Genetics & Applications

Institute for Genetic Engineering and Biotechnology University of Sarajevo

Research article Open access

Phylogenetic analysis of *Colchicum* (Colchicaceae) genus by molecular markers from nuclear and chloroplast genome

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DOI: 10.31383/ga.vol4iss1pp1-11

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Received
January, 2020
Accepted
March, 2020
Published
June, 2020

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Abstract

We evaluated the patterns of genetic variation of 16 *Colchicum* species, including 37 different genotypes, using RAPD marker and *trnL–trnF* chloroplast DNA sequence. A total of 861 polymorphic alleles through RAPDs showed a mean of 33.88 ± 3.80 alleles per primer, while mean major allele frequency was 0.067 ± 0.05. The sequence length of *trnL–trnF* ranged from 1022 bp to 1081 bp. The phylogenetic tree was constructed to understand the relationship between *Colchicum* species and the discrimination power of the nuclear and chloroplast genome for species. The results showed that *trnL–trnF* gene region grouped *Colchicum* species well in comparison with RAPD analysis. This data was also supported by haplotype network analysis, structure analysis and PCA (Principle Component Analysis). This study showed that there is a need for a characterization that contains more molecular and morphological methods to correctly distinguish *Colchicum* species.

Keywords

Chloroplast genome, Colchicum L., genetic diversity, RAPD

Introduction

Colchicum L. is a geophyte member of a flowering plant family, namely Colchicaceae with a widespread distribution. Nineteen genera naturally present throughout Asia, Eurasia, Africa and North America (Vinnersten & Reeves, 2003). The major speciation and diversification center for the genera is thought to be the Balkans and Turkey due to high frequencies of species and endemics (Persson, 1993). Turkey harbours 39 taxa of the family *Colchicaceae*, eighteen of which are endemic to Turkey (Brickell, 1984; Akan & Satil, 2005; Persson, 2000, 2005, 2007). Colchicaceae was firstly used as family name by a researcher, Candolle, in 1805 (Vinnersten & Reeves, 2003). Since then, the

placement of several genera taxonomically within Colchicaceae shows uncertainty (Kahraman & Celep, 2010).

Some studies suggested that, two genera (*Bulbocodium* L. and *Merendera Ramond*) separated depending on their style and sepal characteristics from *Colchicum* L. while they are commonly included with Colchicaceae (Kahraman & Celep, 2010). Investigation of morpho-anatomical characteristic of different *Colchicum* species has previously been conducted several times (Akan & Eker, 2005; Persson, 2005; Düşen & Sümbül, 2007; Kahraman & Celep, 2010; Sevgi & Kucuker, 2011). Additionally, *Colchicum* is a problematic genus so classifying the species in different periods of flowering and foliage is difficult. The characterization of *Colchicum* species, however, has not been fully realized. In such a problematic genus, a morphological or molecular single parameter is not sufficient to accurately describe the species and due its complicated structure it should be evaluated based on different research methods for diversity analysis. Different types of molecular marker techniques throughout the plant genome are usable for determining genetic diversity and evolutionary relationships.

In this study phylogenetic studies including nuclear (RAPDs) and chloroplast (*trnL–trnF*) genome markers due to their different modes of inheritance and rates of mutation were determined along the phylogenetic tree. Random Amplified Polymorphic DNA (RAPD) markers are non-specific markers which are very popular tools for the investigation of nuclear genome and the determination of the genetic fingerprint data. RAPD can help to identify phylogenies among closely related species or at the intraspecific level and detect high levels of polymorphism, without needing any previous information on genome (Williams et al., 1990). The "non-coding intergenic spacer" regions of chloroplast genome are widely used for molecular characterization and phylogenetic analysis in plant genomes.

Chloroplast DNA (cpDNA) is inherited maternally in plants and particularly useful for phylogenetic studies due to its high degree of base sequence conservation (Palmer, 1987; Taberlet et al., 1991; Hodkinson et al., 2000; Zhang et al., 2001). Although cpDNA is highly conserved within species, non-coding regions within the chloroplast genome have higher rates of mutation (Shaw et al., 2005). The non-coding intergenic spacer *trnL* (leucine) and *trnF* (phenylalanine) regions are more convenient to use at taxonomic levels since they have less limited sequences functionally and therefore less chances of change because phylogenetically, they have more informative characters (Shaw et al., 2005; Downie & Palmer, 1992; Soltis et al., 1990). The analysis of non-coding cpDNA regions was extremely useful for the determination of phylogenetic relationships in lower taxonomic levels, between closely related species and even within the species. So far, they have been widely used for reconstructing phylogenetic inference at the intra- and inter-specific levels in Colchicaceae family (Vinnersten & Reeves, 2003; Türktaş et al., 2012). Particularly in the latest phylogenetic analysis of the *Colchicum* genus, Persson et al. (2011) used *Colchicum* morphology and six plastid regions (*trnL* intron, *trnL-trnF* IGS, *trnY-trnD* IGS, *trnH-psbA*, IGS, *atpB-rbcL* IGS, *rps16* intron) to reconstruct *Colchicum* phylogeny and they have revealed important results about phylogenetic relationship between *Colchicum* species.

In this study, we seek to characterize phylogenetic relationships of genus *Colchicum* collected from all regions of Turkey, including 16 species and 37 genotypes, using RAPD markers and sequence data of the chloroplast intergenic region *trnL–trnF* (c-f region) markers to resolve relationships in the genus.

Material and methods

Plant Materials

Leaf samples from 32 various locations (at the seven different regions) of 37 *Colchicum* L. genotypes, including 16 different species throughout Turkey flora, were obtained from the Republic of Turkey, Ministry of Agriculture and Livestock General Directorate of Agricultural Research and Policy, Atatürk Central Research Institute of Gardening Plants (ACRIGP) at Yalova (Table 1). The samples consist out of: 10 autumn species including 18 genotypes; 6 spring species including 19 genotypes and 6 endemic species including 16 genotypes.

Table 1. *Colchicum* L. species and genotypes, sample codes, their geographical locations and endemism data used in this study

No of Species	Species	No of Genotypes	Genotype Code - Location (province and region in Turkey)	Endemism
1	**C. atticum* (Spruner) Boiss&Spruner	**10**	C2604-Eskişehir-Central Anatolia	-
		19	C4305-Kütahya-Aegean	
		1	C0736 B-Antalya- Mediterannean	
2	***C. balansae* Planchon	**33**	C7001-Karaman-Central Anatolia	Endemic
		34	C7008-Karaman-Central Anatolia	
3	***C. bivonae* Guss.	**9**	C2602-Eskişehir-Central Anatolia	-
4	***C. boissieri* Orph.	**21**	C4502-Manisa-Aegean	-
5	***C. chalcedonicum* Azn. ssp. *chalcedonicum*	**36**	C7701-Yalova-Marmara	Endemic
6	***C. decaisnei* Boiss.	**2**	C0763-Antalya- Mediterannean	-
		20	C4406-Malatya-Eastern Anatolia	
		23	C4703 B-Mardin-Southeastern Anatolia	
7	***C. kotschyi* Bornm.		C4904-Muş-Eastern Anatolia	-
		24	C6511-Van-Eastern Anatolia	
		31		
8	**C. leptanthum* K.Perss.	**8**	C2506-Erzurum-Eastern Anatolia	Endemic
9	***C. micrantuhum* Boiss.	**13**	C3405-İstanbul-Marmara	Endemic
10	***C. polyphyllum* Bois. &Heldr.	**37**	C7901-Kilis-Mediterannean	-
11	**C. raddeanum* K.Perss.	**30**	C6503-Van-Eastern Anatolia	-
		5	C2301 B-Elazığ-Eastern Anatolia	
	**C. serpentinum* Woron. ex.	**7**	C2403-Erzincan-Eastern Anatolia	
12	Miscz.	**18**	C4202 A-Konya-Central Anatolia	Endemic
		25	C5102-Niğde-Central Anatolia	
		26	C5203-Ordu-Black Sea	
13	***C. speciosum* Steven	**28**	C6101-Trabzon-Black Sea	-
		32	C6901-Bayburt-Black Sea	
		6	C2402-Erzincan-Eastern Anatolia	
		11	C2803-Giresun-Black Sea	
14	**C. szovitsii* Fisch. Et Mey. ssp. *szovitsii*	**12**	C2901-Gümüşhane-Black Sea	Endemic
		14	C3601-Kars-Eastern Anatolia	
		22	C4606-Kahramanmaraş-Mediterannean	
		4	C1802-Çankırı-Central Anatolia	
		15	C3801-Kayseri-Central Anatolia	
15	**C. triphyllum* G. Kunze	**17**	C4201-Konya-Central Anatolia	-
		27	C5803-Sivas-Central Anatolia	
		29	C6401-Uşak-Aegean	
		3	C0808-Artvin-Black Sea	
16	***C. umbrosum* Steven	**16**	C3901-Kırklareli-Marmara	-
		35	C7507-Ardahan-Eastern Anatolia	

*Spring flowering 6 *Colchicum* L. species, **Autumn flowering 10 *Colchicum* L. species

DNA Extraction

CTAB (hexadecyltrimethylammonium bromide) protocol (Doyle & Doyle, 1987) was used for the genomic DNA isolation from *Colchicum* leaf tissues. DNA quantity was measured using Qubit 2.0 fluorometer and diluted for PCR reactions. Qualities of isolated DNAs were visualized at the 0.8% agarose gel.

Genotyping by Molecular Markers from Nuclear Genome

We generated RAPD fingerprints, using the primers synthesized by Invitrogen Technologies based on the Operon Technologies. We used 51 RAPD primers in this study (Table 2). The optimal reaction for RAPD analysis was set, using the following conditions: 25 µl containing 1× buffer, 0.2 mM dNTP mix, 3.75 mM

$MgCl_2$, 0.025 U μl^{-1} Taq DNA Polymerase, 1.8 µM primer and 1µl gDNA. The PCR program was carried out by the following procedure: initial denaturation for 3 min at 94 °C, denaturation for 1 min at 94 °C, 1 min for annealing at the temperature determined for each primer, 1 min at 72 °C for elongation for 40 cycles following by a final elongation step at 72 °C for 5 min. We run the PCR products in agarose gel containing 1.5% agarose dissolved in 0.5×TBE buffer and 2 µl 100 ml^{-1} red safe for 90 minutes in 150V. We visualized the gels by gel documentation system under UV light and the images were analyzed by Genosoft version 3.8.2 software.

Table 2. Statistics of RAPD marker analysis

Marker Name	Ta (°C)	Band size* (bp)	MAF	Allele No	Gene Diversity	PIC	Marker Name	Ta (°C)	Band size* (bp)	MAF	Allele No	Gene Diversity	PIC
OA 20	43	240-1170	0.054	35	0.970	0.969	OPC 19	46	295-1120	0.054	32	0.966	0.965
OH 12	43	205-972	0.054	35	0.970	0.969	OPE 1	44	340-1270	0.081	31	0.963	0.962
OH 15	44	300-1375	0.054	35	0.970	0.969	OPE 19	43	230-1050	0.027	37	0.973	0.972
OPA 01	40	300-1455	0.054	33	0.967	0.966	OPF 3	42	375-1535	0.054	36	0.972	0.971
OPA 02	42	270-1365	0.054	35	0.970	0.969	OPF 8	41	295-1500	0.027	37	0.973	0.972
OPA 03	42	210-1580	0.081	33	0.966	0.965	OPF 12	44	210-1230	0.162	27	0.944	0.941
OPA 04	42	185-920	0.054	36	0.972	0.971	OPF 13	40	430-1255	0.108	31	0.960	0.958
OPA 07	42	155-1230	0.054	34	0.969	0.968	OPH 4	42	250-1225	0.027	37	0.973	0.972
OPA 08	43	275-1165	0.054	36	0.972	0.971	OPH 11	40	360-1330	0.054	33	0.967	0.966
OPA 09	41	430-1330	0.162	18	0.919	0.913	OPH 19	40	330-1050	0.054	35	0.970	0.969
OPA 10	42	270-1375	0.054	36	0.972	0.971	OPJ 9	43	270-1760	0.054	35	0.970	0.969
OPA 11	41	130-1175	0.054	36	0.972	0.971	OPJ 10	44	310-1530	0.027	37	0.973	0.972
OPAB 6	43	300-1050	0.081	32	0.964	0.963	OPJ 20	41	290-1150	0.351	24	0.858	0.853
OPAC 12	44	205-1245	0.054	36	0.972	0.971	OPM 11	41.5	120-925	0.081	33	0.966	0.965
OPAD 11	42	285-1410	0.081	35	0.969	0.968	OPR 6	44	255-1035	0.081	32	0.964	0.963
OPAK 14	39	180-1030	0.081	34	0.967	0.966	OPR 12	42	210-1525	0.027	37	0.973	0.972
OPAL 8	40	310-1455	0.054	35	0.970	0.969	OPT 16	41	310-1325	0.027	37	0.973	0.972
OPAL 12	44	265-1350	0.027	37	0.973	0.972	OPT 17	43	210-1340	0.027	37	0.973	0.972
OPAW 7	44	215-1860	0.027	37	0.973	0.972	OPT 18	40.5	305-1740	0.054	34	0.969	0.968
OPB 07	44	315-1250	0.027	37	0.973	0.972	OPW 20	44	360-1150	0.081	32	0.964	0.963
OPB 08	42	235-1650	0.054	34	0.969	0.968	OPY 8	40	305-1530	0.054	36	0.972	0.971
OPB 09	40	230-1430	0.081	33	0.966	0.965	UBC 199	40	350-1250	0.054	35	0.970	0.969
OPC 02	44	170-1200	0.027	37	0.973	0.972	UBC 212	41	440-1550	0.081	31	0.960	0.958
OPC 08	34.5	340-1530	0.054	34	0.969	0.968	UBC 359	41	265-1045	0.054	36	0.972	0.971
OPC 11	40	235-1035	0.216	23	0.918	0.913	UBC 551	41	265-1230	0.054	33	0.967	0.966
OPC 15	40	330-1450	0.027	37	0.973	0.972			Mean	0.067	33.88	0.965	0.963

* Minimum and maximum size of the bands; MAF – Major Allele Frequency; PIC - Polymorphism Information Content

Genotyping by Molecular Markers from Chloroplast Genome

Non-coding region between the *trnL* (UAA) 5' exon (c region) and *trnF* (GAA) (f region) of chloroplast genome was amplified using primers (5'- CGA AAT CGG TAG ACG CTA CG -3') and (5'- ATT TGA ACT GGT GAC ACG AG -3') (Taberlet et al., 1991; Mummenhoff et al., 2001; Figure 1). The amplification reactions were performed using 1×PCR buffer, 2.5 mM $MgCl_2$, 0.025 U Taq DNA polymerase, 0.2 mM dNTPs, 0.3 µM forward primers and 0.5 µM reverse primer, and 4 ng of genomic DNA in final volume of 25 µl reactions. PCR amplifications were carried out following the PCR reactions: initial denaturation at 94 °C for 1 min 30 s, 35 cycles at 94 °C for 1 min, 60 °C for 35 s, 72 °C for 1 min 50 s, and final extension step at 72 °C for 10 min. The PCR products were separated by 1.5% agarose gel with red safe nucleic acid staining solution and photographed. Purification of amplifications obtained from PCR analysis was performed by "charge-switch-Pro PCR clean-up purification" kit. Following purification, cycle sequencing reaction was carried out using *trnL–trnF* fragment with "ABI BigDye Terminator v3.1 Cycle Sequencing" kit. The cycle sequencing reaction mixture with a total reaction volume of 10 µL included 1µL 5× buffer, 2 µL Big Dye terminator, 1.8 µL nuclease-free water, 3.2 µL primer (1 µM), and 2 µL of genomic DNA (4 ng μL^{-1}). Reactions of sequence analysis were carried out with a procedure; at 96 °C for 1 min (denaturing), followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C (final

extension). Using ABI PRISM 310 Genetic Analyzer *trnL–trnF* gene regions of *Colchicum* genotypes were sequenced. The sequences were aligned by BLAST (Basic Local Alignment Search Tool) and they were submitted to NCBI gene bank.

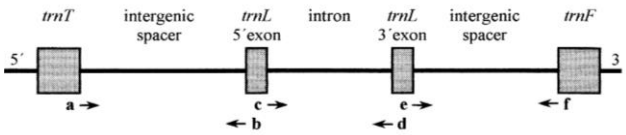


Figure 1. Schematic representation of 6 DNA regions (a-b, a-d, a-f, c-d, c-f, e-f) to be amplified by *trnL-trnF* region specific primers

Data Scoring and Statistical Analysis

After RAPD analysis, obtained band sizes were determined and by Genosoft software version 3.8.2. they were analyzed. The fragments were evaluated statistically as 1-0 data according to their absence or presence. PIC (polymorphism information content) values, major allele frequency and gene diversity were identified by the software POWER MARKER version 3.25 (Liu & Muse, 2005). Model-based software STRUCTURE 2.3.3 was used to infer the population structure. STRUCTURE 2.3.3 software was run using a burn-in the length of 10.000 - 100.000 for the determination of the number of the subpopulations (K) (Mucciarelli et al., 2014). To determine the genetic structure of the 37 *Colchicum* sp. genotypes using RAPD, the Principal Component Analyses (PCA) was performed by GenAlEx software 6.41 (Peakall & Smouse, 2012). For this application pairwise genetic distances of each marker were calculated and the principal components were extracted from the pairwise genetic distance matrix. Cluster analysis was performed, and phylogenetic tree was constructed by Phylip V 3.695 software using neighbor joining method with 100 bootstrap replicates (Felsenstein, 2005) for RAPD markers. Phylogenetic tree was visualized by TREEVIEW program (Page & Holmes, 1998). Thirty-seven *Colchicum* genotypes having been sequenced, alignments for *trnL–trnF* gene region were carried out using MEGA 6.06 (Tamura et al., 2013). Single Nucleotide Polymorphism (SNP) extraction was conducted by MEGA 6.06 and SNPs were detected as loci after alignments were performed. The population structure was evaluated by STRUCTURE 2.3.3 software with SNP data as 0 and 1. Genetic diversity assessment was carried out by PCA. Using Mega 6.06 software program, pairwise distance was computed. We exported the computed genetic distance data into excel format. GenAlEx 6.4 was run to examine PCA. Phylogenetic analysis of *trnL–trnF* chloroplast intergenic region sequence data was performed in MEGA 6.06 and neighbor joining-based dendrogram was constructed. So as to supply the phylogenetic tree of *trnL–trnF* chloroplast region, haplotype median joining network was constructed using PopART program (Bandelt, 1999).

Results and Discussion

Genotyping Results from Nuclear and Chloroplast Genome

RAPD analyses were carried to assess 37 genotypes gathered from 32 different locations all over Turkey. Annealing temperatures, number of bands per primer and minimum-maximum band sizes acquired by amplification of 51 RAPD primers are listed in Table 2. A total of 861 polymorphic alleles were found through RAPD marker analyses. Minimum fragment size observed was 120 base pairs by OPM 11 primer, the maximum fragment size observed were 1870 base pairs by OPAW 7 primer. All alleles were weighted equal and subjected to statistical analyses with Power Marker 3.25 software. Analyses showed a mean of 33.88 ± 3.80 alleles per primer, while mean major allele frequency was found 0.067 ± 0.05. Mean of gene diversity was found 0.964 ± 0.018 and, PIC was found as 0.963 ± 0.19. The results of the summary statistics were given in Table 2. The sequence length of *trnL–trnF* ranged from 1022 bp to 1081 bp. The 37 sequences obtained from chloroplast intergenic region *trnL–trnF* of 37 *Colchicum* genotypes were submitted to NCBI GenBank (accession numbers: KY860481-KY860517).

Phylogenetic Analysis

Phylogenetic tree was constructed with RAPD markers and the sequencing data of *trnL–trnF* region for 37 *Colchicum* L. genotypes (Figure 2A, B) showed differences from one another. Dendrogram constituted with 51 RAPD markers data indicated

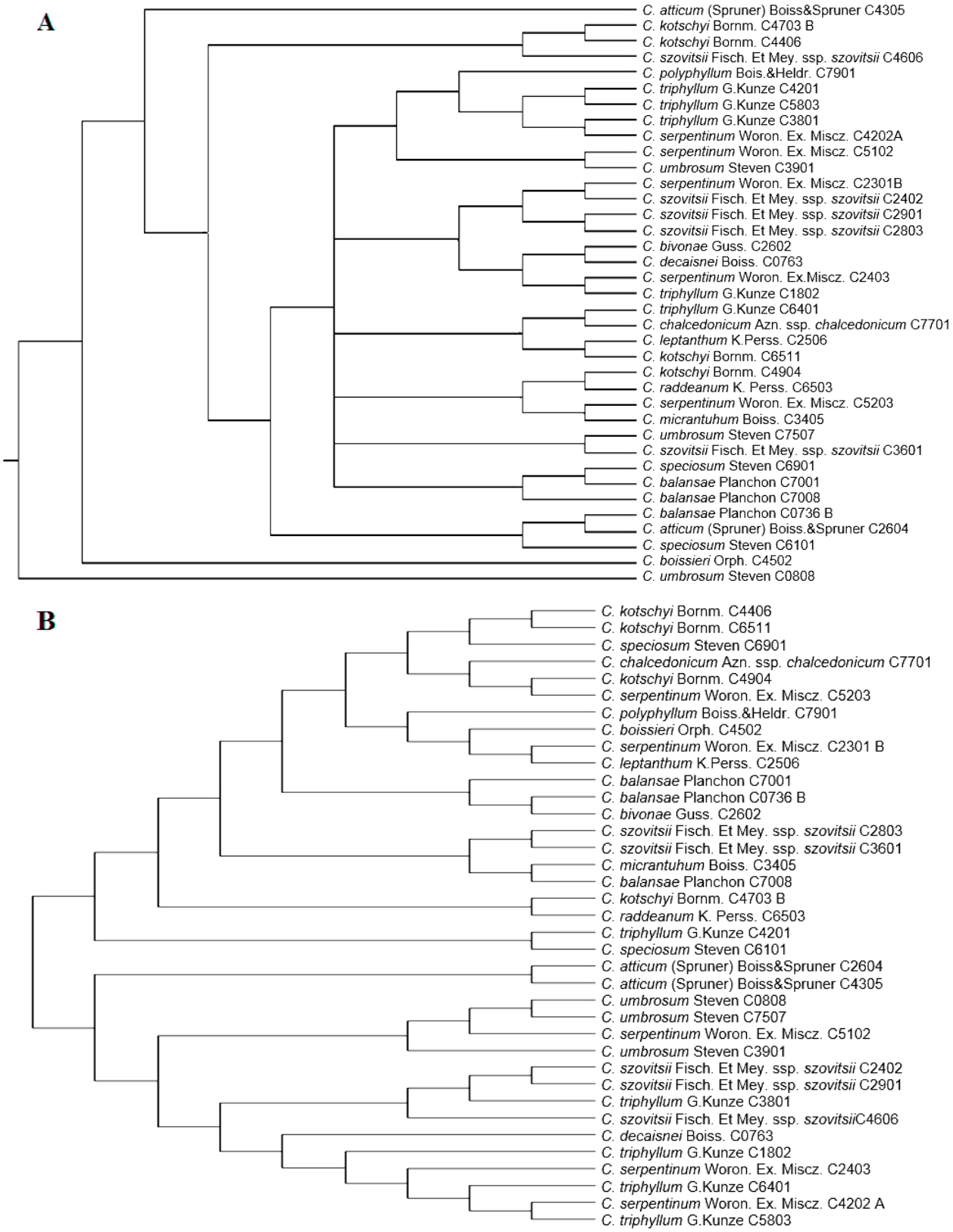


Figure 2. Neighbor-joining tree computed **A** by Phylip 3.695 for RAPD markers data, **B** by Mega 6.06 for sequencing data of *trnL-trnF* region in 37 *Colchicum* L. genotypes

that 37 *Colchicum* L. genotypes were separated as three outgroups and two main groups, one of which contains eight subgroubs. Other phylogenetic tree, constructed with sequencing data showed two main groups, and each of which contains two subgroups. There are eight species represented by a single genotype.

Two of them (*C. boissieri* Orph., *C. polyphyllum* Boiss. & Heldr.) appear in one of the main out groups of the phylogenic tree by RAPD results and four of them (*C. boissieri* Orph., *C. chalcedonicum* Azn. ssp. *chalcedonicum*, *C. decaisnei* Boiss. and *C. polyphyllum* Boiss. & Heldr.) appear in one of the main outgroups of the phylogenic tree by *trnL-trnF* region results. It has been revealed through both RAPD and *trnL-trnF* region phylogenetic tree results that, as the single represented species, *C. boissieri* Orph. and *C. polyphyllum* Boiss. & Heldr. were observed in one main group.

Each marker system has its own advantages and disadvantages for phylogenetic analysis. It was observed that some genotypes belonging to the same species were grouped well by RAPD analysis and some genotypes with *trnL-trnF* region analysis (Figure 2A, B). It is quite difficult to determine the relationships between the species using only a marker system. In addition, haplotype network analysis was performed to support the accuracy of the phylogenetic tree generated by sequence knowledge of the *trnL-trnF* gene region. The haplotype network of all haplotypes visualizes their relationships as well as their relative abundance. In haplotype network (Figure 3), haplotypes are represented by circles. Two haplotypes are connected by a line if they are separated by one mutation; each additional mutation is indicated by black lines. Configuration of the haplotype network is consistent with the *trnL-trnF* chloroplast gene region phylogenetic tree.

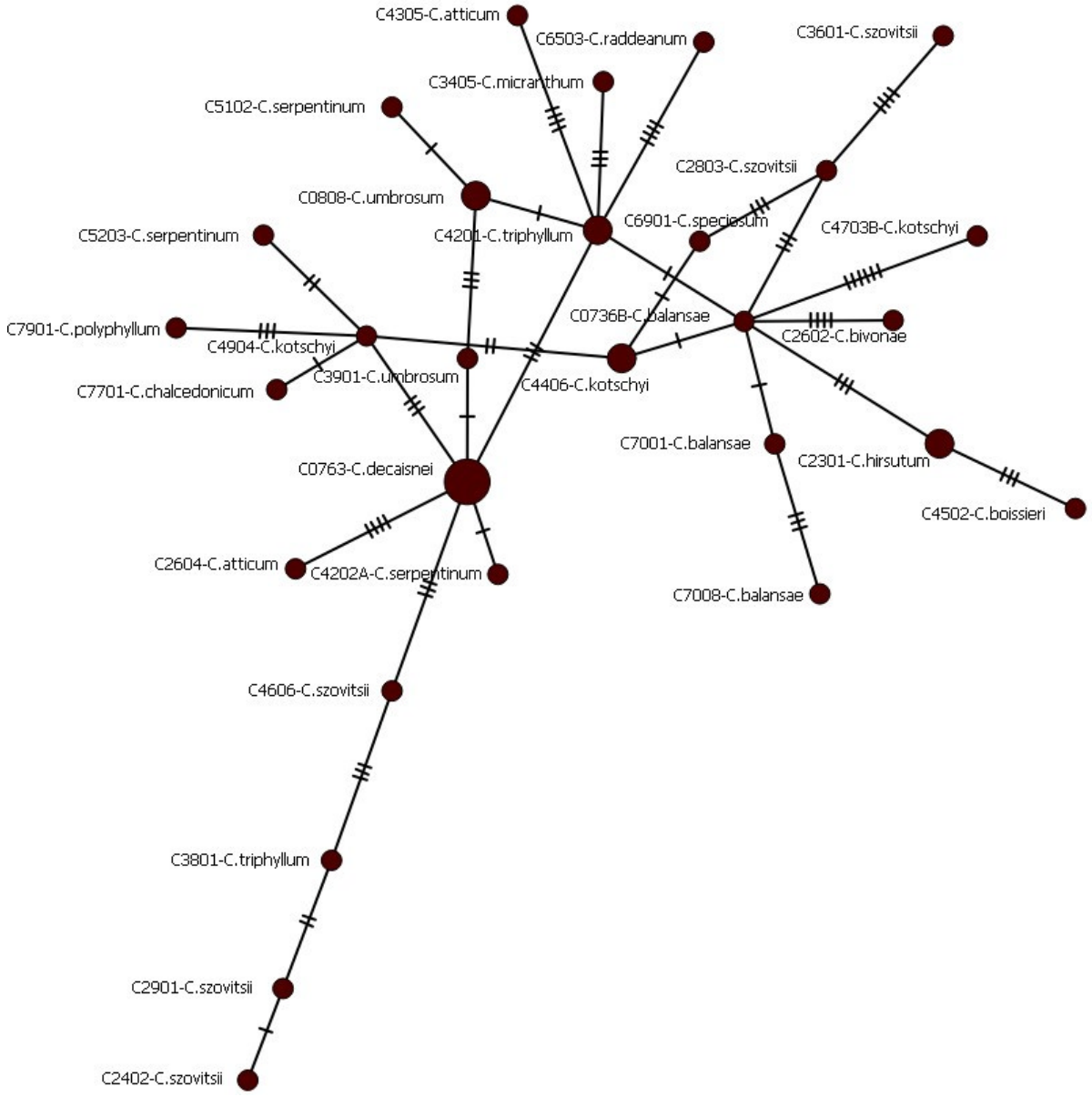


Figure 3. A median-joining network showing the relationships among chloroplast DNA haplotypes found in 37 *Colchicum* L. genotypes

Structure Analysis

STRUCTURE software was used for the estimation of possible distributions of *Colchicum* genotypes. The structure of genotypes was evaluated by optimal K value which shows the most probable subgroup estimation. Variations of RAPD and *trnL–trnF* markers in 37 genotypes of *Colchicum* L. resulted in four and two meaningful clusters (K=4, K=2) respectively (Figure 4A, B), with the exploration of the meaningful clustering by statistics developed by Evanno et al. (2005). In Figure 4A, deltaK showed high peak so it was decided that genotypes are divided into 4 groups. In Figure 4B, deltaK value was observed at only K=2 point. The structure results were taken into consideration in the light of Structure Harvester (Earl and Vonholdt, 2012) and the four and two groups were seen as in the Figure 4C, D respectively. Subpopulations structured by RAPD (yellow, red, green and blue) and *trnL–trnF* (red and green) marker data indicated that the 37 genotypes of *Colchicum* L. genotypes were not clustered well.

The Principal Component Analysis (PCA)

PCA of the RAPD marker data indicated that 36.81% of the variation was represented by the coordinates 1 (18.76%) and 2 (18.05%). The first three principle coordinates represented 53.57% of the total variation (Table 3). PCA data of sequencing analysis of *trnL–trnF* showed that 56.58% of the variation was represented by the coordinates 1 (37.67%) and 2 (18.91%). The first three principle coordinates represented 71.37% of the total variation (Table 3). The PCA graph of RAPD markers dataset indicated that 37 genotypes of *Colchicum* L. were not divided into any groups (Figure 5A), while the PCA graph of sequencing analysis of *trnL–trnF* dataset indicated that 37 genotypes of *Colchicum* L. were divided into three main groups (Figure 5B).

By using RAPD markers and chloroplast intergenic region *trnL–trnF* to characterize the genus *Colchicum*, this study performed to show the phylogenetic relations, genetic diversity, and population structure among 37 *Colchicum* L. genotypes belongs to 16 species.

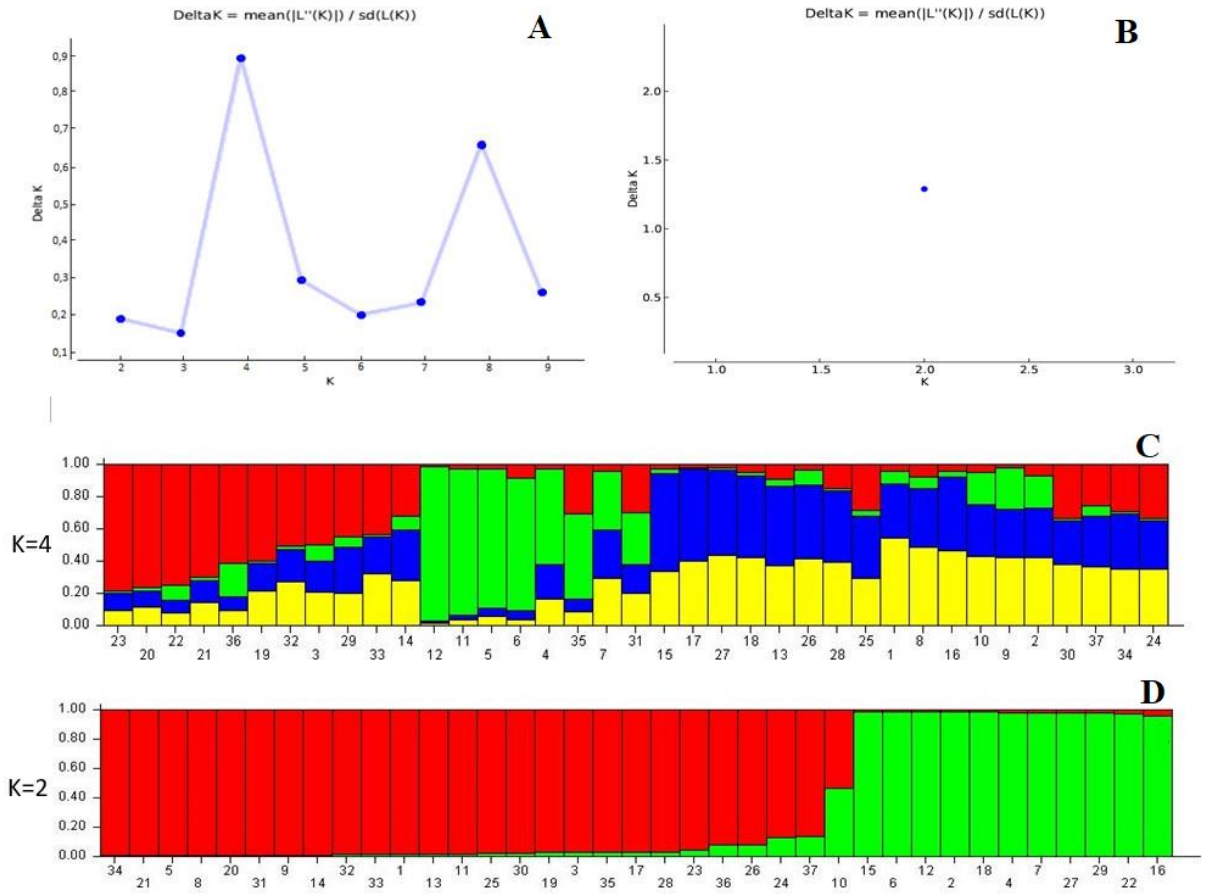


Figure 4. Results of the Bayesian model-based clustering STRUCTURE analyses **A** and **B** ΔK value estimated by structure harvester with using RAPD and sequence analysis of *trn*L-*trn*F. **C** and **D** Structure output of 37 *Colchicum* L. genotypes with RAPD and sequence analysis of *trnL-trnF* data respectively

Table 3. Eigen values, percentage and cumulative percentage of variation in RAPD and *trn*L(UAA)-*trn*F(GAA) PCA data explained by first 3 axes

Axis	Eigen Value	Percentage of Variation	Cumulative Percentage of Variation
RAPD			
1	34.255	18.76	18.76
2	32.962	18.05	36.81
3	30.612	16.76	53.57
***trn*L(UAA)–*trn*F(GAA)**			
1	0.005	37.67	37.67
2	0.002	18.91	56.58
3	0.002	14.79	71.37

The PCA results of RAPD markers showed that 37 genotypes of *Colchicum* L. were not divided into any groups while the PCA results of *trnL–trnF* showed that 37 genotypes of *Colchicum* L. were divided into three main groups. There was no correlation between RAPD markers and *trnL–trnF*, according to PCA results.

Though very few phylogenetic studies on *Colchicum* have been performed, there are many studies related to Colchicaceae family, particularly with *Androcymbium* species. In these studies, covering some *Colchicum* and *Androcymbium* species, Manning et al. (2007) used *rps16* intron, the *atpB-rbcL* spacer and the *trn*L-F region and Del Hoyo & Pedrola-Monfort (2008) used *trn*L intron, *trnL-trnF IGS, trn*Y-*trn*D *IGS, trn*H-*psbA IGS* and RNA*pol2* intron 23 specific markers and they created a phylogenetic tree of *Colchicum* species based upon both *trnL-trnF* sequences and morphology data. The result of this study reported that, the clades were in many cases not very well supported and the origin of the *Colchicum* clade at the side of the Androcymbium group basis indicates that the history of this dispersion event occurred early.

Arzate-Fernández et al. (2005) evaluated the molecular characterization using RAPD, ISSR and cpDNA markers for the selection of *Lilium maculatum* var. *bukosanense* species, a significant correlation between the gene diversity and the population size was reported, and these species should thus be taken into *in-situ* conservation and breeding program. The latest phylogenetic analysis of the genus *Colchicum* including *Bulbocodium* and *Merendera* was published in 2011 by Persson et al. (2011) studied phylogenetic relationships among *Colchicum* species by using six plastid regions, morphological and chromosomal characteristics and reported that the phylogeny of almost all known *Colchicum* species based on sequence data of non-coding chloroplast DNA (cpDNA) was used for the elucidation of phylogenetic relationships among species. According to their results, in a group of phylogenetic, consisting of species with ideal data sampling would include a combination of morphology, plastid sequence data and parentally inherited single-copy nuclear sequence data. The study of phylogenetic relationships between 14 *Colchicum* taxa spreading throughout Turkey performed by fluorescent-based AFLP technique reported that the genetic diversity among these Turkish genotypes is relatively high (Metin et al., 2014). These researchers grouped 14 *Colchicum* taxa into 3 clusters.

Each marker system has its advantages as well as its disadvantages, so it is difficult to clearly determine the phylogenetic relationships of species with only one type of marker. *Colchicum* L., species should be characterized by multiple aspects such as agronomic traits, molecular and cytogenetics data to resolve the phylogeny of genus (Del Hoyo et al., 2009).

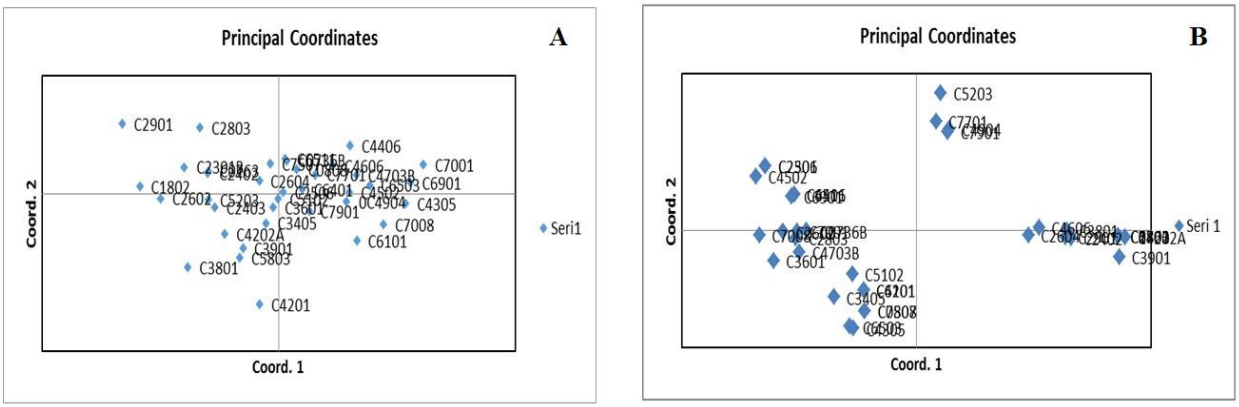


Figure 5. Principal Component Analysis (PCA) for in 37 genotypes of *Colchicum* L. genotyped A with RAPD markers, B with *trnL-trnF* region

Conclusion

Phylogenic data obtained in this study may provide significant contribution to the future studies to increase the power of the resolution in the phylogenetic pattern of *Colchicum*. As the species possess different levels of ploidy, the addition of plastid sequence data to better resolve the phylogenetic classification of the genus for future investigations is important. Additional data such as selected morphometric characteristics and the single-copy nuclear DNA sequence data, will be required to resolve all the relationships and correlations among the *Colchicum* genus.

Acknowledgement

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (Project No: 111T854) and Marmara University Research Foundation (Project No: FENC-DRP-41112-0336). Authors are thankful to Erdal KAYA from Republic of Turkey Ministry of Agriculture and Livestock General Directorate of Agricultural Research and Policy, Atatürk Central Research Institute of Gardening Plants (ACRIGP) for providing plant material.

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