Celal Bayar University Journal of Science
Volume 13, Issue 4, p 913-917

Genetic diversity by AFLP analysis within *Tulipa orphanidea* L. (Liliaceae) populations in Manisa

Hande Morgil[1], Levent Şık[2]*, Osman Erol[3]

[1]İstanbul University Science Faculty Department of Biology Vezneciler-İstanbul, 02124555700, handemorgil@gmail.com
[2]Manisa Celal Bayar University Faculty of Science and Letters Department of Biology Muradiye, Yunusemre-Manisa, 02362013259, levents@cbu.edu.tr
[3]İstanbul University Science Faculty Department of Biology Vezneciler-İstanbul, 02124555700, erol@istanbul.edu.tr
*Corresponding Author
levents@cbu.edu.tr

Received: 22 September 2017
Accepted: 21 November 2017
DOI: 10.18466/cbayarfbe.334831

Abstract

Tulipa orphanidea shows high morphologic diversity throughout Eastern Mediterranean. The populations of *Tulipa orphanidea* L. in Manisa province, are observed in terms of genetic diversity. For this purpose, leaf samples were taken from individuals in different populations and AFLP analyses were carried out by taking the related protocols into consideration. It is concluded that the Bardakçı and Spil populations are genetically distant from other populations and that the individuals in these populations are in speciation process.

Keywords — Türkiye, Manisa, Tulipa orphanidea, Genetic diversity, AFLP

1. Introduction

Tulipa L. (tulip), which is a member of *Liliaceae* family, is one of the highest awareness of geophytes in the world and Turkey. The most taxa of the genus *Tulipa* is found in Central Asia and the Caucasus (65 species). This is followed by Iran (36 species) and Turkey (17 species) [1]. These data show that the diversity center of the plant is Central Asia and the Caucasus.

Tulipa orphanidea L., has a high morphological variation. In the west of Turkey, the distribution of the taxon along the coastline can be observed. However, according to the available records, the largest area of its distribution is within the borders of Manisa province [2,3]. The plant generally prefers *Pinus nigra* Arn. (black pine) forests, near fields and road sides. It is possible to find vegetation in a wide range of altitudes from sea level, up to 1700 meters.

In 2015, an action plan was prepared with the request of the 5th Regional Directorate of the Ministry of Forestry and Water Affairs, the General Directorate of Nature Conservation and National Parks and the Manisa Provincial Directorate [4]. In this study, taking into consideration the populations within the Manisa province borders, some distinct morphological differences were observed in different populations. This study aimed to find out whether the differences mentioned are meaningful in the genetic aspect.

2. Materials and Methods

The material of the study was taken from different populations of *T. orphanidea* which naturally grows within the boundaries of Manisa province. The Species Action Plan which was implemented in 2015 was used for the determination of these areas, [4].
The specimens were collected in such a way as not to damage the populations of plants and that the results obtained from the study may be statistically significant. The information on the localities where the material is provided and the leaf samples taken are given in Table 1.

Table 1. Information about the leaf samples and their place of origin.

Queue number	Code	Locality	Leaf sample codes
1	**KL** Kula	Manisa: Kula Eroğlu Mahallesi 910 m, 10.05.2016	KL-1, KL-2, KL-3, KL-4, KL-5
2	**SPL1** Spil Mountain 1	Manisa: Spil Dağı, göletin güneyi 1250 m, 11.05.2016	SPL11, SPL12, SPL13, SPL14, SPL15, SPL16, SPL17, SPL18,

			SPL19, SPL110
3	**SPL2** Spil Mountain 2	Manisa: Spil Dağı, yangın kulesi yolu 1418 m, 11.05.2016	SPL21, SPL22, SPL23, SPL24, SPL25, SPL26, SPL27, SPL28, SPL29, SPL210
4	**BRD1** Bardakçı 1	Manisa: Demirci, Bardakçı Köyü 1305 m, 20.05.2016	BRD11, BRD12, BRD13, BRD14, BRD15, BRD16, BRD17, BRD18, BRD19, BRD110
5	**ALYK1** Alayaka 1	Manisa: Alaşehir, Alayaka 1325 m, 24.05.2016	ALYK11, ALYK12, ALYK13, ALYK14
6	**ALYK2** Alayaka 2	Manisa: Alaşehir, Alayaka 1395 m, 24.05.2016	ALYK21, ALYK22, ALYK23, ALYK24, ALYK25
7	**ALYK3** Alayaka 3	Manisa: Alaşehir, Alayaka 1602 m, 24.05.2016	ALYK31, ALYK32, ALYK33, ALYK34, ALYK35

For AFLP analysis 30 samples of 30 different plant leaves were taken. The leaves were dried in silica gel and then stored at -80 ° C.

2.1 DNA Isolation

All samples (equivalent mass = 100 mg) were homogenized with the help of liquid nitrogen using mortar. The resulting powder was subjected to DNA isolation using the GeneSpin DNA Isolation kit (catalog number: 52224400621, Eurofins) according to the manufacturer's instructions. The DNA was eluted twice with 100 microliters of elution (2 x 50 microliters) as a total volume of eluate.

2.2 Measurement of quantity and purity of DNA

The amount and purity of DNA was checked using Nanodrop-ND-2000 at 260/280 nm UV. Samples were set up to participate in a maximum of 200 ng of sample DNA in subsequent reactions.

Generally, restriction and PCR reactions perform with 200 ng of total DNA and 4 µl DNA sample is added in every reaction due to that the concentrations were diluted to 50 ng (Table 2).

Table 2. Quantity and purity measurements of DNA samples

Specimen	Specimen	260/280	Concentration
01	ALYK 2-1	1,9	184
02	ALYK 1-1	1,93	73,3
03	ALYK 1- 4	1,97	206,8
04	ALYK 3-1	1,97	171,6
05	ALYK 3-5	1,89	153,5
06	ALYK 2-4	1,97	149,2
07	ALYK 3-4	1,97	136,7
09	ALYK 2-5	1,96	122,2
10	ALYK 3-2	1,94	104,4
12	ALYK 1-3	1,91	145,7
13	ALYK 2-3	1,96	100,5
14	ALYK 2-2	1,93	97,5
15	KL4	1,87	112,8
16	KL2	1,96	147,6
20	SPL1-3	1,96	157,6
22	SPL2-4	2,01	216,4
24	SPL1-8	1,78	243,4
28	SPL1-5	1,94	137,5
29	SPL1-2	1,94	179,6
32	SPL2-1	1,93	146,7
33	KL3	1,92	130,5
34	SPL2-5	2	198
36	SPL1-4	1,93	186,8
37	SPL2-8	1,95	234,6
39	SPL2-6	1,87	181,2
40	BRD1-1	1,99	382
43	BRD1-4	1,98	196
44	BRD1-5	1,98	384
45	BRD1-6	1,96	346
46	BRD1-7	1,95	386

2.3 AFLP Analyses

AFLP assays using 30 *T. orphanidea* leaves were performed according to Pharmawati et al. [5] MseI and EcoRI adapter pairs (EcoRI upper adapter 5'CTCGTAGACTGCGTACC'3, EcoRI base adapter 5'AATTGGTACGCAGTCTAC'3, MseI upper adapter 5'GACGAGAGTCCTGAG'3, MseI base adapter 5'TACTCAGGACTCAT'3) were mixed at a density of 100 µM and incubated at 95 °C for 5 minutes. The working densities of the adapters were reconstituted to be 50 pmol/µl for the MseI adapter and 5 pmol/µl for the EcoRI adapter. Restriction and ligation procedures for DNA samples were performed separately. The ligation was performed using T4 DNA Ligase enzyme.

After incubation, the restriction-ligase samples were diluted with 189µl of $TE_{0.1}$ (20 mM Tris-HCl, 0.1 mM EDTA) for use in PCR reactions. In the first step of the AFLP method, primers were used which matched the adapter sequence but added an additional 1 selective base. In the pre-amplification PCR, primers for EcoRI were used with an additional A-base (5'GACTGCGTACCAATTCA'3) and an additional C-base was added to the primers of MseI (5'GATGAGTCCTGAGTAAC'3).

The final concentration for the pre-amplification PCR samples in 25µl end volume include; 1 X Taq Buffer 1.5 mM MgCl2, 0.12 mM dNTP, 0.25 U/µl Taq polymerase, 0.2 µM primer Mse-C, 0.2 µM primer Eco-A and 100 ng genomic DNA.

PCR program for pre-amplification reaction for AFLP analysis were set up; Initial denaturation at 72 °C for 2 min; denaturation of genomic DNA 94 °C for 30 sec; Annealing temperature was set at 56 °C for 30 sec; Elongation temperature 72 °C for 2 min, after 30 cycles the final elongation was set at 60 °C for 10 min. For an image result 10 µl of the pre-amplification reaction samples were loaded in 1,5% agarose gel.

Samples obtained by the preliminary reaction in the selective amplification reaction was diluted with TE0.1 and used as template. At this step the primer EcoRI (Eco-AGG) were added to the reaction, which are fluorescence labelled and have 3 different bases.

Sequences of fluorescently labelled EcoRI and MseI primers used in the selective amplification are given in Table 3 (fluorescence labeled EcoRI primer "B", are coded as 4 different MseI primers "7-8-9-11" and also primers which are used in PCR reaction were coded as B7, B8, B9, B11.

The final concentration of PCR selective amplication for each sample in a 12,5 µl and 0.2 ml tubes; 1 X Taq Buffer, 2 mM $MgCl_2$, 0.12 mM dNTP, 0.5 U/µl Taq polymerase, 0.04 µM fluorescence labelled primer Eco-AGG, 0.2 µM primer Mse-C** and 8 µg/ml BSA, this content was mixed with 2.5 µl diluted pre-amplification reaction sample.

Table 3. Used primers for selective PCR.

Primer	Sequence (5'-'3)
E- AGG*D4 (B)	CTGCGTACCAATTCAAGG
M-CGA (7)	GATGAGTCCTGAGTAACGA
M-CGT (8)	GATGAGTCCTGAGTAACGT
M-CAT (9)	GATGAGTCCTGAGTAACAT
M-CCA (11)	GATGAGTCCTGAGTAACCA

The used PCR program for selective replication of AFLP analysis has two separate loops set: Initial denaturation for 2 min at 94 °C; the first loop with 13 cycles are set for denaturation of genomic DNA at 94 °C for 30 sec, annealing temperature is decreasing after every cycle in 0.7 °C steps, from 65 °C until 56 °C for 30 sec; and elongation at 72 °C for 2 min; the second loop has 24 cycles for denaturation of genomic DNA at 94 °C for 30 sec and for annealing of primers 72 °C for 2 min; the final elongation is set at 72 °C for 10 min.

2.4 Phylogenetic Analyses

With the obtained reaction product after selective PCR, the bi-directional fragment analysis was performed with the Applied Biosystems® brand BigDye® Terminator v3.1 Cycle Sequencing Kit. The obtained fragment lengths were converted to 1-0 data using the RAWGONZO program. The scored band results of the AFLP analysis were converted in a suitable form for data entry in the "PHYLIP" program. The data were analysed in the "PHYLIP" program. A phylogenetic tree was created using the Maximum Parsimony method with the "PHYLIP" program. For using the 1-0 data from the AFLP analysis to obtain the Maximum Parsimony tree, it is necessary to prepare the distance matrix as a first step. For this purpose, the "Phytols (Phylogenetic Tools for Comparative Biology)" program was utilized. The bootstrap value was selected as 100 while the trees were being hand made. The trees were displayed using the "Treeview" program.

3. Results and Discussion

3.1 DNA Isolation

The isolation of the genomic DNA of the *T. orphanidea* strain was carried out with the protocol of Eurofins GeneSpin DNA Extraction Kit. The agarose gel image after the isolation is shown in Figure 1.

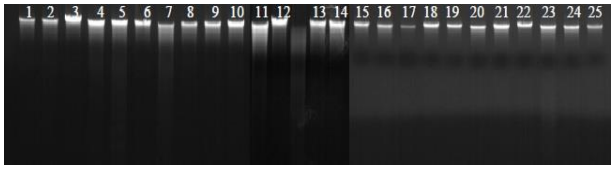


Figure 1. Some of the DNA samples visualized in agarose gel electrophoresis after isolation. Numbers above the bands represent sample codes.

3.2 Data Results of ALFP Analysis

3.2.1 AFLP Analysis with pre-amplification PCR

The isolated DNAs were amplified by PCR using the AFLP pre-amplification. The agarose gel image after the PCR is shown in Figure 2.

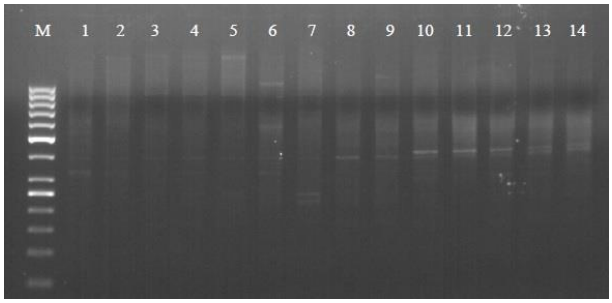


Figure 2. Image of some samples in agarose gel after AFLP pre-amplification step. The numbers above the lines represent the sample codes.

3.2.2 Selective Replication PCR performed in AFLP Analysis

Following the AFLP pre-amplification step a selective amplification PCR was done. The PCR was performed using the primer combinations given in Table 10 and subsequently visualized by agarose gel electrophorese (Fig. 3). B7, B8, B9 and B11 show the primer combinations. The procedure has been applied to all samples, only two samples with the codes 2 and 10 are shown. (Figure 3).

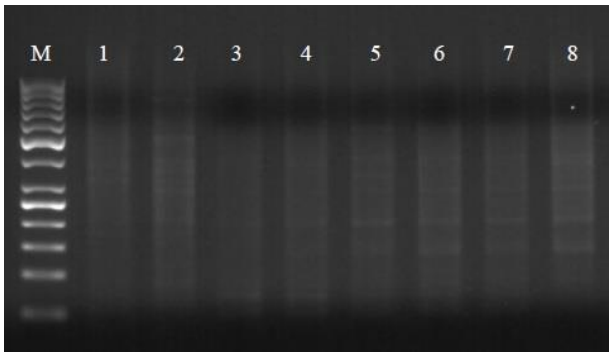
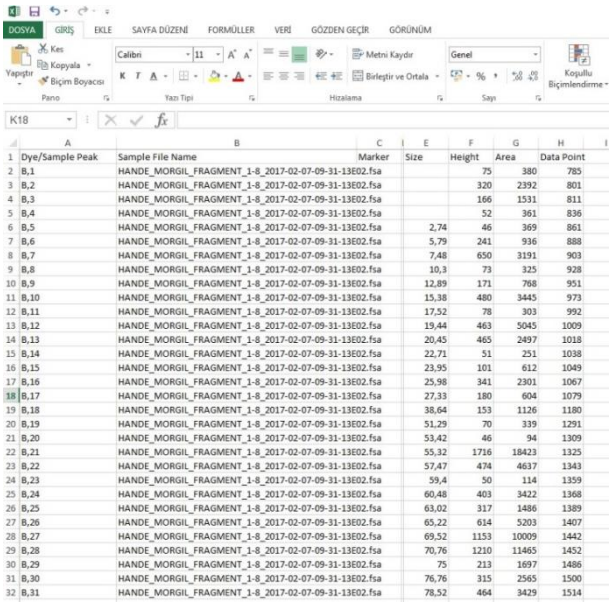


Figure 3. Selective amplification PCR after the AFLP pre-amplification step. The sample codes 2 and 8 show the different primer combinations of B7, B8, B9 and B11, respectively.

3.2.3 Processing of AFLP Data

As result of fragment analysis, the obtained lengths of bands for each primer combination are shown in Fig. 4. The data of lengths is converted to 1-0 data using the RAWGENO program. The band lengths for all samples with different primer combinations are shown in Figure 5. The graphics that indicates the frequency from the gel image of the bands are obtained with the RAWGENO program (Figure 6).



	A	B	C	E	F	G	H
1	Dye/Sample Peak	Sample File Name	Marker	Size	Height	Area	Data Point
2	B,1	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa			75	380	785
3	B,2	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa			320	2392	801
4	B,3	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa			166	1531	811
5	B,4	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa			52	361	836
6	B,5	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		2,74	46	369	861
7	B,6	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		5,79	241	936	888
8	B,7	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		7,48	650	3191	903
9	B,8	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		10,3	73	325	928
10	B,9	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		12,89	171	768	951
11	B,10	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		15,38	480	3445	973
12	B,11	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		17,52	78	303	992
13	B,12	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		19,44	463	5045	1009
14	B,13	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		20,45	465	2497	1018
15	B,14	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		22,71	51	251	1038
16	B,15	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		23,95	101	612	1049
17	B,16	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		25,98	341	2301	1067
18	B,17	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		27,33	180	604	1079
19	B,18	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		38,64	153	1126	1180
20	B,19	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		51,29	70	339	1291
21	B,20	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		53,42	46	94	1309
22	B,21	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		55,32	1716	18423	1325
23	B,22	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		57,47	474	4637	1343
24	B,23	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		59,4	50	114	1359
25	B,24	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		60,48	403	3422	1368
26	B,25	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		63,02	317	1486	1389
27	B,26	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		65,22	614	5203	1407
28	B,27	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		69,52	1153	10009	1442
29	B,28	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		70,76	1210	11465	1452
30	B,29	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		75	213	1697	1486
31	B,30	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		76,76	315	2565	1500
32	B,31	HANDE_MORGIL_FRAGMENT_1-8_2017-02-07-09-31-13E02.fsa		78,52	464	3429	1514

Figure 4. Results obtained from AFLP fragment analysis.

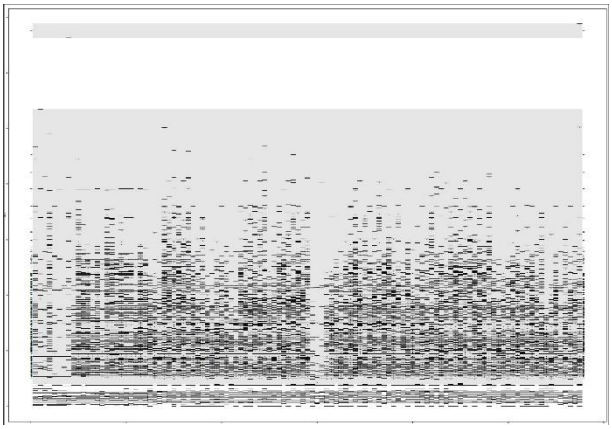


Figure 5. The band lengths for all samples with different primer combinations.

The band data, obtained as a result of AFLP analysis, was converted into a suitable data to enter in the "PHYLIP" program. The data were analysed in the "PHYLIP" program. The maximum Parsimony method was used to create a phylogenetic tree, with the PHYLIP program (Figure 7).

Using the 1-0 data obtained from AFLP analysis, it is necessary firstly to prepare the distance matrix, in order to obtain the Maximum Persimmon tree. Like this the "Phytols (Phylogenetic Tools for Comparative Biology)" program was utilized. The bootstrap value was selected as 100 while the trees were being hand made. The trees were displayed using the "Treeview" program.

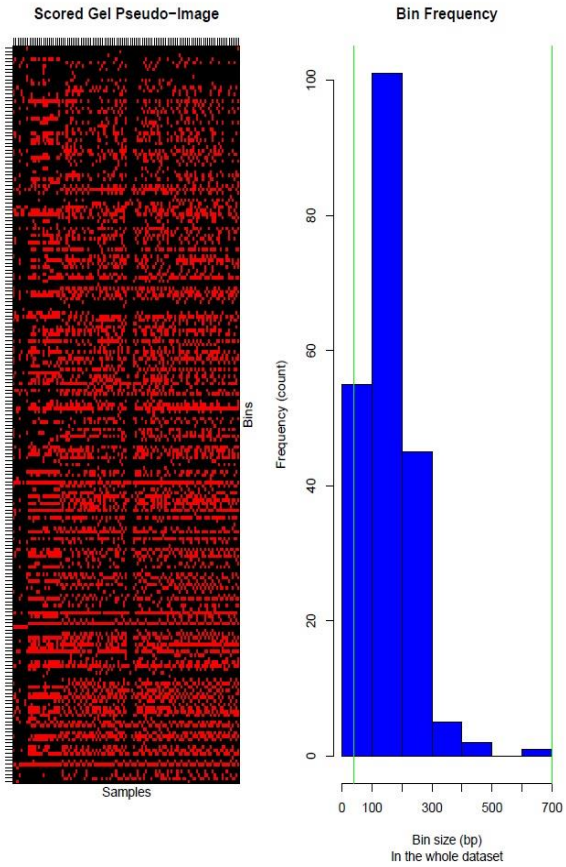


Figure 6. Gel image and frequency ratio of bands scored by RAWGENO program.

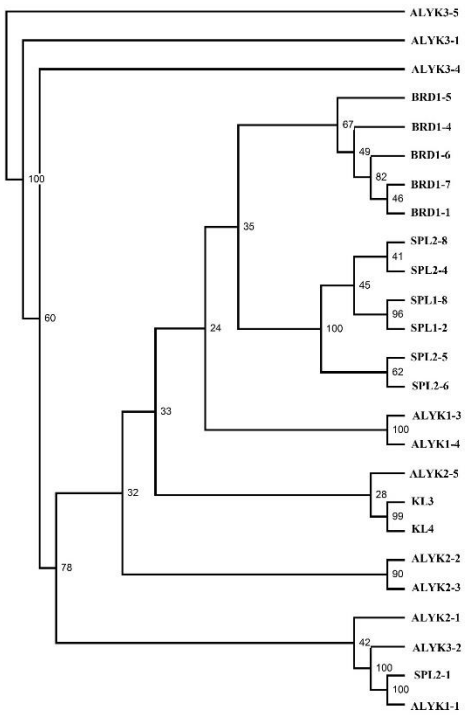


Figure 7. Maximum Parsimony tree obtained from AFLP analysis of *Tulipa orphanidea* L. samples.

4. Conclusion

When the results of AFLP fragment analysis are analysed in terms of nucleotide polymorphism, two main findings are obtained. According to the phylogenetic tree in figure 7, the ALYK population is relatively expanded and has the capacity to exchange genes with other populations. It is also seen that the geographical area, which is grouped by the BRD and SPL populations and is localized in contrast to the ALLC population, seems to maintain its general flow within itself.

It is assumed that the ALYK population has a wide distribution and that gene exchange with other populations causes similarity in nucleotide diversity within and between populations.

The grouping of the BRD population in the phylogenetic tree shows that intra-population nucleotide polymorphism differs from other populations. This also partly applies to SPL population. The possible explanation of this situation is that the spread of BRD and SPL populations are partially isolated within the geographical area. Both molecular biology data and morphological observation show that the BRD and SPL populations are different from other populations, which are studied. This data reinforces the idea that BRD and SPL populations may be from separate taxa.

Although the difference between genetic diversity and populations in *Tulipa orphanidea* taxon, which is distributed in Manisa province is reveald, the plant has a wide spreading range (South West Anatolia, North West Anatolia, Bulgaria nad Greece) it would be inaccurate in terms of scientific methodology to only examine data within the borders of Manisa province.

Acknowledgement

We thank the Ministry of Forestry and Water Affairs, General Directorate of Nature Conservation and National Parks IV. and the Regioanl Directorate, which supported this study financially. We also indebt Dr. Vahap ELDEM for its kind help about data processing.

References

1. Eker, I, Babac, MT, Koyuncu, M, Revision of the genus *Tulipa* L.(Liliaceae) in Turkey. *Phytotaxa* 2014; 157(1), 001-112.

2. Marais, W, *Tulipa* L. in Davis, PH (ed.) Flora of Turkey and the East Aegean Islands. 1984; 8 Edinburgh.

3. Güner, A, Özhatay, N, Ekim, T, Başer, KHC, Flora of Turkey and the East Aegean Islands. 2000; 11 (supp.2) Edinburgh.

4. Şık, L, Erol, O, Aktaş, K, Manisa Lalesi (*Tulipa orphanidea* L.) Tür Eylem Planı. 2015; Orman ve Su İşleri Bakanlığı, Doğa Koruma ve Milli Parklar Genel Müdürlüğü, Ankara.

5. Pharmawati, M, Yan, G, Finnegan, PM, Molecular variation and fingerprinting of *Leucadendron* cultivars (Proteaceae) by ISSR markers. *Annals of Botany* 2005; 95(7), 1163-1170.