

# Use of fluorescent-based amplified fragment length polymorphism to resolve phylogenetic relationships of *Colchicum* species from Turkey

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**ABSTRACT.** The study of phylogenetic relationships between 14 *Colchicum* taxa spread throughout Turkey was performed using a fluorescent-based amplified fragment length polymorphism (AFLP) technique. Five primer pair combinations were used in AFLP reactions. The data set was analyzed statistically using the NTSYS 2.1 software, and the neighbor-joining and maximum parsimony methods were implemented to generate phylogenetic trees. These analyses clustered the samples into 3 main clades. Both the neighbor-joining and maximum parsimony analyses resulted in similar topologies. Furthermore, supporting the phylogenetic trees, a similar grouping of 14 taxa was generated by principal component analysis. The AFLP analysis with 5 primer combinations was carried out to assess 14 taxa. Fragment sizes ranged from 54 to 462 bp in length for each primer combination. The average was 166 fragments per primer pair,

primer B2 generated the highest number of bands (200), and primer B3 produced the lowest number of bands (112). A total of 834 polymorphic bands were scored. The cophenetic correlation coefficient between the data matrix and the cophenetic matrix for AFLP data was 0.72. Based on this molecular data, we concluded that the genetic diversity among these Turkish accessions is relatively high.

**Key words:** *Colchicum*; Amplified fragment length polymorphism; Phylogenetic relationship; Principal component analysis; Neighbor-joining method; Maximum parsimony

## INTRODUCTION

Colchicaceae is a family of flowering plants showing a widespread distribution. Africa, Asia, Eurasia, and North America are the natural habitats of the 19 genera (Vinnersten and Reeves, 2003). Because of the high frequencies of species and endemics, Turkey and the Balkans are the major centers of diversity and speciation (Persson, 1993). The genus *Colchicum* L. is represented by 39 taxa, of which, 18 are endemic to Turkey (Brickell, 1984; Persson, 2000, 2005, 2007; Akan and Satil, 2005; Düşen and Sümbül, 2007).

*Colchicum* species have been used as a medicinal plant for more than 3000 years (Franková et al., 2005). Modern medicine uses *Colchicum* as a source of therapeutically active alkaloids called colchicinoids. These are poisonous alkaloids and include colchicine (Baytop, 1999). Colchicine was isolated by Pelletier and Caventou in 1820. However, its complete structure was not determined until the 1950s (Goodman and Gilman, 1956). Geophytes are used not only in medicine but also in ornamental industries (Çelik et al., 2004). Many of them are grown in parks and gardens as ornamental plants because of their beautiful flowers.

Candolle was the first researcher to use the family name Colchicaceae in 1805 (Vinnersten and Reeves, 2003). Since then, the taxonomic status of several genera within Colchicaceae has remained uncertain (Kahraman and Celep, 2010). For instance, Colchicaceae often includes the genera *Bulbocodium* L. and *Merendera* Ramond. However, some authors separate 3 genera based on style and sepal characteristics (Kahraman and Celep, 2010). The subgeneric taxa of the genus *Colchicum* including a few genera such as *Bulbocodium*, *Fouha*, *Merendera*, *Monocaryum*, and *Synsiphon* were listed by Persson (2007). Anatomical features of different *Colchicum* species have been investigated several times (Akan and Eker, 2005; Persson, 2005; Düşen and Sümbül, 2007; Kahraman and Celep, 2010). Fridlender et al. (2002) studied genome size of the genus *Colchicum* using flow cytometry. Smith and Waldren (2010) used the amplified fragment length polymorphism (AFLP) genetic fingerprinting technique to clarify a question over the native status of the Irish *Colchicum* species to assess its conservation status by comparison with populations throughout northwestern Europe. Recently, Persson et al. (2011) used nucleotide sequences from 6 plastid regions and 33 morphological, life history, and chromosomal characteristics to identify phylogenetic relationships among *Colchicum* species. The *trnL-trnF* sequence of non-coding chloroplast DNA (cpDNA) is one of the most preferable regions to elucidate phylogenetic relationships among species. The phylogeny and classification of genera belonging to the Colchicaceae family was studied analyzing these regions (Vinnersten and Reeves, 2003; Türkteş et al., 2012). However, the power of this region fails to resolve the phylogenetic assessments (Després et al., 2003). AFLP emerges as an efficient technique to solve difficulties whereas cpDNA is not sufficient to identify phylogenies, particularly among

closely related species or at the intraspecific level (Hodkinson et al., 2000; Koopman et al., 2001; Xu and Sun, 2001; Zhang et al., 2001; Karakaş et al., 2013). AFLP analysis can detect high levels of polymorphism and has high repeatability and speed of analysis. These markers have a quite high diversity index, resulting in a limited number of primer combinations that are required to screen an entire genome. In addition, they have been applied to develop a system for the fingerprinting of an organism (Faccioli et al., 1999) and for map expansion (Castiglioni et al., 1998).

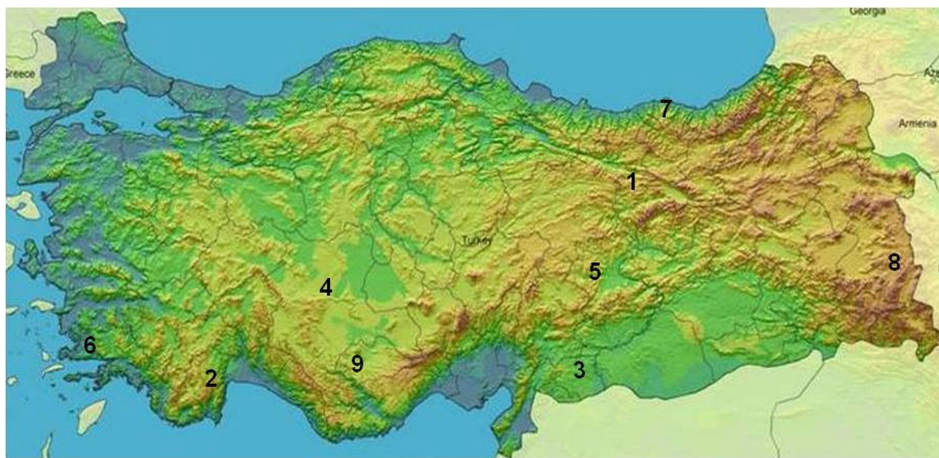
In this study, we determined phylogenetic relationships between *Colchicum* species using AFLP markers. We discussed results of AFLP data to infer phylogenetic relationships among closely related species. The phylogeny was assessed with phenetic (neighbor-joining), maximum parsimony, and principal component analysis (PCoA) methods. The results are comparable with the previous phylogenetic studies for known *Colchicum* species. These data would be a valuable source for the taxonomy of *Colchicum* species distributed in Turkey, and it will supply further insights into the taxonomy of this complex genus.

In addition, the recently published Persson et al. (2011) report that inferred the phylogeny of almost all known *Colchicum* species based on sequence data of 6 plastid regions provided a phylogenetic hypothesis of the relationships within species in the genus. Nonetheless, because of the low resolution in some clades in the Persson et al. (2011) study, the inclusion of AFLP data in this manuscript will advance information to broaden the current understanding of *Colchicum* spp.

## MATERIAL AND METHODS

### Plant materials

Fourteen *Colchicum* taxa were randomly collected from natural wild populations that were found throughout Turkey (Figure 1). The assessment of phylogenetic relationships among *C. dolichantherum*, *C. kotschyi*, *C. szovitsii*, *C. balansae*, *C. decaisnei*, *C. polyphyllum*, *C. cilicicum*, *C. inundatum*, *C. heldreichii*, *C. serpentinum*, *C. chalconicum* ssp. *punctatum*, *C. macrophyllum*, and *C. speciosum* was determined (Table 1).



**Figure 1.** Approximate locations of collected materials in Turkey. Sites were pointed with numbers. Numbers correspond to samples as listed in Table 1.

**Table 1.** List of the 14 *Colchicum* taxa analyzed, with voucher information and GenBank accession numbers of newly generated sequences.

<i>Colchicum</i> species	Place of collection (Turkey)	Collector	Collector No.	Accession No.
<i>Colchicum decaisnei</i> Boiss., Fl. Or. 5: 157 (1882)	Erzincan (1)	E. Kaya	CBT1	JX012296
<i>Colchicum dolichantherum</i> K.M. Perss. in Edinb. J. Bot. 56: 126 (1999)	Antalya (2)	E. Kaya	CBT2	JX012297
<i>Colchicum polyphyllum</i> Boiss. & Heldr. in Boiss., Diagn. ser. 2, 4: 121 (1859)	Gaziantep (3)	E. Kaya	CBT3	JX012298
<i>Colchicum szovitsii</i> Fisch. & C.A. Mey. in Ind. Sem. Horti Petrop. 1: 24 (1835)	Konya (4)	E. Kaya	CBT5	JX012299
<i>Colchicum inundatum</i> K.M. Perss. in Edinb. J. Bot. 56: 99 (1999)	Konya (4)	E. Kaya	CBT6	JX012300
<i>Colchicum heldreichii</i> K.M. Perss. in Edinb. J. Bot. 56: 98 (1999)	Konya (4)	E. Kaya	CBT7	JX012301
<i>Colchicum serpentinum</i> Woron. ex Misch. Fl. Cauc. Crit. 2: 114 (1912) (a)	Malatya (5)	E. Kaya	CBT8	JX012302
<i>Colchicum balansae</i> Planchon in Ann. Sci. Nat. Ser. 4, 4: 145 (1855) (a)	Muğla (6)	E. Kaya	CBT9	JX012303
<i>Colchicum chalcedonicum</i> subsp. <i>punctatum</i> K.M. Perss. in Candollea 53: 405 (1998)	Muğla (6)	E. Kaya	CBT10	JX012304
<i>Colchicum macrophyllum</i> B.L. Burt in Kew Bull. 5: 433 (1951)	Muğla (6)	E. Kaya	CBT11	JX012305
<i>Colchicum speciosum</i> Steven in Nouv. Mém. Soc. Imp. Mosc. 1: 265 (1829)	Trabzon (7)	E. Kaya	CBT12	JX012306
<i>Colchicum kotschyi</i> Boiss., Diagn. ser. 1 (13): 38 (1853)	Van (8)	E. Kaya	CBT13	JX012307
<i>Colchicum serpentinum</i> Woron. ex Misch. Fl. Cauc. Crit. 2: 114 (1912) (b)	Karaman (9)	E. Kaya	CBT14	JX012308
<i>Colchicum balansae</i> Planchon in Ann. Sci. Nat. Ser. 4, 4: 145 (1855) (b)	Karaman (9)	E. Kaya	CBT15	JX012309

## DNA isolation

Total genomic DNA was isolated using the cetyltrimethylammonium bromide extraction method modified from Doyle and Doyle (1990) by washing the DNA pellet in 70% ethanol and suspending it in nuclease-free water. Each genomic DNA was diluted to 100 ng/μL with nuclease-free water. DNA samples were quantified using the Nanodrop ND-1000 spectrophotometer. In addition to that, their qualities were evaluated by electrophoresis on 0.8% agarose gels according to Sambrook et al. (1989).

## AFLP analysis

AFLP fingerprints were generated using adaptors and fluorescently labeled primers from the AFLP Amplification Core Mix Module Analysis (Applied Biosystems, CA, USA) based on manufacturer instructions. Briefly, enzyme master mix was prepared to perform the restriction-ligation reactions for a DNA sample using 10X T4 DNA Ligase Buffer, 0.5 M NaCl, 1 mg/mL bovine serum albumin (BSA), 1 U *Mse*I, 25 U *Eco*RI, and 2 U T4 DNA Ligase. The restriction-ligation reactions created the template for adaptors. Then, adaptor pairs were ligated to the prepared template DNA. DNA digestion was carried out using *Eco*RI and *Mse*I (BioLabs, MA, USA); 0.5 μg genomic DNA was added to a reaction mixture containing 10X T4 DNA ligase buffer that included ATP, 1 mg/mL BSA, 5 μM *Eco*RI adaptor, 50 μM *Mse*I adaptor, 1 μL enzyme master mix, and water to a final volume of 10 μL. After incubation for 2 h at 37°C, 189 μL TE<sub>0.1</sub> buffer [20 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA)], pH 8.0 was added to each restriction-ligation reaction. The DNA template that was prepared by restriction-ligation was diluted 20-fold with TE<sub>0.1</sub> buffer, and about 4 μL DNA template was amplified in a final volume of 20 μL in a mixture containing AFLP Core Mix (Applied Biosystems, CA, USA) and 1 μL AFLP pre-selective primer pairs. The polymerase chain reaction (PCR) was performed with the following program: 2 min at 72°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C; and 10 min at 60°C. The presence of the pre-amplified products was verified through electrophoresis on a 1.5% agarose gel in 1X Tris,

boric acid, EDTA buffer. Pre-selective PCR amplification products were 20-fold diluted with TE<sub>0.1</sub> buffer. The selective amplifications were carried out using 5 primer pair combinations [*EcoRI* AGG\*D4-*MseI* CAA (B2), *EcoRI* AGG\*D4-*MseI* CTT (B3), *EcoRI* AGG\*D4-*MseI* CAT (B8), *EcoRI* AGG\*D4-*MseI* CAC (B9), and *EcoRI* AGG\*D4-*MseI* CCA (B10)], which were selected on the basis of the quality and quantity of bands produced. These were used in AFLP reactions on all 14 samples. The PCR mixture was the same as the pre-amplification reaction, except for the primers [*MseI* (Primer-Cxx) at 5 µM and 1.0 µL *EcoRI* (Dye-primer-Axx) at 1 µM]. The amplification program was: 1 cycle at 94°C for 2 min; 13 cycles of 30 s at 94°C, 30 s ramping from 65 to 56°C (0.7°C per cycle), and 1 min at 72°C; 24 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C; and 1 cycle at 60°C for 30 min. About 2.5 µL amplification product was added to 17.5 µL sample loading solution mixed with the DNA size standard-400 and overlaid with mineral oil. AFLP detection via capillary electrophoresis was performed using fluorescently labeled primers. Fluorescently labeled final PCR products were separated, detected, and precisely quantified by the GeXP system. The separation conditions on the GeXP system were as follows: capillary temperature 50°C, denaturation at 90°C for 120 s, injection for 30 s at 2.0 kV, and separation at 6.0 kV for 35 min.

### Data scoring and statistical analysis

Each PCR product was assumed to represent a single locus, and only reproducible polymorphic bands were scored automatically by the GeXP system as present (1) or absent (0). All fragments were given equal weights. Only fragments between 54 and 462 bp were taken into account to avoid scoring problems due to excess primer peaks near the front of the electrophoresed fragments and a decreasing signal for fragments longer than 500 bp.

Neighbor-joining (NJ) and maximum parsimony (MP) methods were implemented for phylogenetic analyses. MP analysis was conducted with the Phylip v.3.69 software (Felsenstein, 1989). MP trees were built using the Fitch (1971) method. The level of support for branches was tested using bootstrap analysis with 1000 replicates (Felsenstein, 1985). The NJ analysis was performed utilizing the Phylip v.3.69 software, and it was conducted by calculating Kimura's (1980) 2-parameter distance. The binary matrix of AFLP data was converted to a distance matrix using the PhylTools package (Buntjer, 1997). Genetic similarities among samples were calculated using the Jaccard similarity index. A phylogenetic tree was generated using the NJ method of Saitou and Nei (1987) and implemented by the Phylip v.3.69 software (Felsenstein, 1989). Trees were viewed with the Tree View v.32 program (Page, 1996).

The Mantel test (Mantel, 1967) with the random permutation number of 1000 was performed to determine the extent of distortion from converting the data into the dendrogram by comparing the original similarity matrix with the cophenetic value matrix that was calculated from the AFLP dendrogram. PCoA was carried out using NTSYS-pc package version 2.1 (Rohlf, 2000). In addition, the samples were plotted as points in a 3-dimensional continuous space.

## RESULTS

### AFLP primer combination evaluation and AFLP divergence

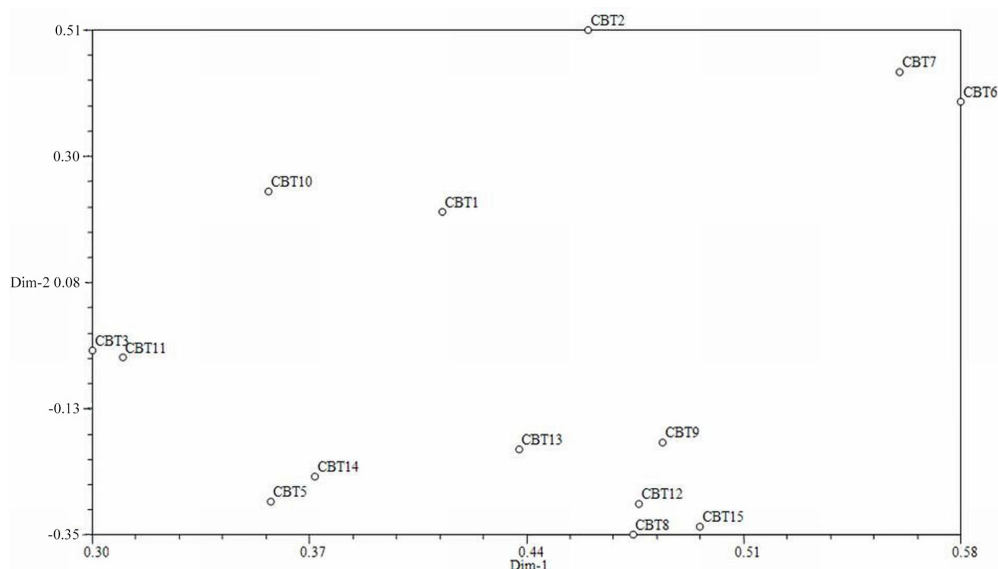
The AFLP analysis with 5 primer combinations was carried out to assess 14 taxa. Fragment sizes ranged from 54 to 462 bp in length for each primer combination (Table 2). The average was

166 fragments per primer pair, primer B2 generated the highest number of bands (200), and primer B3 produced the lowest number of bands (112). A total of 834 polymorphic bands were scored.

**Table 2.** Statistics of fragments generated by each amplified fragment length polymorphism primer pair.

Primer pair combinations	Primer pair code	Minimum fragment length (bp)	Maximum fragment length (bp)	No. of fragments
<i>EcoRI</i> AGG*D4- <i>MseI</i> CAA	B2	54.15	461.88	200
<i>EcoRI</i> AGG*D4- <i>MseI</i> CTT	B3	54.28	447.08	112
<i>EcoRI</i> AGG*D4- <i>MseI</i> CAT	B8	54.19	454.15	172
<i>EcoRI</i> AGG*D4- <i>MseI</i> CAC	B9	54.27	460.05	173
<i>EcoRI</i> AGG*D4- <i>MseI</i> CCA	B10	54.19	458.67	177
Total				834

The genetic relationships between samples were investigated using PCoA. The first 2 axes accounted for 28.26% of the variation in the dataset, and their eigenvectors were plotted in Figure 2. The first axis accounted for 19.24% of the variation in the whole dataset, and the second axis accounted for 9.01% of the variation.

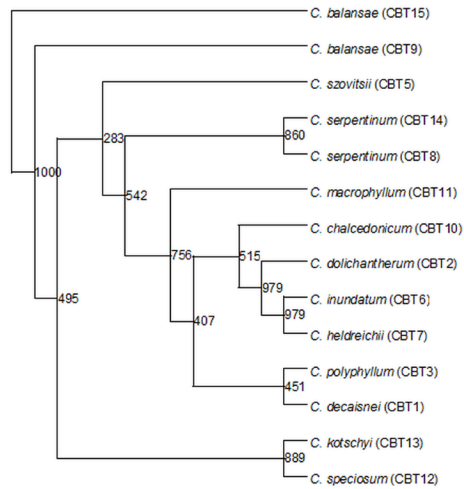


**Figure 2.** Plot of first two axes from a principal coordinates analysis. Axis 1 (Dim-1) accounts for 19.24% of the variation in the dataset, and axis 2 (Dim-2) accounts for 9.01%. The numbers correspond to individuals as indicated: 1) *C. decaisnei*, 2) *C. dolichantherum*, 3) *C. polyphyllum*, 4) *C. szovitsii*, 5) *C. inundatum*, 6) *C. heldreichii*, 7) *C. serpentinum* (a), 8) *C. balansae* (a), 9) *C. chalconicum* subsp *punctatum*, 10) *C. macrophyllum*, 11) *C. speciosum*, 12) *C. kotschyi*, 13) *C. serpentinum* (b), 14) *C. balansae* (b).

The species were divided into 3 groups by PCoA (Figure 2). The cophenetic correlation coefficient (r-value) between the data matrix and the cophenetic matrix for AFLP data was 0.72.

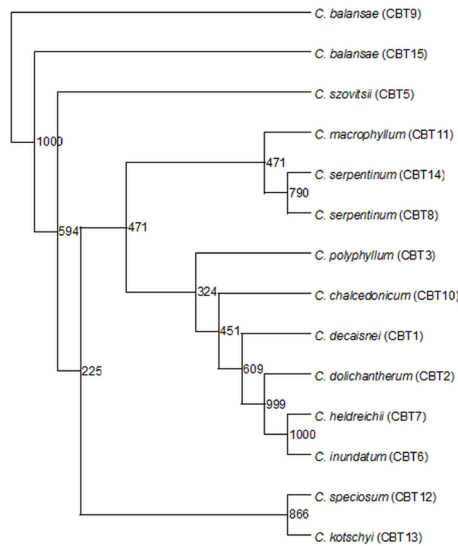
The NJ tree based on AFLP data identified 3 main groups (Figure 3). *C. balansae* (CBT15) and *C. balansae* (CBT9) generated clade 1. The second clade was formed by 10 *Colchicum* species. Two of the *C. serpentinum* isolates (CBT14 and CBT8) were collected from different regions in Turkey and formed the second clade with *C. macrophyllum*, *C. chalconicum*, *C. kotschyi*, *C. speciosum*, *C. heldreichii*, *C. polyphyllum*, *C. szovitsii*, *C. decaisnei*, and *C. dolichantherum*.

*donicum* subsp *punctatum*, *C. dolichantherum*, *C. inundatum*, *C. heldreichii*, *C. polyphyllum*, and *C. decaisnei*. The last group included only *C. kotschy* and *C. speciosum*.



**Figure 3.** Neighbor-joining tree based on AFLP data.

The MP tree revealed that 3 major clades arose among the samples, and clade 2 was further divided into 2 subgroups (Figure 4). *C. balansae* (CBT9 and CBT15) and *C. szovitsii* comprised clade 1. *Colchicum serpentinum* (CBT14 and CBT8) and *C. macrophyllum* formed the first subgroup of clade 2. *C. polyphyllum*, *C. chalconicum* subsp *punctatum*, *C. decaisnei*, *C. dolichantherum*, *C. heldreichii*, and *C. inundatum* created the second subgroup of clade 2 MP analysis. The last group included *C. speciosum* and *C. kotschy*.



**Figure 4.** Maximum Parsimony tree based on AFLP data.

## DISCUSSION

Phylogenetic relationships within the genus *Colchicum* L. have been studied several times since Baker's original report (1879). Because of the high morphological uniformity and low number of diagnostic characteristics among species, the phylogeny of the genus has always been a matter of question (Persson et al., 2011).

As a result of random sorting of polymorphic alleles in different lineages, gene trees do not always correspond to the true phylogeny of species (Després et al., 2003). The advantages of the AFLP technique can be observed in the large number of fragments that are revealed and the highly reproducible results. In addition to a similar accuracy degree of AFLP and sequence data, AFLP showed higher resolution (Althoff et al., 2007; García-Pereira et al., 2010). Therefore, in this study, AFLP was applied to obtain accurate phylogenetic relationships among species. We observed that the phylogenetic tree that was obtained from the AFLP analysis had good resolution. It supported supplementary data on relationships among 14 *Colchicum* taxa.

NJ and MP analyses divided 14 *Colchicum* taxa into 3 main groups. The results of PCoA mainly corresponded to those of the NJ and MP analyses along with some inconsistencies. The analyses placed *C. decaisnei*, *C. dolichantherum*, *C. polyphyllum*, *C. inundatum*, *C. heldreichii*, and *C. chalconicum* far from the others, indicating a clear divergence of those species. Although *C. macrophyllum* was placed in a different clade in the MP tree, it was weakly supported [bootstrap (BS) value of 471]. Similar to our findings, Persson et al. (2011) reported that *C. inundatum*, *C. heldreichii*, and *C. decaisnei* were found close to each other with a clear resolution. In that study, *C. dolichantherum* was also included in the same clade, although its position was not fully resolved within the clade; however, AFLP data clearly showed the placements of those species on different branches with high support. Any relationships between the topology of analyzed species and their distribution were observed. However, all the species that were clustered in that clade were fall-blooming species, suggesting a flowering time-based grouping.

Based on sequences of 6 plastid regions, *C. serpentinum* was placed in a separate clade than the others (Persson et al., 2011). Similarly, it was placed on a different branch in the AFLP-based phylogenetic tree with moderate support (BS value of 542 BS in the NJ tree and 471 in the MP tree). On the other hand, although *C. serpentinum* was placed on a different branch, its divergence from *C. szovitsii* was weakly supported in the NJ tree (BS value of 283). Supporting that, these 2 species were clustered together in the PCoA. The MP tree showed their separation with moderate support (BS value of 594). Therefore, the relationship between these species remained not fully solved.

*C. kotschyi* and *C. speciosum* were placed in an unsupported clade that was founded on the plastid region sequence analysis (Persson et al., 2011). Taking advantage of analysis of multiple loci, AFLP data successfully showed the divergence of *C. kotschyi* and *C. speciosum* in the NJ and MP trees with fairly high support (BS values of 889 and 866, respectively). Supporting that, the PCoA separate these 2 species.

*C. balansae* was found to be in the same clade as *C. macrophyllum* with weak support (Persson et al., 2011). Similar to the previous case, AFLP analysis depicted another ambiguous relationship between these *Colchicum* spp. Both species were clearly diverged from each other with very high support in both trees (100%), which was also observed by PCoA.



As it was discussed above, the classification of the genus *Colchicum* L. has always posed a great dilemma for researchers. It is hard to determine the exact phylogenetic positions of species based on 1 marker type. Each marker system has its own advantages and disadvantages. Manning et al. (2007) constructed a phylogenetic tree of *Colchicum* species based on combined data of *trnL-trnF* sequences and morphology. Vinnersten and Reeves (2003) analyzed 3 DNA regions to determine the phylogeny of the same *Colchicum* species. Because the analysis of several regions did not produce a better resolution in that study, DNA sequence analysis remains insufficient to study the *Colchicum* genus. Recently, Persson et al. (2011) discussed the phylogenetic relationships of *Colchicum* species by analyzing 6 plastid regions. Here, we included AFLP markers to provide further resolution. Providing an opportunity to analyze multiple loci, AFLP analysis succeeded to species identification when sequence evaluation failed. Thus, AFLP appeared to be an appropriate method to study *Colchicum* species. To resolve the phylogeny of the genus *Colchicum* L., species should be examined based on several characteristics, such as molecular, morphological, karyological, and phytochemical characteristics. In addition, the combination of all this information should be considered in species identification.

## CONCLUSIONS

Turkey and the Balkans are the major centers of diversity and speciation of the *Colchicum* L. genus. Genetic resources and their conservation and utilization are very important for breeding new varieties. In this study, we determined phylogenetic relationships within the genus *Colchicum* L. using the AFLP molecular fingerprinting method. We successfully determined the phylogenetic position of 14 *Colchicum* taxa. These genotypes can be used as candidates for breeding lines. With further supporting findings, our analysis presented valuable data to fill the gaps in phylogenetic relationships among *Colchicum* spp and improved our knowledge of *Colchicum* spp.

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