TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2018) 42: 227-236

doi:10.3906/tar-1711-37

Genetic and phenotypic variation among Turkish terrestrial orchid species as revealed by RAPD and morphological characteristics

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Received: 08.11.2017 • **Accepted/Published Online:** 03.01.2018 • **Final Version:** 07.08.2018

Abstract: Terrestrial orchid species are natural sources of salep and a closely related group of plant species widely distributed throughout Turkey. The phylogenetic relationship among fourteen different tuber-producing orchid species was investigated after analyzing phenotypic and genetic variation within and among the natural population through fifteen morphometric traits and ten random amplified polymorphic DNA (RAPD) primer combinations. Statistical analyses (principal component analysis (PCA), principal coordinate analysis (PCoA), and cluster analysis) using the generated data identified taxonomic and genetic distance within the studied plant samples. The results of PCA from morphological traits show that there are no major groupings within and among different species instead somehow overlapping with few distinctly characterized species. In addition, the UPGMA-based phenogram with Euclidean distance (0–1) produces five major clusters among the studied orchid species according to their taxonomic status with few exceptions. On the other hand, PCoA and the phylogenetic dendrogram with the coefficient (0.56–0.79) from RAPD band profiles determine the true genetic diversity of those species. Although both combinations of genetic and phenotypic characteristics reveal the phylogenetic relationship of some those studied species very effectively, they are not clear for others. These results suggest that in the natural population of terrestrial orchid species significant amounts of gene flow are ongoing at intra/interspecies level. Therefore, it is recommended that conservation studies of these groups of orchid species should be done as a geographical unit rather than according to taxonomic status.

Key words: Characterization, terrestrial orchids, Turkey, principal component analysis, principal coordinate analysis, random amplified polymorphic DNA

1. Introduction

Orchidaceae is one of the largest families, along with Asteraceae and Fabaceae, containing approximately 20,000 species distributed in 899 genera, representing 7% of total flowering plants species all over the world (Judd et al., 2008). Being one of the advanced complex groups and having an intense association with mycotrophic fungi for nutrition (at least some stage of the life cycle) have made this entire family a target group for research by biologists and plant scientists for more than one hundred years (Harvais and Hadley, 1967; Batty et al., 2002). In addition to some outstanding features of orchids like colorful flowers and unusual seeds, they can survive in quite diverse habitats both terrestrial and epiphytic (Rasmussen and Rasmussen, 2007). Besides the aesthetic and ornamental value the economic importance of orchid species has been intensified because of their therapeutic value (Hossain, 2010).

The geographical location of Turkey makes it rich in terrestrial orchid species. About 150 taxa of terrestrial orchid species have been recorded from this region and 85% of them are tuberous, most of them belonging to the genera *Orchis*, *Serapias*, *Ophrys*, *Anacamptis*, *Dactylorhiza*, *Cephalanthera*, *Epipactis*, and some other genera (Sezik, 2002). The tubers of terrestrial orchid species are very valuable due to the presence of glucomannose and are used to produce a special drink and ice cream having particular aroma and rheological properties (Kaya and Tekin, 2001; Dalar and Konczak, 2012, 2013). Although consumption of this drink is high, these plants are not cultivated and hence are collected from nature, leading to even disappearance of these groups of species from some areas (Kasparek and Grimm, 1999; Şekercioğlu et al., 2011).

Although a significant number of research studies focused on the pharmaceutical properties, biology, propagation method, and genetic and phenotypic structure of these common groups of terrestrial species have been carried out, morphological and physiological characteristics of these species are still ambiguous in

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natural conditions (Steinbrück, 1986; Jacquemyn et al., 2009a, 2009b; Balestrini et al., 2014; Deniz et al., 2015; Molnár et al., 2017). Cytological characterization has been done for selected species although not covering all of the available species in the Turkish and Mediterranean region (Kliphuis, 1963; D'Emerico, 1996a, 1996b; Kretzschmar et al., 2007). Allozyme characteristics of some terrestrial orchid species show distinction among them and their hybrids (Arduino et al., 1995, 1996).

Genetic divergence and population studies prove that interspecies and intraspecies hybridization is still ongoing, which is why in the population level of most of the species shows morphological plasticity, therefore being difficult to identify (Scacchi et al., 1990; Arduino et al., 1996). Recent employment of DNA barcoding on 133 traded tuberous taxa mainly focused on 490 nrITS, *trn*L-F spacer, and *mat*K gene sequencing has been carried out to identify each tuber and their parental heredity. The result, however, does not reflect the true genetic background and diversity of morphologically diversified natural populations efficiently (Ghorbani et al., 2017). Genome size variation analysis in the genus *Cephalanthera* reveals that 2C DNA content varies from 27.49 pg to 36.33 pg in different species of this genus (Ahmadian et al., 2017). Despite the availability of some disperse information on different terrestrial orchid species the overall variation in the common morphologically ambiguous natural species are still not fully genetically characterized, which is very important for conservation and later improvement of these economically important plant species.

Different PCR-based molecular markers (RAPD, SSR, ISSR) are being used to produce DNA fingerprints. These simple techniques are high throughput, easily available, low cost, and efficient to get the genetic profile and to generate a phylogenetic dendrogram. The method has been successfully used for the characterization of many plants and animals to reveal their true genetic background and substantial research on this area is still ongoing (Sultana et al., 2013; Pareek et al., 2017; Punja et al., 2017).

The efficiency of molecular characterization can even be improved with morphological data to have an overall view of plants' genetic background and related morphological plasticity (Curuk et al., 2016; Yıldız et al., 2016). Several multivariate statistical methods like principal component analysis (PCA), principal coordinate analysis (PCoA), and cluster analysis are widely used today to deduct the actual morphological, genetic, and biochemical diversity within and between populations. Generally, different types of data (like discrete, continuous, and binomial) can be analyzed with this group-based technique (Aremu, 2017).

In the present study, to identify the genetic variation of the wild terrestrial orchid species, PCR-based genetic fingerprinting was carried out with RAPD markers. Morphological data were collected on several distinct characteristics (leaf, tuber, plant length, flower). Data generated through this research were used as input data for statistical analysis, producing a phylogenetic dendrogram and genetic distance.

2. Materials and methods

Terrestrial orchid species belong to the family Orchidaceae were collected from a different part of Niğde Province and its surroundings in Turkey in 2015 and 2016. Availability of these species was recorded as the Demirkazık area in Niğde, Çamardı district, Pınarbaşı village, Çukurbağ village, Maden village, and Aladağ Mountains in different literature. Therefore, plant materials were collected by field expedition during the flowering time around that area and identified and the identification was confirmed by botanists at the Department of Biology, Niğde Ömer Halisdemir University (Table 1).

Different morphological traits of leaf, stem, body, spike, and flowers were determined at the same time in millimeters with the exception of centimeters for plant length. Fresh young leaf tissue from each sample was collected and preserved at –80 °C for genomic DNA extraction. Finally, plants were transferred to the greenhouse conditions of Ayhan Şahenk Faculty of Agricultural Sciences and Technologies of Niğde Ömer Halisdemir University, Niğde, Turkey, and monitored throughout. Genomic DNA was extracted using a CTAB mini-prep according to the protocol described by Dellaporta et al. (1983). The quality and concentration of the extracted DNA were checked using a Shimadzu BioSpec-nano spectrophotometer and 1% agarose gel electrophoresis.

A set of 10 RAPD primers, OPAA 8, OPAA 20, OPAA 09, OPAA 05, OPAA 15, OPAI 05, OPAI 11, OPAJ 05, OPAK 17, and OPB 10, was used to generate RAPD fingerprinting. PCR amplification was done in 10 µL of reaction volume containing 10 µM of primer, 0.5 mM of each dNTP, 1.5 µL of 10X Dream Taq buffer (with 25 mM of $MgCl_2$), 0.12 µL of Dream Taq polymerase, and 50 ng of genomic DNA.

PCR conditions were 94 °C for 3 min followed by 55 cycles of 94 °C for 30 s, 1 min at 37 °C, 72 °C for 1 min, and followed by a 10-min final extension at 72 °C. The completed reactions were held at 4 °C (Labcycler, Sensoquest, Göttingen, Germany). The amplified PCR fragment was separated by 2% agarose gel electrophoresis in 1X TAE buffer (40 Mm Tris-Acetate, 1 Mm EDTA, pH 8.0) for 2 h at 80 V using 1 kb and 100 bp DNA ladder. The RAPD banding pattern was photographed under UV light after immerging in ethidium bromide solution for 30 min. The repeatability of the profiles was checked by using two replicates where no discrepancies were found for the clear bands scored. The RAPD band profiles were scored as 1 for

Table 1. List of fourteen terrestrial orchid species (salep) and their sampling locations in Turkey.

Species group number	Species	Number of plants studied	Place name	Latitude	Longitude	Geographical region	Elevation (m)
1	*Orchis mascula*	2	Çamlıyayla	37.25110065	34.55406189	Mediterranean	2483
2	*Orchis anatolica*	4	Çamlıyayla	37.25110065	34.55406189	Mediterranean	680
3	*Serapias vomeracea*	12	Manavgat	36.786869	31.441282	Mediterranean	53
4	*Ophrys isaura*	2	Alata	36.6098525	34.3171552	Mediterranean	9
5	*Serapias vomeracea*	3	Alata	36.6098525	34.3171552	Mediterranean	9
6	*Anacamptis laxiflora*	2	Konya, Beyşehir	37.679796	31.724299	Central Anatolia	1131
7	*Orchis purpurea*	3	Konya, Beyşehir	37.679796	31.724299	Central Anatolia	1131
8	*Anacamptis laxiflora* subsp. *dinsmorei*	3	Emli Vadisi	37.7839036	35.0766142	Central Anatolia	1699
9	*Dactylorhiza romana*	3	Emli Vadisi	37.7839036	35.0766142	Central Anatolia	1699
10	*Orchis boryi*	3	Emli Vadisi	37.7839036	35.0766142	Central Anatolia	1699
11	*Orchis palustris*	3	Niğde	38.0993086	34.6856509	Central Anatolia	1522
12	*Anacamptis laxiflora*	6	Maden	38.4443966	39.6270779	Eastern Anatolia	1152
13	*Ophrys reinholdii*	3	Çamliyayla	37.25110065	34.55406189	Mediterranean	2483
14	*Cephalanthera longifolia*	4	Maden	38.4443966	39.6270779	Eastern Anatolia	1152
15	*Epipactis helleborine*	1	Maden	38.4443966	39.6270779	Eastern Anatolia	1152
16	*Epipactis purpurata*	3	Maden	38.4443966	39.6270779	Eastern Anatolia	1152

presence of a particular band and 0 for absence and a table was generated for further statistical analysis.

Different software packages were used for statistical analysis of data generated through morphological and molecular characterization. Mean and standard deviation of the morphological data were calculated using Microsoft Excel. PCA, score plot, and clustering using unweighted pair group method with arithmetic mean (UPGMA) and Euclidean distance were carried out using Minitab 17. For the analyses of RAPD data, they were recorded as 1 for the presence of a band and 0 for its absence to generate a binary matrix. Only reproducible bands were scored for all the accessions tested. PCoA and cluster analyses were performed using NTSYS. For these analyses, a similarity matrix was generated using Jaccard coefficients. For cluster analysis, UPGMA was used to construct dendrograms.

3. Results

Comparative morphological traits of 57 plants from fourteen different species indicate that a significant difference is present among the natural populations of terrestrial orchid species. Table 2 shows the mean and standard deviation of nine quantitative morphological characters (leaf length (LL), leaf width (LW), ratio of leaf length and width (RLLW), tuber length (TL), tuber width (TW), ratio of tuber length and width (RTLW), plant length (PL), diameter of spike (DSE), diameter of stem (ST), leaf number (LN)).

The highest leaf length and width observed were 117.22 ± 11.27 and 79.17 ± 20.67 in *Cephalanthera longifolia*. The lowest leaf length and width value were 9.58 ± 0.80 and 45.58 ± 0.13 in *Dactylorhiza romana* and *Ophrys isaura*, respectively. The overall ratio of leaf length and width varied from 1.21 to 5.53. The results show that leaf characteristics are highly variable with wide standard deviation among all of the orchid species.

By contrast to leaf characteristics, tuber characteristics were more stable with less standard deviation. While the highest tuber length and width (39.55 ± 7.03 and 40.39 ± 16.01) were observed in *Epipactis purpurata* and *Serapias vomeracea* collected from Alata showed the lowest values 11.27 ± 1.47 and 8.70 ± 1.39 for tuber length and width, respectively. However, the ratio of tuber length and width ranged from 0.98 to 2.07.

On the other hand, highest and lowest plant length were 41.00 and 16.67 ± 5.13 recorded in *Epipactis helleborine* and *Serapias vomeracea* from Alata. In all of the studied species, stem diameter ranged between 8.24 ± 3.18 and 2.25 ± 0.27 and total leaf number varied from 2.33 ± 0.58 to 8.33 ± 2.08 (Table 2).

In addition, six quantitative and one qualitative flower morphological traits (flower width (FW), flower length (FL), ratio of flower width and length (RFWL), flower number (FN), diameter of spike (DSP), and spike length (SL)) were also analyzed in a similar fashion (Table 3). The data indicate that flower traits are more consistent within the population

Table 2. Comparison of plants morphological traits (means and standard deviations) among fourteen terrestrial orchid species (salep) collected from different regions of Turkey (Measurement scale millimeter except for plant length where it is in centimeter).

Scientific name	Leaf width	Leaf length	Leaf length/ Width	Tuber width	Tuber length	Tuber length/ Width	Plant length	Diameter of stem	Leaf number
Orchis mascula	10.92 ± 3.68	58.84 ± 2.31	5.39	15.08 ± 5.43	20.42 ± 6.46	1.35	18.25 ± 6.72	5.01 ± 1.53	6.50 ± 0.71
Orchis anatolica	11.66 ± 3.62	71.06 ± 23.20	6.10	11.80 ± 1.48	13.83 ± 1.64	1.17	17.75 ± 8.14	3.62 ± 1.55	4.00 ± 0.82
Serapias vomeracea (Manavgat)	15.94 ± 4.47	91.85 ± 17.79	5.76	15.52 ± 3.40	25.31 ± 5.16	1.63	26.67 ± 6.49	6.25 ± 1.98	5.67 ± 1.56
Ophrys isaura	21.97 ± 17.54	45.58 ± 0.13	2.07	15.77 ± 1.32	20.98 ± 6.27	1.33	30.50 ± 5.66	2.89 ± 0.49	2.50 ± 0.71
Serapias vomeracea (Alata)	38.68 ± 12.07	46.78 ± 18.96	1.21	8.70 ± 1.39	11.27 ± 1.47	1.30	16.67 ± 5.13	2.25 ± 0.27	2.33 ± 0.58
Anacamptis laxiflora (Konya, Beyşehir)	19.88 ± 4.48	109.84 ± 46.46	5.53	14.12 ± 4.28	27.09 ± 10.08	1.92	31.25 ± 6.72	7.38 ± 2.03	5.00 ± 1.41
Orchis purpurea	35.36 ± 15.00	129.35 ± 13.47	3.66	18.35 ± 3.58	37.81 ± 8.40	2.06	31.00 ± 3.61	8.24 ± 3.18	5.33 ± 0.58
Anacamptis laxiflora subsp. *dinsmorei*	30.39 ± 8.66	109.14 ± 17.51	3.59	19.67 ± 5.61	29.35 ± 6.69	1.49	42.67 ± 14.19	5.04 ± 0.29	5.33 ± 0.58
Dactylorhiza romana	9.58 ± 0.80	114.33 ± 29.38	11.93	18.35 ± 4.10	31.75 ± 6.45	1.73	28.00 ± 6.08	7.16 ± 0.56	8.33 ± 2.08
Orchis boryi	28.67 ± 2.75	98.76 ± 4.09	3.44	16.23 ± 1.96	30.60 ± 7.01	1.89	31.33 ± 2.84	7.22 ± 0.50	4.33 ± 0.58
Orchis palustris	20.05 ± 4.52	106.86 ± 4.98	8.02	13.94 ± 4.76	20.58 ± 7.30	1.48	40.00 ± 5.57	8.18 ± 1.50	6.67 ± 2.08
Anacamptis laxiflora (Maden)	23.85 ± 7.61	89.26 ± 23.63	3.74	16.91 ± 3.82	23.75 ± 7.61	1.40	35.67 ± 8.94	7.10 ± 1.62	5.67 ± 0.82
Ophrys reinholdii	18.98 ± 3.24	89.67 ± 2.21	4.73	16.52 ± 1.60	21.36 ± 2.75	1.29	37.67 ± 11.02	4.64 ± 2.60	2.33 ± 0.58
Cephalanthera longifolia	79.17 ± 20.67	117.22 ± 11.24	1.48	23.51 ± 2.37	33.9 ± 2.71	1.44	36.25 ± 4.19	2.92 ± 0.53	5.00 ± 0.82
Epipactis helleborine	22.59 ± 0.0	55.46 ± 0.0	2.46	10.41 ± 0.0	21.57 ± 0.0	2.07	41.00 ± 0.0	2.54 ± 0.0	8.00 ± 0.0
Epipactis purpurata	28.96 ± 5.71	57.67 ± 4.27	1.99	40.39 ± 16.01	39.55 ± 7.03	0.98	27.83 ± 6.05	3.12 ± 1.51	7.33 ± 1.15

Table 3. Comparison of six quantitative (means and standard deviations) and one qualitative (flower color) traits of flower among fourteen terrestrial orchid species collected from different regions of Turkey.

Scientific name	Flower width	Flower length	Flower length/width	Flower number	Diameter of spike	Spike length
Orchis mascula	8.05± 2.59	11.81± 3.51	1.47	8.00 ± 4.24	1.87 ± 0.76	7 ± 0.0
Orchis anatolica	7.60 ± 5.39	19.92 ± 10.59	2.62	4.00 ± 2.45	1.61 ± 0.22	10 ± 0.
Serapias vomeracea (Manavgat)	5.34 ± 0.97	22.28 ± 4.02	4.18	4.75 ± 2.09	2.23 ± 0.86	11.50 ± 4.81
Ophrys isaura	6.16 ± 4.81	10.87 ± 0.01	1.76	5.00 ± 1.41	2.03 ± 0.18	12 ±-0.0
Serapias vomeracea (Alata)	4.58 ± 0.16	15.34 ± 2.89	3.35	3.67 ± 0.58	1.49 ± 0.42	12 ±-0.0
Anacamptis laxiflora (Konya, Beyşehir)	7.80 ± 2.75	17.63 ± 4.63	2.26	35.00 ± 0.00	3.82 ± 0.30	12.00 ± 1.41
Orchis purpurea	6.09 ± 0.61	13.79 ± 2.08	2.27	123.33 ± 25.17	3.91 ± 0.74	6.33 ± 1.53
Anacamptis laxiflora subsp. *dinsmorei*	10.38 ± 1.52	15.55 ± 4.57	1.50	35.33± 5.03	2.92 ± 0.76	16.00 ± 7.21
Dactylorhiza romana	6.96 ± 0.76	15.75 ± 1.41	2.26	18.00 ± 5.29	2.27 ± 0.49	8.00 ± 2.65
Orchis boryi	12.06 ± 4.81	15.78 ± 4.91	1.31	35.67 ± 4.04	2.34 ± 0.45	9.83 ± 1.89
Orchis palustris	8.41 ± 2.93	28.48 ± 3.50	3.39	25.67 ± 6.03	4.96 ± 1.15	13.33 ± 2.89
Anacamptis laxiflora (Maden)	10.02 ± 3.28	21.14 ± 5.95	2.11	35.67 ± 4.32	3.94 ± 0.99	12.83 ± 4.71
Ophrys reinholdii	19.14 ± 3.20	24.06 ± 5.31	1.26	5.33 ± 3.21	2.90 ± 1.12	13.33 ± 5.51
Cephalanthera longifolia	6.79 ± 2.67	26.59 ± 2.97	3.92	9.50 ± 3.32	2.45 ± 0.47	11.88 ± 4.63
Epipactis helleborine	4.95 ± 0.0	31.57 ± 0.0	6.38	9.00 ± 0.0	1.71 ± 0.0	14.00 ± 0.0
Epipactis purpurata	8.99 ± 2.51	18.33 ± 3.93	2.04	6.67 ± 1.53	1.53 ± 0.79	2.00 ± 0.00

with less standard deviation. While the highest flower length belonged to *Epipactis helleborine*, 31.57, the lowest was 11.81 ± 3.51 in *Orchis mascula*. On the other hand, the highest width was found in *Anacamptis laxiflora* subsp. *dinsmorei* (10.38 ± 1.52) and the lowest belonged to *Serapias vomeracea* from Alata. However, the ratio of flower length and width ranged from 1.26 to 6.38 in *Ophrys reinholdii* and *Epipactis helleborine*, respectively. Nevertheless, number of flowers is one of the most distinct characters among flower morphology. The highest flower number was 123.33 ± 25.17 in *Orchis purpurea* with the lowest in *Epipactis purpurata*, 6.67 ± 1.53. The highest diameter of spike and spike length recorded were 4.96 ± 1.15 and 14.00 in *Orchis palustris* and *Epipactis helleborine*, respectively. In contrast, *Epipactis purpurata* gave the lowest values for diameter of spike and spike length, 1.53 ± 0.79 and 2.00, respectively (Table 3).

PCA using total 15 morphological characters allows a better illustration of the effectiveness of each trait of the total structure of variation of 57 plants belonging to fourteen different orchid species. The analysis of 15 principal components proves that PC1 is the main and most effective component, accounting for about 26% total variation with eigenvalue 3.95 and cumulative value 0.26. However, the next three principal components, PC2 (eigenvalue 2.14), PC3 (eigenvalue 2.07), and PC4 (eigenvalue 1.83), account for about 14%, 13%, and 12% of total variation, respectively (Table 4). All together PC1, PC2, PC3, and PC4 stand for about 65% of total morphological variation. LL and DSP with coefficient 0.4 caused the most important traits of PC1 and the other effective traits are DST (0.38) and PL (0.39). By contrast, LW (0.5), TW (0.3), and FW (0.3) are the most important traits for PC2. Moreover, FL (0.4) and SL (0.4) are the most significant traits for PC3 and RFLW is the only most effective trait of PC4 (Table 4). Score plot analysis of the 57 plants' species against PC1 and PC2 shows that a highly significant difference is present for both inter/intraspecies level and no apparent grouping was possible (Figure 1).

UPGMA cluster analysis on average morphological traits for each species produced a total of five different clusters. Euclidean distance for total variation ranged between 0.0 and 1.0. The phenogram shows that *O. mascula*, *O. anatolica*, *O. isaura*, and *S. vomeracea* belong to cluster 1; *A. laxiflora* subsp. *dinsmorei*, *A. laxiflora*, *O. boryi*, and *O. reinholdii* belong to cluster 2; *E. purpurata* is the only species in cluster 3; *O. purpurea*, *D. romana*, and *O. palustris* belong to cluster 4; and *C. officinalis* and *E. helleborine* belong to cluster 5. From this phenogram it is further evident that these terrestrial orchid species had a wide range of morphological variation within and among the genus and the grouping of those species is not solely based on taxonomic classification (Figure 2).

Table 4. Coefficients and eigenvalues for first three principal components of PCA analysis for 16 collected terrestrial orchid species from different regions of Turkey.

Variable	PC1	PC2	PC3
Leaf width	0.07	0.51	–0.20
Leaf length	0.43	–0.03	0.08
Ratio of leaf length and width	0.13	–0.48	0.20
Tuber width	0.10	0.31	–0.28
Tuber length	0.29	0.19	–0.43
Ratio of tuber length and width	0.22	–0.14	–0.27
Plant length	0.37	0.29	0.18
Flower number	0.27	–0.16	–0.29
Flower width	0.14	0.06	0.03
Flower length	0.12	0.31	0.41
Ratio of flower length and width	–0.12	0.20	0.28
Diameter of spike	0.40	–0.07	0.13
Diameter of stem	0.39	–0.25	0.08
Leaf number	0.24	–0.02	0.00
Spike length	0.16	0.18	0.44
Eigenvalue	3.96	2.15	2.08
Proportion	0.26	0.14	0.14
Cumulative	0.26	0.41	0.55

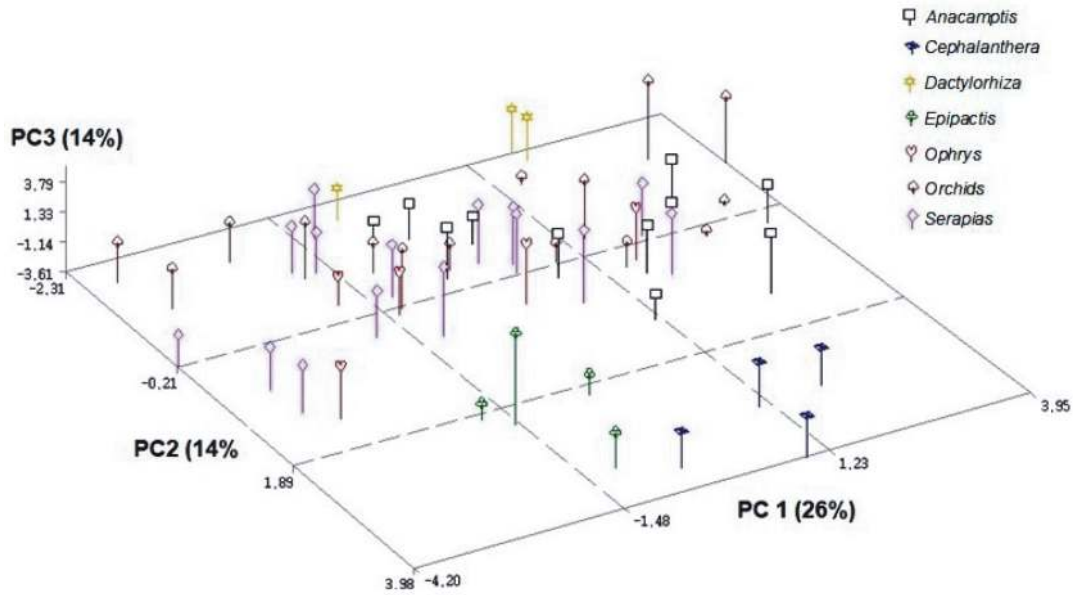

Figure 1. Score plot of the first two principle components (1 and 2) illustrating overall morphological variation among 57 plants belonging to fourteen different terrestrial orchid species based on 15 morphological characters.

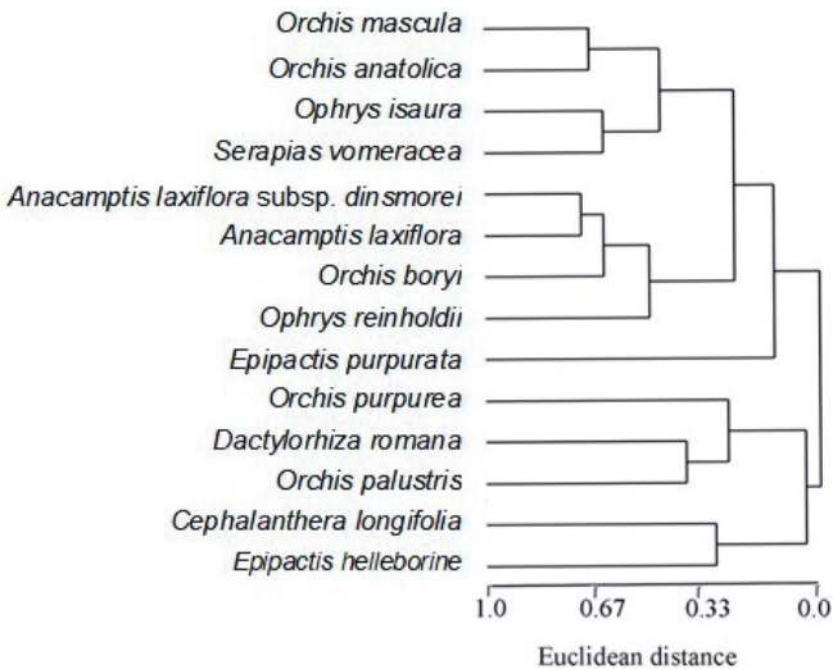

Figure 2. UPGMA phenogram of fourteen collected terrestrial orchid species based on average 15 quantitative morphological traits distance.

The 10 arbitrary RAPD primers used for fingerprinting of the 14 different species amplified 504 clear scorable bands with 100% polymorphism. The maximum number of bands was yielded by OPAI11 (80) and the minimum was by OPAA 05 (2). The band analyses from agarose gel confirmed that some of those polymorphic bands were unique and only scored in certain species. The numbers of unique bands were 4 for OPAA 8 and OPAA 20; 3 for OPAA 15, OPAI 05, and OPAI 11; 1 for OPAA 09 and OPAJ 05; and 2 for OPB 10. However, OPAA 05 and OPAK 17 showed no unique band in the studied plant species. Therefore, the total number of unique bands was 21 specific to each species (Table 5).

Table 5. Arbitrary RAPD primers code, sequences, the sizes of the amplified fragments, number of polymorphic and unique bands, and percentage of polymorphism.

Primer code	Sequence (5' to 3')	Size (bp)	No. of amplified bands with 100% polymorphism	No. of unique bands
OPAA 8	TCCGCAGTAG	200–2000	14	4
OPAA 20	TTGCCTTCGG	375–6500	16	4
OPAA 09	AGATGGGCAG	250–2500	15	1
OPAA 05	GGCTTTAGCC	5000	1	0
OPAA 15	ACGGAAGCCC	250–2500	15	3
OPAI 05	GTCGTAGCGG	375–6000	16	3
OPAI 11	ACGGCGATGA	250–6000	16	3
OPAJ 05	CAGCGTTGCC	300–2250	15	1
OPAK 17	CAGCGGTCAC	200–2500	13	0
OPB 10	CTGCTGGGAC	250–2000	11	2

By contrast to the phenogram, the UPGMA dendrogram generated through a binary matrix of RAPD bands produced phylogenetic clustering with cophenetic coefficient 0.56–0.79 with two major clusters. Figure 3 shows cluster one only represents *O. mascula*, which is separated from rest of the species. Cluster two is further subdivided into two major subclusters. Subcluster one is made up of *O. anatolica*, *O. reinholdii*, and *D. romana* and the rest of the species belong to subcluster two. Moreover, subcluster two can be further divided in four small groups where *O. purpurea*, *O. palustris*, *E. helleborine*, *C. officinalis*, and *E. purpurata* belong to group one; *O. boyri*, *A. laxiflora*, and *A. laxiflora* subsp. *dinsmorei* belong to group two; and groups three and four have one species, *S. vomeracea* and *O. isaura*, respectively. The results indicate that although the clustering generated from RAPD analysis is in agreement in some points with the phenogram generated through morphological data, they

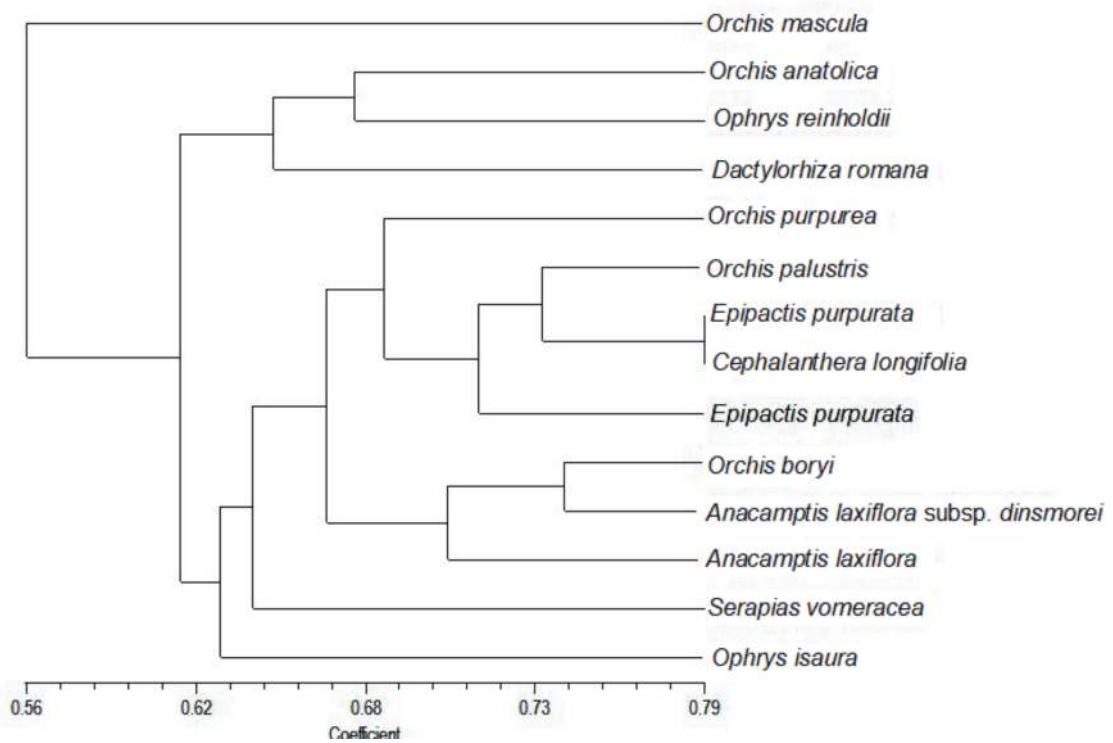

Figure 3. UPGMA dendrogram of RAPD bands generated by 10 arbitrary oligonucleotide primers.

did not totally match (Figures 2 and 3). In addition to cluster analysis, PCoA using the RAPD band data plotted in a three-dimensional scale supplied the supplementary information of the species relationship (Figure 4).

4. Discussion

The overall record of morphological traits with higher standard deviation indicates a high level of morphological plasticity is present among those orchid species. Therefore, to identify the real structure of the entire collection, PCA was performed. Since first four principal components estimated about 65% of the morphological variation, the representing morphometric traits (DST, PL, LW, TW, FW, FL, SL, RFLW) are recommended to consider as useful taxonomic characteristics for further studies. Our results are supplementary to those reported by Jakubska-Busse and Gola (2010), who suggest that not a single trait but joined ones can be useful as a taxonomic value for identification and characterization of terrestrial orchid genera.

The consistent clustering derived from phenotypic and genotypic data proves the close relationship among *O. palustris*, *O. purpurea*, *C. longifolia*, *and E. helleborine*. This finding was supported by the previous record by Ehlers et al. (2002), who showed that *E. helleborine* is a widely distributed species with high morphological variability. The other group of orchids following a similar pattern are *A. laxiflora* subsp. *dinsmorei*, *A. laxiflora*, and *O. boryi* according to the phenogram and dendrogram. Although the taxonomic status of several species was contradictory, it appeared that the RAPD-based UPGMA dendrogram and PCoA could be used for the identification and characterization of them efficiently. Although the presence of natural hybrids among different species of *Orchis*, *Epipactis*, and *Serapias* has been already studied, the information available was not extensive to draw a spatial relationship among them (Caputo et al., 1997; Jakubska-Busse and Gola, 2010; Hršak et al., 2011).

The intermingling characteristics of these species within the taxonomic ground while covering the board environmental region with somewhat narrow genetic distance proves that the difference comes from sampling artifact, from environmental variation, habitat fragmentation, or natural hybridization (Aybeke et al., 2010; Altundağ et al., 2012; Sevgi et al., 2012). Therefore, a detailed investigation with improved experimental set-up is necessary to solve that question.

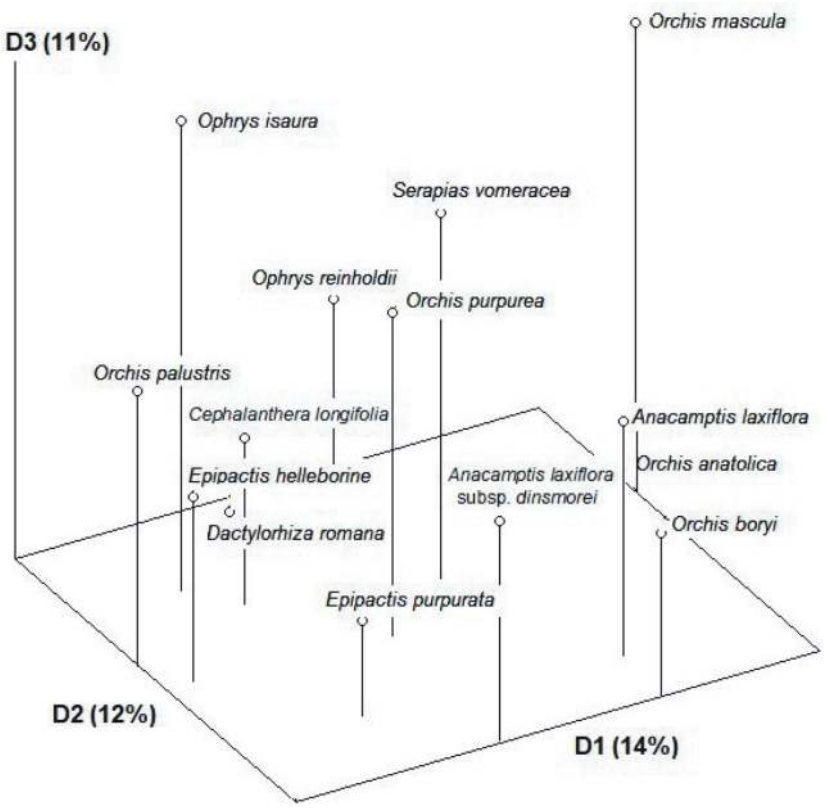

Figure 4. Principal coordinate analyses of RAPD bands generated by 10 arbitrary oligonucleotide primers for terrestrial orchid species.

In conclusion, despite some fragmented information available in the literature reported by several scientists during the past year, our results could serve as valuable data for estimation of the ecological distribution and conservation strategies of the genus and Turkish terrestrial orchid species as a broad taxonomic group.

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