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Internal transcribed spacer (ITS) polymorphism in the wild *Primula* (Primulaceae) taxa of Turkey

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Abstract: Twenty-three populations belonging to 10 wild *Primula* L. (Primulaceae) taxa of Turkey, some of which are morphologically quite similar, were investigated based on nrDNA ITS regions. The plant materials were collected from different geographical areas of Anatolia in vegetation periods of 2005 and 2006. Total genomic DNAs were isolated from the healthy leaves of each population. The entire ITS regions of the populations were amplified by universal primers with the aid of a polymerase chain reaction (PCR), and then the PCR products were sequenced. Neighbour-Joining (NJ) and Maximum Parsimony (MP) trees were constructed in order to identify the relationships among *Primula* taxa. According to ITS data, 724 characters were determined among the aligned sequences of the populations and the divergence values were found to be between 0.0% and 20.9%. ITS sequences from 23 specimens provided a number of variable and sufficient characters to explore the relationships. As a result, it was determined that the dendrograms obtained by NJ and MP analysis are concordant with the traditional taxonomical order at subgeneric level.

Key words: Turkey, ITS PCR, nrDNA, phylogeny, *Primula*

Türkiye'de doğal olarak yayılış gösteren *Primula* (Primulaceae) taksonlarının ITS polimorfizmi

Özet: Bu çalışmada, morfolojik olarak birbirine oldukça benzeyen 10 doğal *Primula* L. (Primulaceae) taksonuna ait 23 populasyon nrDNA ITS bölgeleri bakımından incelenmiştir. Bitki örnekleri 2005-2006 vejetasyon döneminde Anadolu'nun farklı coğrafik alanlarından toplanmıştır. Her populasyonun genomik DNA'ları sağlıklı yapraklardan elde edilmiştir. ITS bölgeleri evrensel oligonükleotidler kullanılarak PCR yardımıyla elde edilmiş ve baz dizin analizleri yapılmıştır. *Primula* taksonları arasındaki filogenetik ilişkiyi belirlemek için Neighbour-Joining (NJ) ve Maksimum Parsimoni (MP) ağaçları çizilmiştir. Populasyonlara ait baz dizin verilerinin hizalanmasıyla 724 karakterlik bir veri matriksi elde edilmiş ve divergens değerlerinin de % 0,0-20,9 arasında değiştiği bulunmuştur. ITS baz dizinleri 23 örnekte akrabalık ilişkilerini açıklamada yeterli veri sağlamaktadır. Sonuç olarak, NJ ve MP analizlerinden elde edilen dendrogramların altcins seviyesinde geleneksel taksonomik verilerle uyumlu olduğu bulunmuştur.

Anahtar sözcükler: Türkiye, filogeni, ITS PCR, nrDNA, *Primula*

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Introduction

The genus *Primula* L. (Primulaceae) includes about 400 species belonging to 6 subgenera and 37 sections (Richards, 1993). It is widely distributed outside of the Asian highlands and in the high altitudes of North America, Europe, and the eastern Sino-Himalayan region, considered the primary centre of diversity for this genus (Hu & Kelso, 1996). Many *Primula* species are also widely cultivated throughout the world as ornamental plants (Mizuhiro et al., 2001). Plant collectors have supplied enough materials for horticultural and scientific purposes and have played an important role in recognising the genus (Hu & Kelso, 1996; Zhang & Kadereit, 2004) and this genus has been recently studied by many taxonomists, ecologists, geneticists, and gardeners (Mast et al., 2001), but it is still not well known in the wild. Heterostyly, recognised as a complex reproductive syndrome with significant ecological and evolutionary implications, is a well-known process for the genus *Primula* (Barrett et al., 2000). Heterostyly has played an important role in systematic treatments of the genus *Primula* at the generic and infrageneric levels (Richards, 1993). The suggested base chromosome number is x = 11, but it varies from x = 8 to x = 12 (Wendelbo, 1961; Mast et al., 2001; Abou-el-Enain, 2005). Systematic investigations of this genus have also shown that the chromosome numbers are an important character in its classification (Wendelbo, 1961; Richards, 1993).

A few characters used in the traditional systematic studies for the genus *Primula*, besides heterostyly and homostyly, are the chromosome base number, leaf vernation, and pollen exine morphology (Mast et al., 2001). Despite their putative importance in elucidating relationships among *Primula* taxa, the characters listed here do not align consistently with one another (Mast et al., 2001), and therefore, molecular sampling might provide an independent systematic value (Conti et al., 2000). The internal transcribed spacer (ITS) region and many other molecular markers have been used in numerous plant systematic studies at the family, generic, and specific levels (Baldwin et al., 1995; Ogundipe & Chase, 2008). This region is often used in closely related species for phylogenetic analyses in several plant genera (Anderberg & Stahl, 1995) and also in the genus *Primula* (Conti et al., 2000; Kovtonyuk & Goncharov, 2009). Conti et al. (2000) used nuclear DNA sequences to reconstruct the infrageneric phylogeny of *Primula*, while Kovtonyuk and Goncharov (2009) analysed the sequences of nuclear DNA and confirmed Richards' (2003) sectional rank proposal. Mast et al. (2001) examined the specific chloroplast DNA regions (the *atpB*, *ndhF*, and *rblC* genes) and stressed that subg. *Aleuritia* needs a revision of its delimitation. Analysis of the ITS sequences provided new insights into the phylogeny of *Primula* (Conti et al., 2000; Martins et al., 2003). According to Wendelbo (1961) and Richards (1993), involute leaf vernation is an ancestral trait, based on phenetic analysis, but Conti et al. (2000) stress that the revolute traits are ancestral, based on ITS data. Therefore, we thought that performing molecular analyses based on nuclear rDNA could provide additional useful information about the *Primula* taxa of Turkey.

According to Lamond (1978), the genus *Primula* is represented by only 8 species in Turkey. Of these, 2 members, *P. davisii* W.W.Sm. and *P. longipes* Freyn & Sint, are endemic to Turkey, and they are assessed as vulnerable (VU) and endangered (EN), respectively (Ekim et al., 2000). Turkish representatives of *Primula* have been the subject of morphological, anatomical (Beyazoğlu, 1989), cytotaxonomical (Hayırlıoğlu-Ayaz & İnceer, 2003), and palynological (Pınar et al., 2005) studies that have improved our understanding of the systematics of the genus, but the *Primula* taxa of Turkey have not been evaluated by analysis of ITS region, except for *P. davisii*, which was used as an outgroup to investigate the phylogeny and biogeography of *Dionysia* Fenzl (Trift et al., 2004). Thus, the main purpose of the current research was to determine the ITS polymorphism among the taxa of *Primula* distributed naturally in Turkey and to elucidate their relationships.

Materials and methods

Plant Material

All plant materials used in this research were collected from various regions of Anatolia during field work in 2005 and 2006. For each population, information related to the collection region and the subgenera are shown in Table 1. The samples were

Table 1. Locality information.

Pop. no.	Subg.	Taxa	Locality
1		*P. vulgaris* Huds. subsp. *vulgaris*	A7 Trabzon: Beşikdüzü, Yeni Camii village, 01.v.2005, 970 m, *Uzuner* P11, KTUB
2		*P. vulgaris* Huds. subsp. *vulgaris*	A7 Trabzon: Beşikdüzü, Yeni Camii village, 01.v.2005, 970 m, *Uzuner* P12, KTUB
3		*P. vulgaris* Huds. subsp. *sibthorpii* (Hoffmanns.) W.W.Sm. & Forrest	A7 Trabzon: Beşikdüzü, Yeni Camii village, 01.v.2005, 970 m, *Uzuner* P13, KTUB
4		*P. vulgaris* Huds. subsp. *vulgaris*	A4 Kastamonu: Bozkurt, 11.v.2005, 1200 m, *Uzuner* P15, KTUB
5		*P. vulgaris* Huds. subsp. *vulgaris*	A4 Kastamonu: Bozkurt, 11.v.2005, 1200 m, *Uzuner* P16, KTUB
6		*P. vulgaris* Huds. subsp. *sibthorpii* (Hoffmanns.) W.W.Sm. & Forrest	A4 Kastamonu: Bozkurt, 11.v.2005, 1200 m, *Uzuner* P17, KTUB
7	*Primula* L.	*P. megaseifolia* Boiss. & Bal.	A7 Trabzon: Beşikdüzü, Şahmelik village, 10.iv.2005, 450 m, *Uzuner* P6, KTUB
8	*Primula* L.	*P. megaseifolia* Boiss. & Bal.	A8 Trabzon: Araklı, Sularbaşı village, 14.iv.2005, 1120 m, *Uzuner* P7, KTUB
9	*Primula* L.	*P. elatior* (L.) Hill subsp. *meyerii* (Rupr.) Valentine & Lamond	A8 Rize: İkizdere, Ovit Mountain, 24.vii.2005, 2850 m, *Uzuner* P26, KTUB
10		*P. elatior* (L.) Hill subsp. *meyerii* (Rupr.) Valentine & Lamond	A7 Trabzon: Çaykara, Demirkapı village, 28.v.2005, 2920 m, *Uzuner* P29, KTUB
11		*P. veris* L. subsp. *columnae* (Ten.) Lüdi	A7 Trabzon: Çaykara, Ablaryas Plateau, 01.vi.2005, 2052 m, *Uzuner* P18, KTUB
12		*P. veris* L. subsp. *columnae* (Ten.) Lüdi	A7 Trabzon: Maçka Sümela Monastery, 24.v.2006, *Coşkunçelebi* 700, KTUB
13		*P. veris* L. subsp. *macrocalyx* (Bunge) Lüdi	A9 Artvin: Karagöl, 08.vi.2005, 1950 m, *Uzuner* P22, KTUB
14		*P. longipes* Freyn & Sint.	A8 Rize: İkizdere, Ovit Mountain, 24.vii.2005, 2850 m, *Uzuner* P25, KTUB
15		*P. longipes* Freyn & Sint.	A7 Trabzon: Çaykara, Demirkapı village, 28.v.2005, 3067 m, *Uzuner* P27, KTUB
16	*Aleuritia* (Duby) Wendelbo	*P. auriculata* Lam.	A7 Gümüşhane: Kadırga village, 13.vi.2005, 2150 m, *Uzuner* P20, KTUB
17	*Aleuritia* (Duby) Wendelbo	*P. auriculata* Lam.	A4 Kastamonu: Bozkurt, 1200 m 11.vi.2005, *Coşkunçelebi* 701, KTUB
18	*Aleuritia* (Duby) Wendelbo	*P. auriculata* Lam.	A8 Erzurum: İspir, Moryayla, 21.x.2005, 2450 m, *Uzuner* P30, KTUB
19	*Aleuritia* (Duby) Wendelbo	*P. auriculata* Lam.	A8 Kop Dağı, 2290 m, 18.v.2006, *Coşkunçelebi* 699, KTUB
20	*Aleuritia* (Duby) Wendelbo	*P. algida* Adams	A9 Ardahan: Posof, Gönülaçan, 09.vi.2005, 2130 m, *Uzuner* P23, KTUB
21	*Aleuritia* (Duby) Wendelbo	*P. algida* Adams	A8 Rize: İkizdere, Ovit Mountain, 24.vii.2005, 2850 m, *Uzuner* P24, KTUB
22	*Aleuritia* (Duby) Wendelbo	*P. algida* Adams	A7 Trabzon: Çaykara, Demirkapı village, 28.v.2005, 2920 m, *Uzuner* P28, KTUB
23	*Sphondylia* (Duby) Rupr.	*P. davisii* W.W.Sm	C10 Hakkari: Çukurca, Stream path fork, 7.3 km, limy rock splits, 1200 m, 10.vi.2006, *MF* 10124, KTUB

then identified according to traditional methods, mainly by using *Flora of Turkey* (Lamond, 1978) and *Flora Europaea* (Valentine & Kress, 1972), and stored as herbarium specimens in the herbarium of the Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University.

DNA extraction

Total genomic DNAs were extracted from silica-dried leaves or herbarium materials following the modified CTAB extraction procedure of Doyle and Doyle (1987). The gDNAs were resuspended in TE (tris HCl-EDTA) and stored at +4 °C. The isolated genomic DNAs were checked in a 1% agarose-TAE (tris, acetate, and EDTA) gel containing 0.5 µg/L of ethidium bromide and examined under UV light.

PCR amplification

The entire ITS regions (ITS1, 5.8S, and ITS2) were amplified using a Biometra Personal Thermal Cycler.

The PCR reactions were performed using universal ITS4 (5′- TCCTCCGCTTATTGATATGC- 3′) and ITS5 (5′- GGAAGTAAAAGTCGTAACAAGG -3′) primers, designed by White et al. (1990). The amplification process was performed in 50 µL of PCR reaction volume, containing 10 mM of Taq polymerase reaction buffer, 2 mM of magnesium chloride ($MgCl_2$), 200 mM of dNTP, 1 µM (ITS4 and ITS5) each of the primer, 1-2 units of Taq DNA polymerase, 2-6 ng (1 µL of 2-6 ng/µL) of total template DNA, and 14 µL of ddH_2O. Reaction mixtures were sealed with 1 or 2 drops of mineral oil to prevent evaporation during thermal cycling. Thermal cycling amplification was performed with an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of strand denaturation at 94 °C for 1 min, annealing at 50 °C for 45 s, and primer extension at 72 °C for 1 min, and a final elongation at 72 °C for 7 min.

Sequence analysis

PCR product purification and DNA sequence analysis were performed by Macrogen Inc. (Seoul, Korea). The sequencing process was conducted with BigDyeTM terminator cycling protocols (Applied Biosystems Inc., Foster City, CA, USA). PCR products were purified using ethanol precipitation and run on an Automatic Sequencer (ABI 3730x1) by a contract laboratory. Sequencing of the 5' end of the ITS region was carried out using the primer ITS4. Sequences with ambiguous sites were resequenced from the 3' end with the primer ITS5. The sequence data were submitted to GenBank under the accession number of EU643642-EU643664 (Table 1).

Data analysis

The nucleotide sequences were automatically aligned using BioEdit v.7.0 software (Hall, 1999). Neighbour-Joining (NJ) and Maximum Parsimony (MP) trees were built using the Molecular Evolutionary Genetics Analysis (MEGA v.3.1) program (Kumar et al., 2004). DNA sequences were analysed based on Kimura's 2-parameter model (K2P). All characters were unordered and equally weighted, and gaps were treated as missing data. The topology of the consensus tree was constructed and evaluated with 1000 bootstrap replications (Felsenstein, 1985) for both the MP and NJ (Saitou & Nei, 1987) analysis. For the phylogenetic analyses of the ITS regions, *Lysimachia nemorum* L. (GenBank: AY855153) and *Anagallis serpyllifolia* Dumort. (GenBank: AY855154) were selected as outgroups.

Results

The total lengths of the ITS (ITS1, 5.8S, and ITS2) regions of the examined populations ranged from 695 to 714 bp. The shortest ITS length among the examined taxa was identified in *P. algida* (Pop. No. 21), collected from A8 Rize, İkizdere, Ovit Mountain. *P. veris* subsp. *macrocalyx*, collected from A9 Artvin, was identified as the population with the longest ITS length, at 714 bp (Table 1). Alignments of entire ITS sequences resulted in 724 characters, except for the outgroup. The entire ITS region contained 153 (21.1%) parsimony informative sites (Table 2), 252 (34.8%) variable sites, and 466 (64.3%) conserved sites.

The NJ tree obtained from the analysis of the ITS regions provided many useful data (Figure 1). All of the investigated taxa settled explicitly into 3 clusters corresponding to subgenera of *Primula*, namely subg. *Sphondylia* (Clade-I), *Aleuritia* (Clade-II), and *Primula* (Clade-III). Clade-I included only one taxon, *P. davisii*, which is the single species of subg. *Sphondylia* in Turkey. Clade-II, with a bootstrap value of 63%, consisted of the populations of *P. algida*, *P. auriculata*, and *P. longipes*, which belong to subg. *Aleuritia*. Among the representatives of subg. *Aleuritia*, *P. auriculata* and *P. algida* were linked to each other and formed a sister group with *P. longipes* (Figure 1). Clade-III contained the taxa *P. vulgaris*, *P. veris*, *P. megaseifolia,* and *P. elatior* of subg. *Primula*, with a bootstrap value of 98% (Figure 1), and this clade also divided into 2 distinct subgroups. The first subgroup was composed of *P. veris* and the second, which was split again into 2 small clusters, was composed of the rest of the members of subg. *Primula*. While *P. megaseifolia* and *P. elatior* were linked to a low bootstrap value of 51%, the taxa of *P. vulgaris* were linked to each other with bootstrap values of 92% in the second subgroup.

Explanatory information related to the parsimony informative sites inferred by MEGA software is given in Table 2. The analysis of the entire ITS sequence of *P. veris* exhibited notable base alterations from the rest

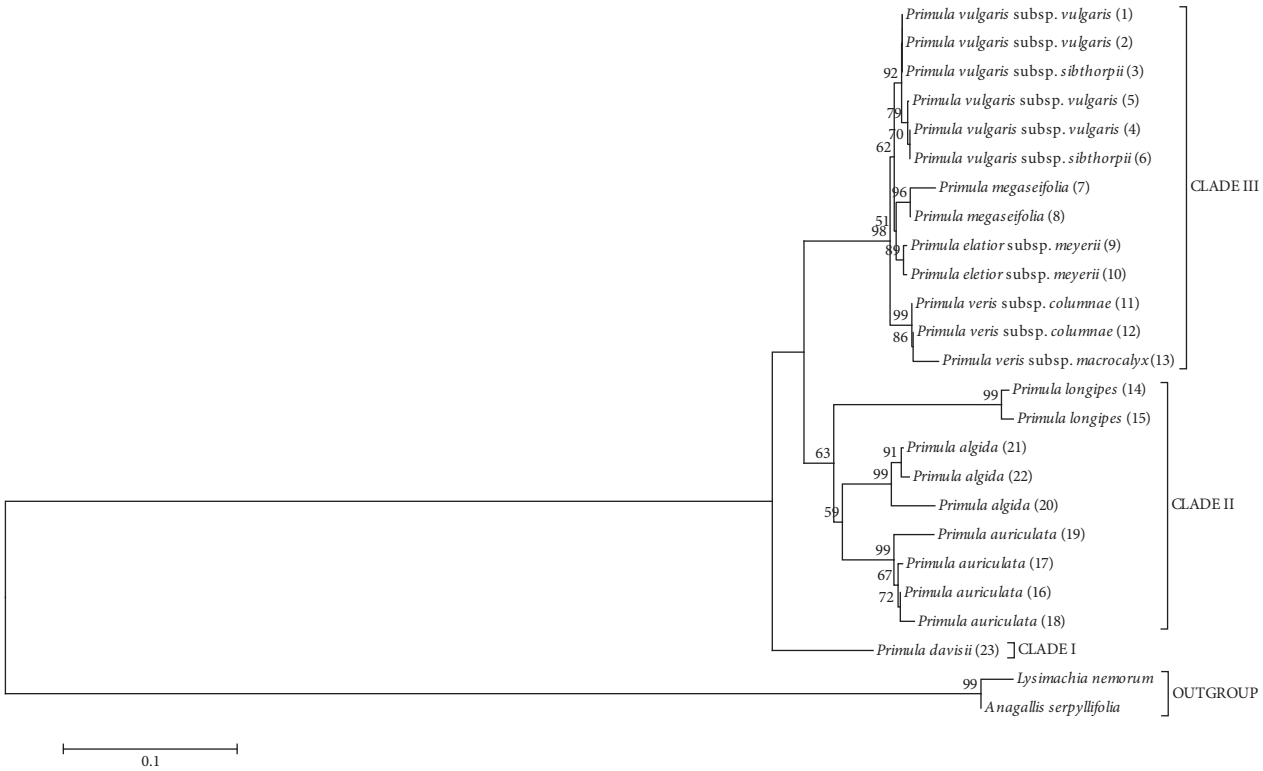

Figure 1. The dendrogram showing the genetic relationships of the Turkish *Primula*. *Primula* species recovered from ITS sequences, evaluated by the neighbour-joining method, with *Lysimachia nemorum* and *Anagallis serpyllifolia* as outgroups. Values above the branch indicate bootstrap values supporting the respective cluster. Values higher than 50% are displayed.

of the taxa of subg. *Primula* in the parsimonic nucleotide sites at the positions of 143, 159, 300, 453, 567, 577, 620, 631, and 672. The examined subspecies of *P. veris* are distinguished from each other by position 659 of their ITS sequences (see Table 2). In addition, the 2 populations of *P. megaseifolia* included different nucleotides at 91, 228, 456, 474, 512, 553, and 600 when compared to the rest of the taxa of subg. *Primula*, and they were also distinguished from each other at the nucleotide positions of 60, 78, 94, and 108. *P. elatior*, however, included fewer parsimony informative sites and was separated from the rest of the members of subg. *Primula* at the positions of 54, 457, 598, and 634. Furthermore, the distinct population of *P. elatior* had only one different base alteration, at the position of 624.

The members of subg. *Aleuritia* (Pop. No. 13-22) displayed the highest parsimony informative sites within all the taxa of the study. In this subgenus, the population of *P. auriculata* displayed a number of base alterations differing from the rest of the taxa of subg. *Aleuritia*, at the positions of 54, 76, 82, 111, 230, 232, 304, 454, 456, 484, 497, 555, 567, 599, 601, 606, and 621 (see Table 2). The populations of *P. auriculata* also exhibited some differences at the positions of 259, 465, 478, 512, 552, 565, 572, 580, 600, 648, and 659. The populations of *P. algida* demonstrated some differences from other populations of subg. *Aleuritia* at the positions of 82, 86, 94, 152, 190, 437, 454, 517, 540, 555, 563, 568, 575, 597, 620, 630, and 672. We also observed some base variations at the positions of 37, 60, 61, 78, 102, 103, 105, 111, 219, 452, 475, and 563 among the populations of *P. algida*. *P. longipes*, with the highest parsimony informative sites, differed from the rest of the species of subg. *Aleuritia* at the positions of 6, 61, 74, 75, 82, 89, 154, 166, 168, 184, 190, 217, 218, 219, 225, 231, 232, 234, 248, 259, 432, 442, 443, 445, 446, 447, 459, 481, 514, 537, 543, 556, 561, 562, 566, 567, 581, 583, 584, 603, 604, 618, 620, 624, 625, 630, 649, 650, 659, 674, 676, 677, 681, and 682. The 2 populations of *P. longipes* also contained

Table 2. Parsimony informative sites' aligned sequences of *Primula* taxa. For the pop. no. explanation, see Table 1.

Pop. no.	6 37 52 53 54 60 61 74 75 76 78 79 82 86 89 90 91 93 94 102 103 105 108 111 116 126 143 152 154 156 159 166 168 184 187 189 190 215 217 218 219 224 225 226 227 228 230 231 232 234 236 239 246 248 259 271 291 300 304 432 437 442 443 445 446 447 449 452 453 454 456 457 459 465 474 475 478
1	TAAGCGCATGGACAACGACTGAAGATGGTAGGTCTGTTAAGAATGCCTATATTTAAGGCTCTTGTAACCTATGGCAG
2	.....
3	.....
4	.....A.....A...
5	.....A.....A...
6	.....A.....A...
7	.....C....T.....A.T...T.C.....T.....T.....G...A..
8	.....A.....C.....T.....T.....G...A..
9	....A.....C-.....T.....C.....
10	....A.....C-.....T.....C.....
11	.....A...A.....A.....T....G.....
12	.....A...A.....A.....T....G.....
13	.....A...A.....A.....T....G.....
14	CG.A..ATA..TT.T..T.A.G..CG..CG.TATAAGATCA.GCA..ATGGG.ATG...A.CGCAC.....CT...A
15	CG.A..ATA..TT.T..T.A.G..CG..CG.TATAAGATCA.GCA..ATGGG.ATG...A.CGCAC.....CT...A
16	.G..A.T..A.TG.GG.T.A.GGACG...G....AA.A...G.C..A.G.GGG..GA.T-...T..G..AGCA....
17	.G..A.T..A.TG.GG.T.A.GGACG...G....AA.A...G.C..A.G.GGG..GA.T-...T..G..AGCA...A
18	.G..A.T..A.TG.GG.T.A.GGACG...G....AA.A...G.C..A.G.GGG.GGA.T-...T..G..AGCA....
19	.G..A.T..A.TG.GG.T.A.GGACG...G....AA.A...G.C..A.G.GGG..GA.T-...T..G..AGCAA...
20	.TCA.CG...TTATGG.TTGCCG.CG.A.G....AAC....G.CA.....GGG..GA..-T..T..G..G.CA.-..
21	.G.A..T....TATGG.TTC.GGTCG.A.G....AAC...TG.CA.....GGG..GA..-T..T..GG.G.CA.-C.
22	.G.A..T....TATGG.TTCAGG.CG.A.G....AAC...TG.CA.....GGG..GA..-T..T..GG-G.CA.-C.
23	.GC.TAT....TA.GG.T.CAGGACG...G....AA.A.....C....TCGGG..G...-.C.T..G....CA.-..

Dashes (-) indicate alignment gaps within the sequence

different nucleotides at the positions of 623 and 631. *P. davisii*, the single population of subg. *Sphondylia* in Turkey, differed from subg. *Primula* and subg. *Aleuritia* at the positions of 54, 60, 234, 566, 612, and 674.

The pair-wise distances obtained by Kimura's 2-parameter model for the examined populations varied from 0.0% to 20.9% (see Table 3). The divergence values within the subg. *Primula* based on ITS sequence variations ranged from 0.0% to 5.4%; for subg. *Aleuritia*, it was determined to range from 0.0% to 19.7%. The pair-wise distance matrix of genetic divergence values among all of the examined species is given in Table 4 and includes various outputs ranging from 0.0% to 18.9%. The base variations of investigated species of subg. *Primula* ranged from 0.0% to 6.0% and varied from 0.0% to 16.1% for the subg. *Aleuritia*. The values of the pair-wise distances of subg. *Sphondylia*, represented by *P. davisii*, to subg. *Primula* and subg. *Aleuritia* are 15.7% and 12.6%, respectively.

Discussion

Internal transcribed spacer (ITS) sequences have been widely used in plant molecular phylogenetics and evolutionary studies (Zheng et al., 2008). Alignment of the nrDNA ITS sequences for many plant taxa has supplied useful data for solving taxonomic problems, especially below the generic levels (Baldwin et al., 1995; Gravendeel et al., 2001).

As an explicit result of the present study, all of the examined taxa were found to be clustered within the 3 main clades of the phylogenetic tree obtained by NJ analysis. The clades, which represent 3 different subgenera, included the closely related taxa congruent with the conventional taxonomic order of Turkey's *Primula* (Table 1). The present data confirmed the tree topologies of Mast et al. (2001), in which subg. *Primula* and subg. *Aleuritia* taxa are clustered in a different clade. The molecular results of these 2 studies supported the view of Lamond (1978) at the subgenera level.

Table 2. Continued.

Pop. no.	481 483 484 490 497 508 512 514 517 533 537 540 541 543 545 550 552 553 555 556 561 562 563 565 566 567 568 569 570 572 573 575 577 580 581 583 584 586 595 597 598 599 600 601 603 604 606 612 613 617 618 620 621 623 624 625 629 630 631 634 636 641 648 649 650 653 654 659 660 667 672 674 676 677 681 682
1	CCAAGGGAGCTCCGATTAACCCCCCCGTGGAAGGACAACGCGAGATGAGAGCCAAGTGACGCATTCAGAAGGAATA
2	.....
3	.....
4	.....A.....
5	.....G.....
6	.....A.....
7	.....AA.....C.....G.....T...G.....
8	.....AA.....C.....G.....-.....T...G.....
9	.....A.....A.....C....T.....T...G.....
10	.....A.....A.....T.....T...G.....
11	.....A.....T.....A.....T.....T.....T...GA.....
12	.....A.....T.....A.....T.....T.....T...GA.....
13	.....A.....T.....A.....T.....T.....T.A.GA.....
14	ATGC...T.TC.TAGCA..ATG..TT--AAG.AATGCGT..A..GATCC.AG..GACCG.A-GAGG.A.G.TTGAG
15	ATGC...T.TC.TAGCA..ATG..TT--AAG.AATGCGT..A..GATCC.AG.GGACC..A-GAGG.A.G.TTGAG
16	.TTCT.A..TG...GCA.C..A.T.A-GAAG-A.CT.GT..T.A..ACCC..T...C...AAG..GGTTG.....
17	.TTCT.A..TG...GCG.C..A.T.A-GAAG-A.CT.GT..T.A..ACCC..T...C...AAG..GGATG.....
18	.TTCT.A..TG...GCA.C..A.T.A-GAAG-A.CT.GT..TGA..ACCC..T...C...AAG..GGTTG.....
19	.TTCT....TG...GCA.C..A...A-GA.G-AACT.GT..T.A..ACCC..T...C...AAT..GGTTG.....
20	.TGC....ATGTT..CG.T..AG...AGAAGGA.CT...A.A....TCCC.T.G..CA..AAG..GGTTGA.....
21	.TGC....ATGTT..CG.T..AG...AGAAGGA.CT...A.A....TCCC.T.G..CA..AAG..GGTTGA.....
22	.TGC....ATGTT..CG.T..AA...AGAAGGA.CT...A.A....TCCC.T.G..CA..AAG..GGTTGA.....
23	.T.CT....T..T.GCG.T..G..GAAGAAGGA.CT.G...-.....TCCA..G..C.....G..GGATGAC....

Dashes (-) indicate alignment gaps within the sequence

According to Lamond (1978), the species of *P. vulgaris*, which does not have well- developed scape structures, morphologically differs from *P. megaseifolia*, *P. elatior*, and *P. veris*. In the present study, it was found that all examined populations of *P. vulgaris* formed a distinct cluster from the rest of the taxa of subg. *Primula*, having well-developed scape structures. As seen in Figures 1 and 2, the examined populations of *P. vulgaris* clustered into 2 lineages with high bootstrap values of 92% and 78%, respectively. These genetic dissimilarities can also be inferred from the pair-wise distance values given in Table 3. The first 3 populations of *P. vulgaris* (Pop. no. 1, 2, and 3), which have diverse corolla colours as the single apparent morphological differentiation, were collected from the same location (A7 Trabzon) and clustered into one subgroup in relation to their ecological closeness. The second 3 populations of *Primula* (Pop. no. 4, 5, and 6; A4 Kastamonu) were also accumulated together in the subgroup due to similar conditions. Although both population members of *P. vulgaris* were collected from 2 distinct regions, they exhibited high similarity (only 2 base diversities; see Table 2) with respect to their ITS sequence data. We also identified several parallel alignments that demonstrate congruency with geographical dispersion within the other examined populations. Many studies have shown that there is a clear affinity between populations and the environments in which they exist (Aston & Bradshaw, 1966). The current and previously introduced data showed that closer geographical distances yield more homology in ITS genotypes (Wardill et al., 2005). On the other hand, the genetic variations within the subspecies could be affected by regional climates (Noyes, 2006).

Both *P. elatior* and *P. veris* have leaves that are rugose, efarinose, and glabrous to densely villous below, and so they carry relatively similar morphological characteristics (Lamond, 1978). Yet, as seen in Figure 1, *P. elatior* is closely linked to *P.*

Table 3. Pair-wise distance matrix of genetic divergence values of 23 populations, according to Kimura's 2-parameter model. For the pop. no. explanation, see Table 1.

Pop. no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	0.000																						
2	0.000																						
3	0.000	0.000																					
4	0.004	0.004	0.004																				
5	0.003	0.003	0.003	0.001																			
6	0.004	0.004	0.004	0.000	0.001																		
7	0.047	0.047	0.047	0.047	0.049	0.047																	
8	0.016	0.016	0.016	0.016	0.018	0.016	0.03																
9	0.013	0.013	0.013	0.013	0.015	0.013	0.046	0.015															
10	0.013	0.013	0.013	0.013	0.015	0.013	0.044	0.015	0.003														
11	0.018	0.018	0.018	0.018	0.020	0.018	0.054	0.023	0.020	0.020													
12	0.018	0.018	0.018	0.018	0.020	0.018	0.054	0.023	0.020	0.020	0.000												
13	0.040	0.040	0.040	0.040	0.041	0.040	0.076	0.044	0.041	0.041	0.021	0.021											
14	0.160	0.160	0.160	0.164	0.162	0.164	0.207	0.170	0.160	0.162	0.164	0.164	0.186										
15	0.162	0.162	0.162	0.166	0.164	0.166	0.209	0.172	0.162	0.164	0.168	0.168	0.190	0.009									
16	0.112	0.112	0.112	0.116	0.114	0.116	0.145	0.112	0.109	0.109	0.119	0.119	0.143	0.138	0.140								
17	0.116	0.116	0.116	0.119	0.117	0.119	0.149	0.116	0.112	0.112	0.123	0.123	0.144	0.138	0.140	0.006							
18	0.119	0.119	0.119	0.123	0.121	0.123	0.149	0.116	0.116	0.116	0.126	0.126	0.150	0.143	0.145	0.006	0.012						
19	0.126	0.126	0.126	0.126	0.124	0.126	0.163	0.130	0.122	0.122	0.133	0.133	0.157	0.154	0.156	0.023	0.029	0.029					
20	0.163	0.163	0.163	0.167	0.165	0.167	0.190	0.171	0.163	0.163	0.165	0.165	0.190	0.197	0.195	0.117	0.120	0.124	0.136				
21	0.117	0.117	0.117	0.121	0.119	0.121	0.155	0.125	0.117	0.117	0.119	0.119	0.143	0.143	0.141	0.067	0.070	0.074	0.085	0.059			
22	0.119	0.119	0.119	0.123	0.121	0.123	0.157	0.127	0.119	0.119	0.121	0.121	0.145	0.145	0.143	0.070	0.073	0.077	0.088	0.060	0.006		
23	0.150	0.150	0.150	0.154	0.152	0.154	0.191	0.158	0.148	0.148	0.154	0.154	0.177	0.173	0.171	0.099	0.097	0.106	0.118	0.154	0.109	0.109	0.000

Table 4. Pair-wise distance matrix of genetic divergence values of 8 taxa, according to Kimura's 2-parameter model.

Subg.	Species	*P. vulgaris*	*P. megaseifolia*	*P. elatior*	*P. veris*	*P. longipes*	*P. auriculata*	*P. algida*	*P. davisii*
	P. vulgaris								
Primula	*P. megaseifolia*	0.032							
	P. elatior	0.014	0.030						
	P. veris	0.040	0.060	0.041					
	P. longipes	0.162	0.189	0.162	0.188				
Aleuritia	*P. auriculata*	0.119	0.135	0.115	0.148	0.144			
	P. algida	0.135	0.154	0.133	0.159	0.161	0.092		
Sphondylia	*P. davisii*	0.152	0.174	0.148	0.177	0.172	0.105	0.124	

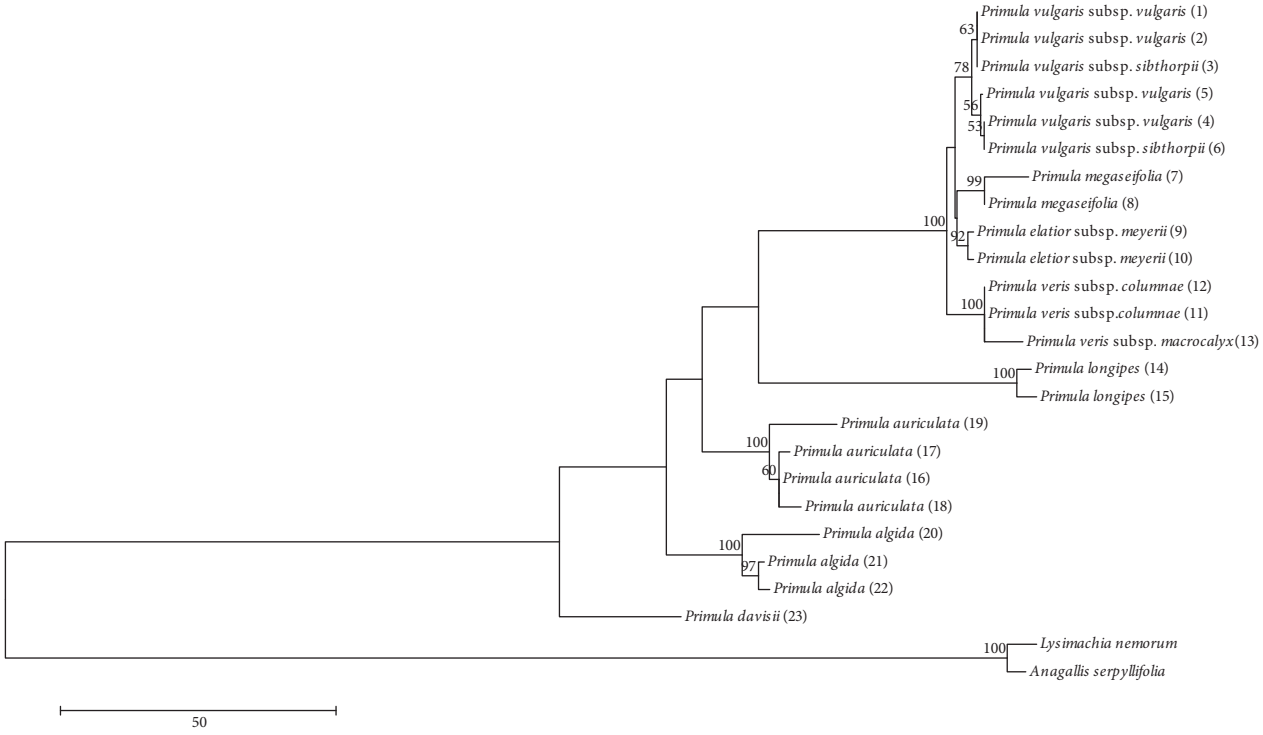

Figure 2. One of 16 parsimony trees for the *Primula* nuclear ITS region. Bootstrap values were obtained by 1000 replicates; numbers above the branches are bootstrap values > 50, tree length 669, consistency index = 0.8714, retention index = 0.9198, rescaled consistency index = 0.8016.

magaseifolia rather than *P. veris*, by virtue of sharing the same character (C) at site 116 (Table 2). This evidence indicates that molecular data may sometimes present findings that are in contrast to morphological data and could not always resolve the relationships in *Primula* (Martins et al., 2003).

Although several hybrids naturally occur between *P. vulgaris* and *P. veris* (Kalman et al., 2004), *P. veris* is a distinct species among the subg. *Primula* based on the NJ analysis (Figure 1). In addition, *P. vulgaris* (Hayırlıoğlu-Ayaz & İnceer, 2003) and *P. veris* (Abou-El-Enain, 2005) have the same (2n = 22) chromosome

number. This is probably due to the few base variations that emerged as a result of geographical and ecological effects.

P. elatior and *P. megaseifolia* are linked to each other with a low bootstrap value (51%) (Figure 1) and they revealed a few more base dissimilarities than the rest of the subg. *Primula* taxa (Table 2). The different corolla colours and chromosome numbers of these species also support their differences. Furthermore, the earlier recorded different chromosome numbers for *P. megaseifolia* (2n = 18) and *P. vulgaris* (2n = 22) (Hayırlıoğlu-Ayaz & İnceer, 2003) can be presented as additional evidence for genetic dissimilarity. Although *P. megaseifolia* morphologically resembles *P. vulgaris* (Lamond, 1978), the results based on phylogenetic analyses showed a distinctive relationship between *P. elatior* and *P. megaseifolia* (Table 4 and Figure 1), despite the differences in their chromosome numbers (2n = 16 and 2n = 18, respectively) (Hayırlıoğlu-Ayaz & İnceer, 2003). The current data also indicate that *P. veris* and *P. megaseifolia* are the most distant species in the subg. *Primula* because of the high base variation (Table 2), which may be caused by ecological effects (Scheiber et al., 2000).

The subg. *Aleuritia* is represented by 3 species in the present study. Among these species, *P. longipes* is the most distinct, based on inflorescence shape and length of scape (Lamond, 1978). The results of the sequence analyses of the entire ITS region also confirmed this taxonomical differentiation (Figure 1). According to the genetic divergence values of the pair-wise distance matrix (Table 4), *P. auriculata* and *P. algida* are the most closely related species, while *P. algida* and *P. longipes* are the most discrete taxa of the subg. *Aleuritia*. *P. longipes* was obviously separated from the other taxa of subg. *Aleuritia* since it contained many different base substitutions (Table 2) compared to *P. auriculata* and *P. algida*, which are the closer taxa in Clade-II (Figure 1). Several earlier studies related to ITS regions have shown that this region supplies sufficient genetic information to explore the relationships at both specific and generic levels (Bain & Jansen, 1995).

Leaf vernation is a useful taxonomic character for Primulaceae (Wendelbo, 1961); however, both revolute and involute leaf vernation can occur in the genus *Primula* (Richards, 1993). According to Wendelbo (1961), the involute vernation is a primitive morphological trait which is one of the distinguishing characters of the subg. *Sphondylia*. The current findings based on the NJ tree and other related data also support the positioning of *P. davisii* as a primitive taxon consisting of traits that are present in the common ancestor (Figure 1).

It is well known that molecular sampling might provide an independent phylogenetic hypothesis (Mast et al., 2001) for traditional taxonomic studies. Thus, our current study can be illustrated as preliminary molecular and phylogenetic research carried out on the wild *Primula* taxa of Anatolia. All the results showed that the ITS region supplies useful molecular information for exploring the relationships among the Turkish *Primula* taxa. However, to reconstruct an accurate phylogenetic relationship, the taxa should be examined with different molecular markers and intensive sampling.

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