahtalı populations located within the Termessos National Park Boundaries maintain a high genetic diversity, and, hence, we propose that these two locations may be the origin of the species.

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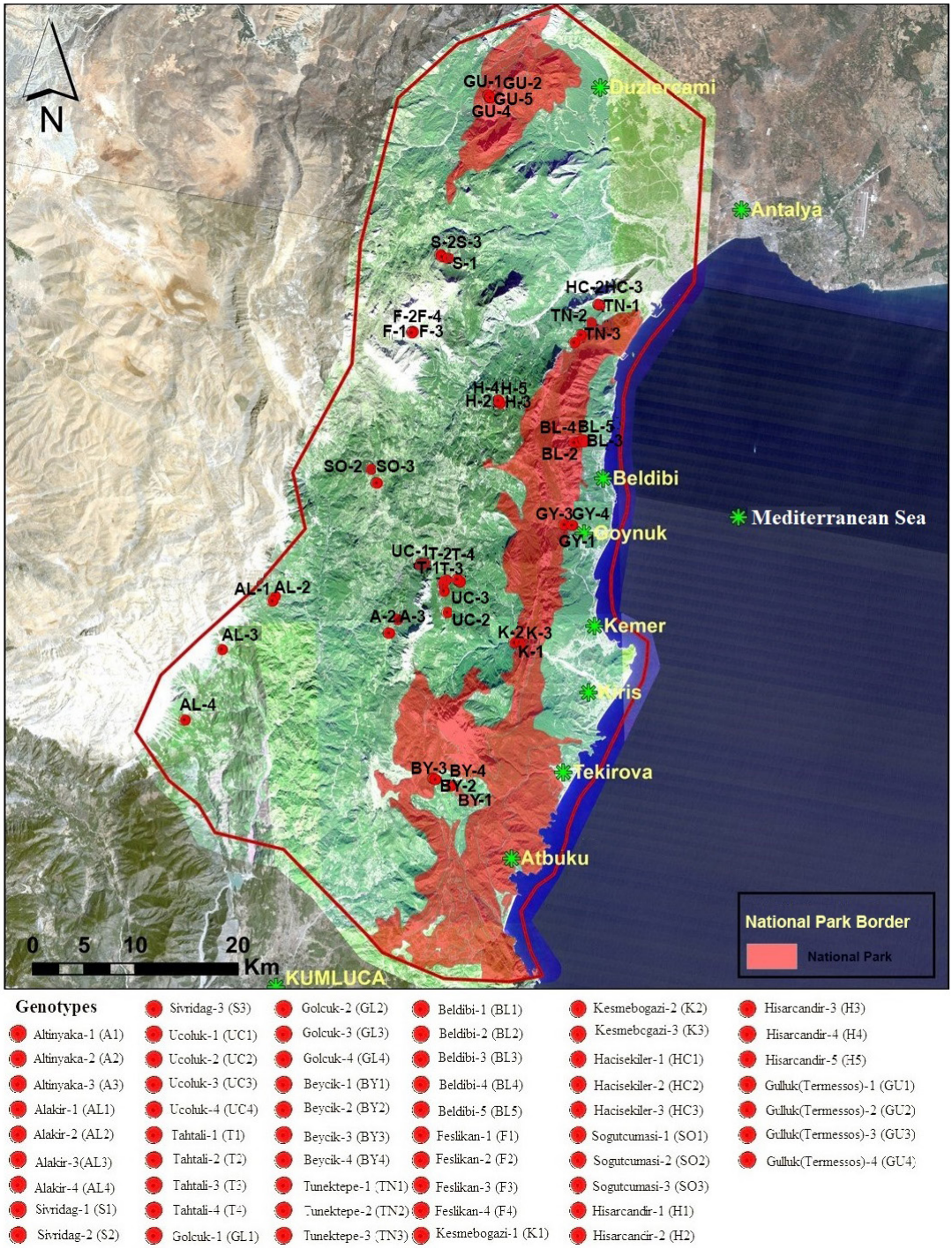
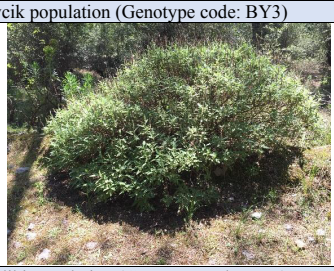
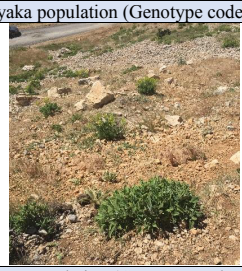
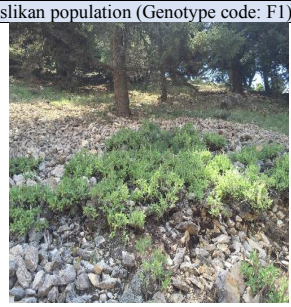


Figure S1. The satellite image indicating the *Dorystaechas hastata* populations. National park boundaries are highlighted in red.

		
Beycik population (Genotype code: BY3)	Altınyaka population (Genotype code: A3)	Güllük population (Genotype code: GU1)
		
Beldibi population (Genotype code: BL5)	Feslikan population (Genotype code: F1)	Gölcük population (Genotype code: GL2)
		
Hisarçandır population (Genotype code: H2)	Söğutcuması population (Genotype code: SO1)	Kesmeboğazı population (Genotype code: K1)

Beldibi population (Genotype code: BL5)

Figure S2. Pictures of *Dorystaechas hastata* plants at different localities under their natural habitats.

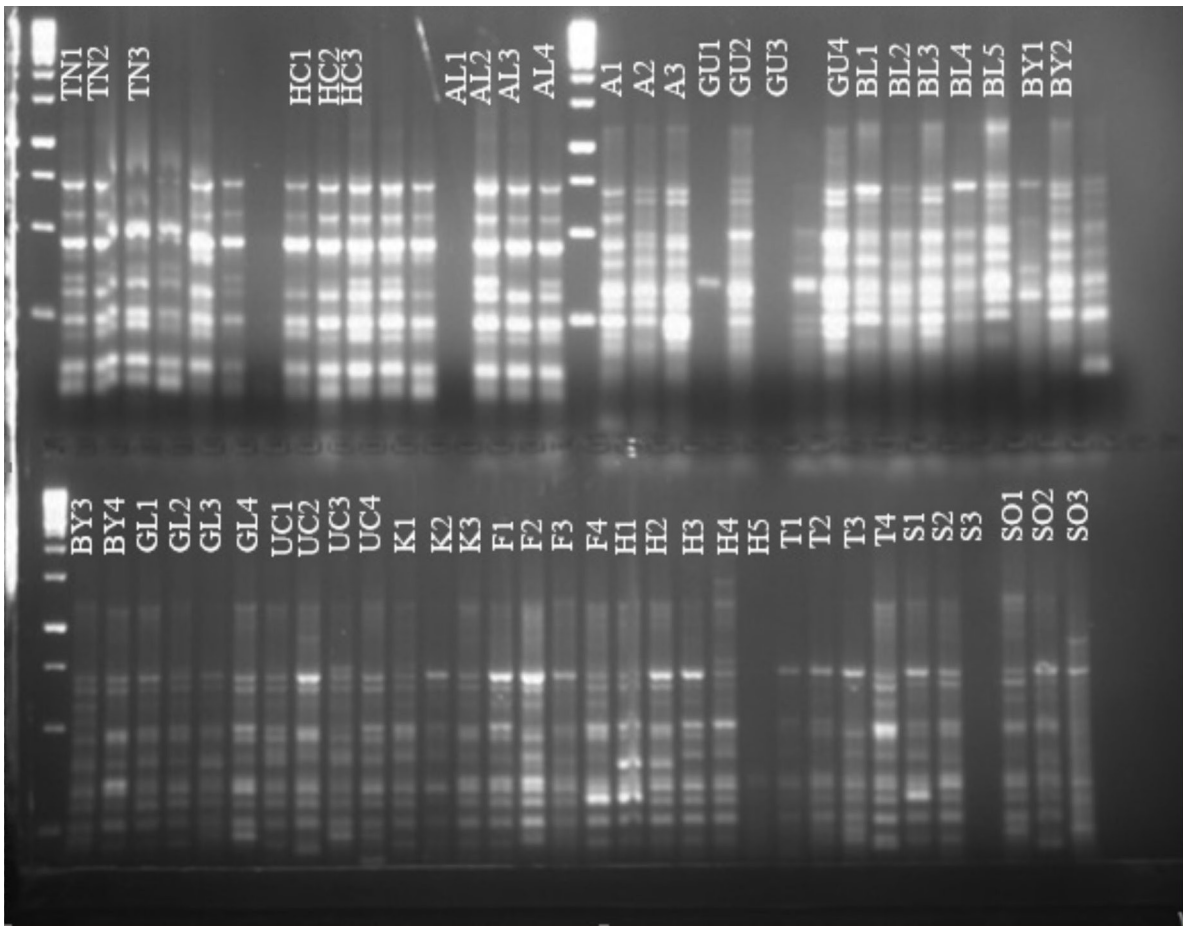


Figure S3a. SRAP profile of 59 *D. hastata* genotypes generated by Em1- Me11 (moleculer marker name/code (Population codes: HC: Hacısekiler, S: Sivridağ, F: Feslikan, TN: Tünektepe, H: Hisarçandır, UC: Üçoluk, BL: Beldibi, K: Kesmeboğazı, BY: Beycik, T: Tahtalı, GL: Gölcük, A: Altınyaka, SO: Söğütcuması, AL: Alakır, GU: Güllük).

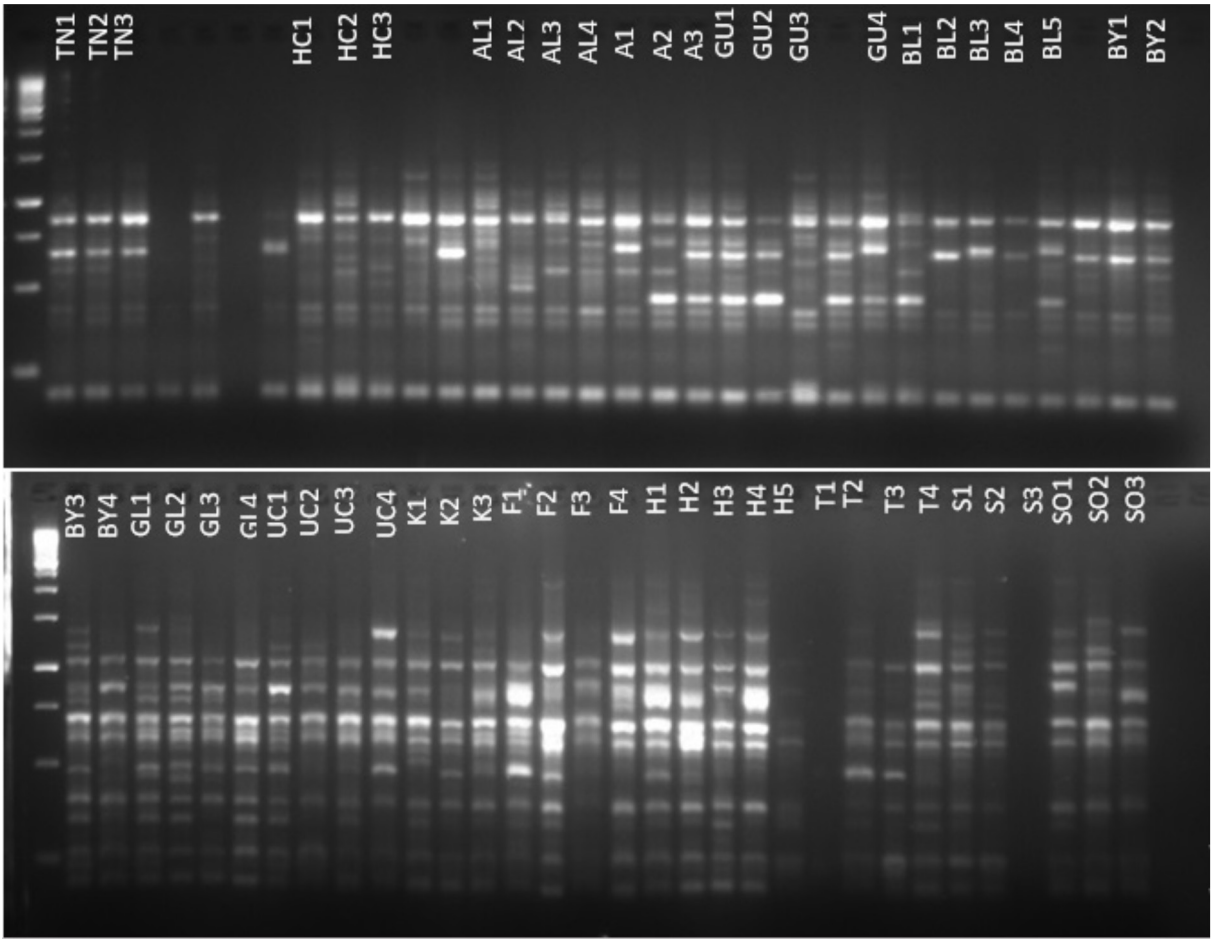


Figure S3b. iPBS profile of 59 *D. hastata* genotypes generated by 2076 and 2375 (moleculer marker name/code) Population codes: HC: Hacısekiler, S: Sivridağ, F: Feslikan, TN: Tünektepe, H: Hisarçandır, UC: Üçoluk, BL: Beldibi, K: Kesmeboğazı, BY: Beycik, T: Tahtalı, GL: Gölcük, A: Altınyaka, SO: Söğütcuması, AL: Alakır, GU: Güllük).