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RESEARCH ARTICLE

Phylogenetic relationships among species of the subsection *Dendrophlomis* Bentham

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Abbreviations:
AMOVA: analysis of molecular variance
ITS: internal transcribed spacer
PCR: polymerase chain reaction
RAPDs: randomly amplified polymorphic DNA
UPGMA: unweighted pair-group method with arithmetic averages

This study used randomly amplified polymorphic DNA markers to determine genetic relationships among species of the subsection *Dendrophlomis*. Twenty accessions of the eleven *Phlomis* taxa were evaluated to determine genetic variability using fourteen ten mer primers selected from a 125 random oligonucleotide set. These 14 selected primers generated 85 RAPD bands that ranged in size from 200 to 1200 base pairs. Of the total bands, 88% (75) were polymorphic among the samples. Genetic distances among accessions were computed to produce a dendrogram based on UPGMA. Genetic distances ranged from 0.133 (between *P. amanica* and *P. monocephala*) to 0.494 (between *P. chimerae* and *P. lunariifolia*). The UPGMA tree based on distances has two major groups. The first comprised 9 taxa that were clustered into two subgroups. The first subgroup consisted of *P. viscosa, P. lycia, P. amanica* and *P. monocephala* while the second comprised *P. lunariifolia, P. bourgaei, P. longifolia* var. *longifolia, P. grandiflora* var. *grandiflora* and *P. grandiflora* var. *fimbrilligera*. The second group comprised 2 species, *P. leucophracta* and *P. chimerae*. Species-specific bands were observed for *P. lycia, P. leucophracta, P. lunariifolia, P. bourgaei, P. chimerae* and *P. longifolia* var *longifolia*.

The genus *Phlomis* L. comprises over 100 species including herbs, shrubs and sub-shrubs of the family Lamiaceae (Albaladejo et al. 2005). The genus is divided into two main sections, *Phlomis* and *Phlomoides* (Moench, 1794). Both sections are spread from the Mediterranean region to central Asia and China; but while species of the section *Phlomoides* occur mostly in central Asia and China, species of the section *Phlomis* appear mainly in the Mediterranean region. Turkey and Iran were indicated as the main centers of diversification in the Mediterranean region for the section *Phlomis* (Hedge, 1986). In particular, southern and eastern parts of the former and north-western part of the latter were proposed as centers of origin of that section. Nevertheless, Turkey has twice the number of species (34) and also nearly twice a higher endemism rate (57%) of species belonging to section *Phlomis* compared to Iran, where the numbers are 18% and 33% respectively (Hedge, 1986).

Measurement of genetic variation within and between plant species is important for several reasons including delimitation of species, conservation of endangered species and construction of phylogenetic relationships among species. Several kinds of methods were used to measure levels and patterns of genetic variation, which range from

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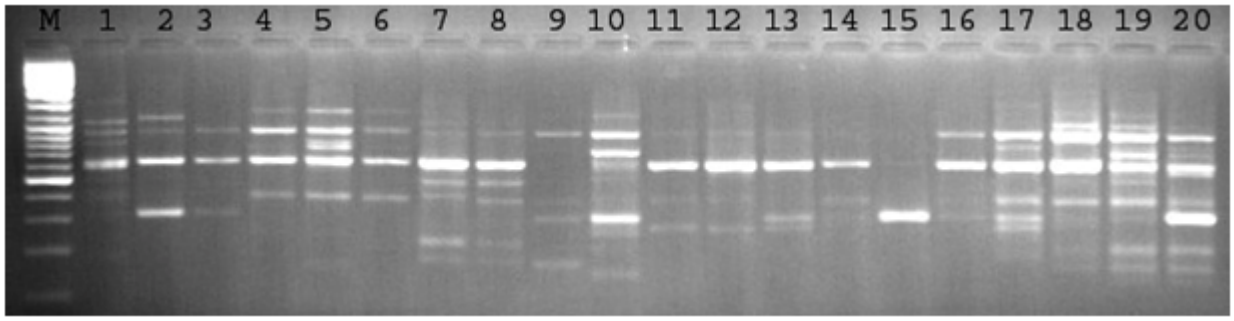

Figure 1. RAPD profile produced from the primer, OPD-12. (M = size marker, and 1-20 refer to the numbers in Table 1).

morphological characterization to various DNA-based markers such as restriction fragment length polymorphism (RFLPs), randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs) and simple sequence repeats (SSRs) (Crawford, 2000; Newton et al. 2002; Martinez et al. 2003; Fontaine et al. 2004; Murtaza, 2006; Nakazawa and Yahara, 2007). RAPD is a useful DNA-based method for assessment of genetic variation in species and genetic relationships between species due to its simplicity, speed and relatively low cost (Williams et al. 1990; Fischer et al. 2000). Due to advantages associated with RAPD, it has been widely used in plants to investigate genetic relationships among species and genetic diversity within species (Esselman et al. 2000; Rout et al. 2003; Castiglioni and De Campos, 2005; Choudhury et al. 2006; Fernandez et al. 2006; Sheng et al. 2006; Yuzbasioglu et al. 2006).

Some studies have been conducted to elucidate relationships among some *Phlomis* species by using morphological, anatomical, palynological and cytological traits (Huber-Morath, 1982; Hedge, 1986; Taylor, 1998). In *Phlomis*, based on these studies, two main sections have been recognized: *Phlomis* and *Phlomoides*, with the former section being subdivided into three subsections, *Gymnophlomis*, *Dendrophlomis* and *Oxyphlomis*. Species in section *Phlomis* have corolla with curved upper lip and trifid lower lip with large median and smaller lateral lobes whereas species in section *Phlomoides* have corolla with straight upper lip and trifid lower lip with sub equal lobes. Bracteoles in subsection *Dendrophlomis* are numerous, linear-subulate to lanceolate and ovate (Azizian and Moore, 1982). Most species in subsection *Oxyphlomis* have numerous rigid, linear-subulate bracteoles, which are sub equal (and sometimes longer) to the calyx. Bracteoles in subsection *Gymnophlomis* are weak, few to many or absent, linear-subulate, small (2-10 mm), free at the base and deciduous (Azizian and Moore, 1982). Natural hybrids between *Phlomis* species are frequently detected in local floras from several countries such as Spain, Iran and Turkey, which has lead to some confusion in differentiating species due to the mix of morphological characters observed in the hybrids and their parental species (Albaladejo et al. 2005). Nevertheless, little is known about genetic relationship among *Phlomis* species at the DNA level. To our knowledge, the study of Albaladejo et al. (2005) dealing with phylogenetic relationships among Iberian *Phlomis* species is the only one conducted in the genus so far. In the Flora of Turkey, the genus *Phlomis* is represented by 34 species, six varieties and ten natural hybrids (Huber-Morath, 1982). Of the 34 species, 4, 13 and 16 were placed under the subsections *Oxyphlomis*, *Gymnophlomis* and *Dendrophlomis*, respectively. Among the 13 species belonging to the subsection *Dendrophlomis*, 9 are endemic of Turkey, including *P. amanica*, *P. bourgaei*, *P. chimerae*, *P. grandiflora* var. *fimbrilligera*, *P. leucophracta, P. longifolia* var. *bailanica*, *P. lycia*, *P. monocephala* and *P. russeliana* (Huber-Morath, 1982). *P. amanica* and *P. grandiflora* var. *fimbrilligera* were considered as endangered and vulnerable, respectively but the others were found under lower risk (Ekim et al. 2000). Moreover, *P. chimerae* and *P. amanica* are known as local endemics, the former only grows in around Cirali, Antalya and the latter in only around Arsuz, Hatay. In Turkey, *Phlomis* species have been named as ballık otu, çalba, şalba and calba in public and also been used as tonic, carminative, appetizer and stimulants in folk medicine (Baytop, 1999; Gurbuz et al. 2003). To date, no study based on DNA markers has been made to investigate phylogenetic relationships among species of the subsection *Dendrophlomis* native to Turkey. The objective of the present study is to determine genetic relationships among species of the subsection *Dendrophlomis* by using RAPD markers.

MATERIALS AND METHODS

Plant material and DNA isolation

Locations, altitudes and collection periods of the plant materials used in this study are given in Table 1. Voucher specimens of samples were kept at the Herbarium of Erciyes University, Faculty of Science and Letters, Kayseri, Turkey. For each taxon, dried leaves of single plants from the herbarium material were ground to powder in porcelain mortars with liquid nitrogen. Genomic DNA was extracted

from 0.150 g powder using a modification of the method of Rogers and Bendich (1988). The powder was transferred into 0.6 ml of CTAB extraction buffer within 1.5 ml tubes containing 100 mM Tris-HCl, 20 mM EDTA, 4 M NaCl, 7% CTAB (Rogers and Bendich, 1988). To this, 2% PVP (polyvinilpyrolidone), 1% 2-mercaptoethanol, 2% ascorbic acid and 1% sodium bisulfate were added and incubated at 65°C in a water bath for 30 min. The mixture was treated with 0.5 ml chloroform / isoamyl alcohol (24:1) and shaked gently by inverting the tubes 50 times. The tubes were put on ice for 20 min to chill out polysaccharides and centrifuged at 9000 rpm for 9 min. Once the supernatant was transferred into new tubes, the 24 chloroform:1 isoamyl alcohol, chilling and centrifugation steps were repeated. After centrifugation, 30 mg / ml RNAase was added and the tubes were incubated in a water bath at 37°C for 1 hr. Then 0.5 ml of isopropanol was added to precipitate DNA overnight at -20°C. The precipitate was centrifuged to pellet DNA. The pellet was washed with 70% and 95% ethanol, air dried, redissolved in TE buffer (50 mM Tris-HCl, 10 mM EDTA) and stored at 4°C.

RAPD procedure

A hundred and twenty five ten mer RAPD primers were obtained from Operon Technologies, (Alameda, California, USA) and tested for amplification in a preliminary study. The primers OPA-4, OPA-10, OPA-17, OPA-18, OPA-20, OPB-17, OPB-18, OPB-20, OPD-6, OPD-8, OPD-10, OPD-12, OPD-18 and OPD-19 were then selected to analyze the genetic variability of the samples because they produced distinct and reproducible bands. DNA amplifications were carried out in 25 µl of final volume containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 4 µM RAPD primer, 150 µM dNTPs, 2 mM $MgCl_2$, 100 ng DNA template and 2 U *Taq* DNA polymerase (Fermentas). The mixture was placed in a T-Gradient (Techne) thermocycler. The PCR profile consisted of an initial step of 2 min at 94°C, followed by 44 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, with a final extension step of 5 min at 72°C.

Amplification products were separated by electrophoresis on 1.6% agarose gels in a 1X TBE buffer. Gels were stained with ethidium bromide and photographed over UV light. Molecular weights were estimated by reference to a Gene Ruler DNA ladder (SMO331, Fermentas).

Data analysis

RAPD bands were scored in a binary manner as either present (1) or absent (0) and entered into a binary data matrix. Only RAPD bands that could be unambiguously scored were included in the analysis. A pairwise similarity matrix was constructed using the simple matching coefficient (SM) (Sokal and Michener, 1958) and NTSYS-pc (Version 1.7, Rohlf, 1992). SM = m/n, where m = shared present fragments (11) + shared absent fragments (00) and n = the total of the obtained fragments. A pairwise genetic distance matrix was produced by subtracting the similarity coefficients from 1. A dendrogram based on the distance matrix was produced using the unweighted pair-group method with arithmetic averages (UPGMA) under the NJ subprogram in the PHYLIP software package version 3.6a3 (Felsenstein, 2002).

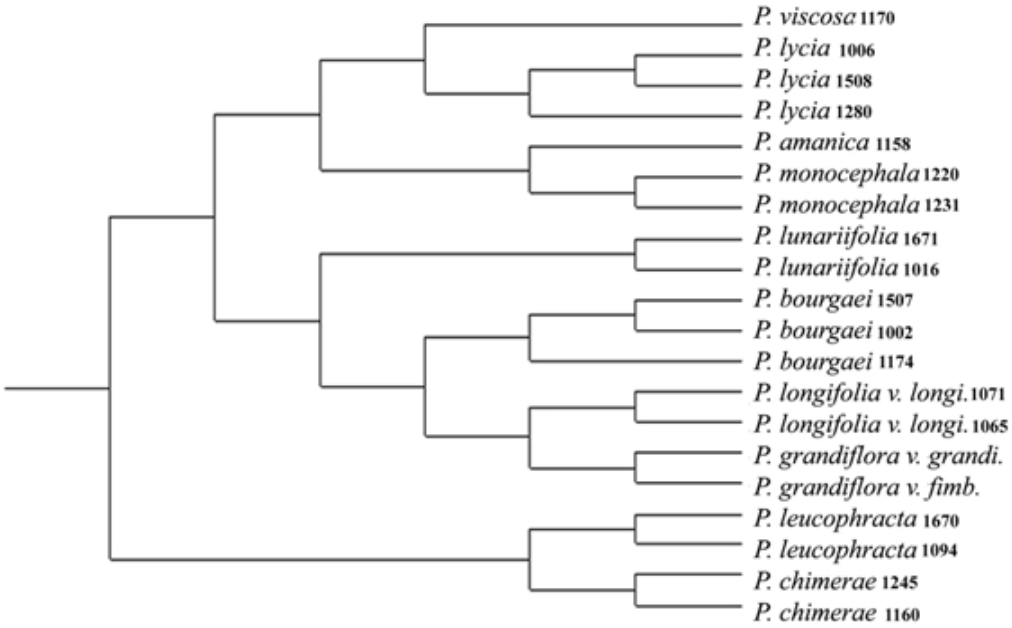

Figure 2. UPGMA phylogram of 11 *Phlomis* taxa in the subsection *Dendrophlomis* based on genetic distances.

RESULTS AND DISCUSSION

RAPD markers have been widely used in the analysis of genetic relationships and genetic diversity in a number of plant taxa because of its simplicity, speed and relatively low cost compared to other DNA-based markers (Esselman et al. 2000; Rout et al. 2003; Sheng et al. 2006; Yuzbasioglu et al. 2006). Nevertheless, dominant inheritance and repeatability of the bands have been the two main limitations with the use of RAPD technique in the assessment of genetic diversity and genetic relationships (Fischer, 2000). However, the limitation relating to the dominant nature of RAPD bands can be compensated to some degree by examining a large number (more than 30) of RAPD loci (Gillies et al. 1999). Reproducibility of RAPD bands can also be improved by isolating pure DNA, selecting primers that have clear amplification patterns and maintaining consistent reaction conditions during amplification (Weising et al. 1995). DNA isolation was optimized in this study by taking these considerations into account and pure DNA was obtained by adding 2% polyvinilpyrolidone (PVP), 2% 2-mercaptoethanol, 2% ascorbic acid, 4 M NaCl and 7% CTAB to the extraction buffer of Rogers and Bendich (1988) because *Phlomis* species were rich in secondary metabolites (Takeda et al. 1999; Calis et al. 2004; Calis and Kirmizibekmez, 2004; Celik et al. 2005) that caused the changes in the DNA colour (from white to yellow, red, brown and black) and weak DNA amplification in PCR (Weising et al. 1995; Yuzbasioglu et al. 2006). The concentrations of magnesium chloride, primer, template DNA, dNTPs and Taq DNA polymerase were optimized to obtain repeatable banding pattern and maintained constant during amplification. Lastly, of the 125 RAPD primers screened for their amplification capacity, fourteen primers produced clear and reproducible RAPD bands across all the species and were chosen and used for the first time to measure genetic relationships among species of the subsection *Dendrophlomis*. These 14 selected primers generated 85 RAPD bands that ranged in size from 200 to 1200 base pairs. Each random primer amplified between 3 and 9 RAPD bands with an average of 6 bands per primer. Of the total bands, 88% (75) were polymorphic among the 20 individuals. Figure 1 illustrates an example of a RAPD profile produced by primer OPD-12.

Genetic relationships among the eleven *Phlomis* taxa are indicated in the UPGMA tree (Figure 2). The topology of the UPGMA tree shows the expected groupings with relation to the taxonomic structure of the taxa used in this study. Based on the UPGMA tree, 11 taxa were divided into two major groups. The first group comprised 9 taxa that were separated into two subgroups. The first subgroup consisted of *P. viscosa*, *P. lycia*, *P. amanica* and *P. monocephala* while the second comprised *P. lunariifolia*, *P. bourgaei*, *P. longifolia* var. *longifolia*, *P. grandiflora* var. *grandiflora* and *P. grandiflora* var. *fimbrilligera*. Apart from *P. viscosa* within the first sub-group, calyx teeth of all are equal or shorter than 2 mm. Within the second sub-group, *P. lunariifolia* makes a separate group with others and it differs from them having calyx with glabrose base while the others have entirely stellate-hairy calyx. Bracteols and calyx are densely long hispid-viscid in *P. bourgaei* but eglandular in *P. longifolia* var. *longifolia*. On the other hand, bracteols in *P. grandiflora* var. *grandiflora* and *fimbrilligera* are glandular-dotted but not hispid-viscid. The second group was represented by two species, *i.e. P. leucophracta* and *P. chimerae*. These two species are similar to each other in terms of their cauline and floral leaves shape (ovate) and calyx teeth length (between 3 and 10 mm). However, *P. leucophracta* differs from *P. chimerae* and from all other species in the tree having galea with brown colour (others yellow). On the other hand, *P. chimerae* also differs from *P. leucophracta* and from all other species in the tree by having clearly branched bracteoles and basal leaves with orbicular shape. The lowest genetic distance (0.133) was observed between *P. amanica* and *P. monocephala* (Table 2) and this finding is in agreement with the result of a morphological study by Taylor (1998), which indicates that *P. amanica* is similar to *P. monocephala* in terms of its habit, leaf shape and hairiness. The hairs of the calyces and bracteoles are only stellate in *P. amanica*, but in *P. monocephala* are long, stellate and form a very dense covering (Taylor, 1998). The highest genetic differentiation (0.494) was found between *P. lunariifolia* and *P. chimerae* in the present study (Table 2) and these two indicate differences in terms of shape, apex and margin of basal, cauline and floral leaves but similarities in terms of base of cauline and floral leaves. *P. lunariifolia* has linear-lanceolate to oblong lanceolate basal leaves which are acute to broadly acute at the apex and cuneate at the base and linear-lanceolate to oblong-lanceolate floral leaves which are acute at the apex and cuneate at the base. On the other hand, *P. chimerae* has orbicular to broadly ovate basal leaves which are obtuse at the apex and obtuse to truncate at the base and ovate floral leaves which are obtuse at the apex and cuneate at the base. The study of Albaladejo et al. (2005) dealing with the genetic relationships among Iberian *Phlomis* species based on DNA markers has been the only one so far. By using three non-coding chloroplast DNA regions and nuclear ribosomal internal transcribed spacer (ITS), they investigated the genetic relationships among *P. crinita* subsp. *crinita* (8 accessions), *P. crinita* subsp. *malacitana* (14 accessions), *P. crinita* subsp. *mauritanica* (3 accessions), *P. lychnitis* (23 accessions) and *P. purpurea* as the outgroup (1 accession) and found contrasting results derived from the nuclear and plastid markers. While the dendrogram produced from the nuclear ITS data revealed two lineages (*crinita* and *lychnitis*), the grouping based on the analysis of chloroplast sequences was geographic rather than taxonomic. In the present study, the accessions grouped into their distinct species clusters and species were also clearly differentiated from each other in the dendrogram.

Classification of several *Phlomis* species based on morphological characters has sometimes been difficult

because they hybridize easily in nature leading to the formation of hybrid plants with a mosaic of morphological characters between the parental phenotypes (Albaladejo et al. 2005; Yuzbasioglu et al. 2008). Hybrization between *Phlomis* species has been indicated by using morphological data (Albaladejo et al. 2004), isozymes (Aparicio et al. 2000) and DNA markers such as ITS (Albaladejo et al. 2005) and RAPDs (Yuzbasioglu et al. 2008). Albaladejo et al. (2004) studied natural hybridization between *P. lychnitis* and *P. crinita* subsp. *malacitana* in Andalusia (south of the Iberian Peninsula) and estimated a hybridization rate of 21.6% within the *Phlomis* populations by using morphometric analysis. Recently, Albaladejo and Aparicio (2007) investigated the population genetic structure and hybridization rate of this complex by using allozymes and found an average hybridization rate of 32%, and concluded that the discrepancy between morphological and genetic hybridization rates could be due to the high occurrence of slightly introgressed individuals having morphology indistinguishable from that of the parental types. In parallel to the findings of Albaladejo et al. (2004) and Albaladejo and Aparicio (2007) in Spain, hybridization between *Phlomis* species has also been detected frequently in Turkey and 12 hybrids were registered in the flora of Turkey (Huber-Morath, 1982; Dadandi, 2003; Dadandi and Duman, 2003). In this study species-specific bands were found for *P. lycia*, *P. leucophracta*, *P. lunariifolia*, *P. bourgaei*, *P. chimerae* and *P. longifolia* var *longifolia* (Table 3). Primers OPD-12, OPA-109 and OPB-18 produced bands of 1030, 450 and 1030 base pairs of size, all of which were absent in *P. longifolia* var. *longifolia, P. monocephala and P. grandiflora* var. *grandiflora* respectively, but present in the other sampled taxa. Some bands (300 and 700 bp from OPA-4; 600 and 700 bp from OPB-17; 300 bp from OPD-8 and 200 bp from OPA-10) were observed in all of 20 samples that could be specific to the subsection *Dendrophlomis*. After checking more individuals within each species, species specific bands can be used for detecting instances of natural interspecific gene introgression between *Phlomis* species, which can provide contribution to the classification of *Phlomis* species made based on morphological characters. Among 13 *Phlomis* species placed under the subsection *Dendrophlomis* in the flora of Turkey, *P. amanica*, *P. chimerae*, *P. bourgaei*, *P. leucophracta*, *P. lycia*, *P. russeliana*, *P. grandiflora*, *P. longifolia* and *P. monocephala* were reported as endemic to Turkey and the species-specific bands observed in some of these endemic species including *P. lycia*, *P. leucophracta*, *P. bourgaei* and *P. chimerae* could also be used to identify *Phlomis* species in danger for preservation purposes. In conclusion, these results demonstrate the utility of using RAPD markers to characterize interspecific relationships and identify unique bands in *Phlomis* species.

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APPENDIX
TABLES

Table 1. Locations, altitudes and collection periods of the plant materials used in RAPD analysis.

Numbers	Code	Species	Location	Altitude (m)	Date
1	1170	*P. viscosa*	B6 Adana; Feke-Adana road 2 km	930	4.vii.1998
2	1671	*P. lunariifolia*	C4 Icel; Aydincik-Gulnar 13 km 36°13'576''N, 33°22'017''E	520	3.vii.2003
3	1016	*P. lunariifolia*	C3 Antalya; Between Manavgat-Alanya, 10 Km North of Kastel.	700	31.v.1997
4	1508	*P. lycia*	C2 Mugla; Marmaris, Bozburun, around Serce limanı 36°34'903''N, 28°03'052''E	71	6.v.2001
5	1280	*P. lycia*	C2 Mugla; Kale-Mugla road 52 km	950-1000	10.vi.1999
6	1006	*P. lycia*	C3 Antalya; Termessos National Park	700-750	2.vi.1997
7	1670	*P. leucophracta*	C4 Icel; Aydincik-Gulnar 13 km 36°13'576''N, 33°22'017''E	520	3.vii.2003
8	1094	*P. leucophracta*	C4 Icel; Ermenek-Mut road 50 km	850-900	24.vi.1998
9	1245	*P. chimerae*	C3 Antalya; Antalya-Kemer road 12 km	50	8.vi.1999
10	1160	*P. chimerae*	C3 Antalya; Kemer, Kesme Bogazi	150	27.vi.1998
11	1002	*P. bourgaei*	C3 Antalya; Termessos National Park	700-750	2.vi.1997
12	1174	*P. bourgaei*	C2 Antalya; Gursu village to Elmalı 6 km	1100	27.vi.1998
13	1507	*P. bourgaei*	C2 Mugla; Bozburun-Marmaris road 10 km, 36°41'773''N, 28°05'323''E	221	5.v.2001
14	1071	*P.longifolia* var. *longifolia*	C6 Hatay; Samandag-Yayladag seaside road, between Coguntu-Gozene villages	380	14.vi.1998
15	1065	*P.longifolia* var. *longifolia*	C6 Hatay; between Gedik and Atik	800	13.vi.1998
16	1225	*P. grandiflora* var. *fimbrilligera*	C4 Antalya; between Anamur-Gazipasa, around Kargıdran stream	20	7.vi.1999
17	1166	*P. grandiflora* var. *grandiflora*	C2 Antalya; Oluk Yaylasi, between Gomuce and Kuruova villages. Kas-Elmali road.	1700	28.vi.1998
18	1158	*P. amanica*	C6 Hatay; Arsuz (Ulucinar), Haymaseki village, around Aktepe	250-300	9.viii.1998
19	1220	*P. monocephala*	C4 Icel; Gulnar-Silifke road 47 km	680	7.vi.1999
20	1231	*P. monocephala*	C4 Icel; Gulnar-Silifke road 47 km	680	7.vi.1999

Table 2. Genetic distances among 11 *Phlomis* taxa in the subsection *Dendrophlomis*. Codes refer to Table 1.

Codes	1170	1671	1016	1508	1280	1006	1670	1094	1245	1160	1002	1174	1507	1071	1065	1225	1166	1158	1220	1231
1170	-																			
1671	0.253	-																		
1016	0.337	0.133	-																	
1508	0.205	0.362	0.398	-																
1280	0.193	0.398	0.434	0.036	-															
1006	0.193	0.374	0.386	0.060	0.048	-														
1670	0.362	0.301	0.386	0.325	0.313	0.313	-													
1094	0.349	0.289	0.398	0.337	0.325	0.325	0.012	-												
1245	0.422	0.386	0.446	0.362	0.398	0.398	0.325	0.313	-											
1160	0.374	0.362	0.494	0.362	0.325	0.349	0.301	0.289	0.145	-										
1002	0.482	0.374	0.362	0.398	0.410	0.410	0.386	0.398	0.398	0.374	-									
1174	0.482	0.374	0.386	0.398	0.434	0.434	0.362	0.374	0.398	0.422	0.121	-								
1507	0.362	0.229	0.289	0.301	0.337	0.313	0.337	0.349	0.349	0.325	0.169	0.169	-							
1071	0.422	0.337	0.398	0.434	0.470	0.446	0.446	0.458	0.410	0.458	0.277	0.301	0.277	-						
1065	0.349	0.313	0.398	0.386	0.398	0.374	0.422	0.434	0.458	0.434	0.349	0.374	0.301	0.121	-					
1225	0.349	0.289	0.374	0.386	0.398	0.374	0.422	0.434	0.434	0.434	0.325	0.398	0.277	0.289	0.241	-				
1166	0.337	0.374	0.434	0.325	0.313	0.313	0.386	0.398	0.398	0.374	0.289	0.386	0.313	0.349	0.277	0.157	-			
1158	0.193	0.301	0.386	0.277	0.265	0.265	0.337	0.325	0.446	0.398	0.386	0.434	0.313	0.349	0.277	0.301	0.241	-		
1220	0.277	0.313	0.374	0.362	0.325	0.349	0.349	0.362	0.482	0.386	0.398	0.422	0.325	0.386	0.289	0.362	0.301	0.133	-	
1231	0.289	0.301	0.362	0.374	0.337	0.362	0.337	0.349	0.470	0.374	0.386	0.410	0.313	0.374	0.277	0.349	0.289	0.145	0.012	-

Table 3. Specific bands observed in *Phlomis* species.

***Phlomis* species**	**Primer**	**Band Size (base pairs)**
P. lycia	OPD-10	250
P. leucophracta	OPD-12	450
	OPD-6	600
P. lunariifolia	OPD-12	300
P. bougaei	OPD-12	300
P. chimerae	OPA-17	600
	OPB-18	250
	OPB-20	700
P. longifolia var. *longifolia*	OPB-20	800