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Araştırma Makalesi

Molecular Phylogeny of Some Geophytes Taxa from Turkey; Systematic Approaches

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Abstract

To determine the evolutionary relationships among some members of geophytes collected from their natural habitat in Kulp-Diyarbakır we carried out molecular phylogenetic analysis of one nuclear DNA (nrDNA) region (internal transcribed spacer, ITS) and one chloroplast DNA (cpDNA) region (intergenic spacer region of *trn*L-F). Despite phylogenetic analysis using maximum likelihood done by two different DNA source show some differences. It was identified that monocotyl petaloid taxa differ from dicotyledone geophyte ones. Also both phylogenetic trees reveal that *Serapias* L.*,* *Anacamptis* Rich.*,* *Cephalanthera* Rich.*,* *Himanthoglossum* Spreng. and *Dactylorhiza* Necker ex Nevski are nested in *Orchis* L. Therefore the infraspecific relationships of these genera should be re-evaluated according to our molecular phylogenetic study results. Dicotyl geophyts show molecular phylogeny in accordance with the classical systematic order in Flora of Turkey and reveal the accuracy of interspecific and infraspecific distinctions of these taxa.

Key words: Geophytes, ITS, *trn*L-F, Molecular phylogeny

Türkiye’ye Ait Bazı Geofit Taksonlarının Moleküler Filogenisi, Sistematik Yaklaşımlar

Öz

Bu çalışmada Kulp-Diyarbakır bölgesindeki doğal habitatlarından toplanan geofitlerin bazı üyeleri arasındaki evrimsel ilişkileri belirlemek için nükleer DNA (nrDNA) bölgesi (internal transkribed spacer, ITS) ve kloroplast DNA(cpDNA) bölgesinin (intergenic spacer, *trn*L-F) moleküler filogenetik analizi gerçekleştirdik. İki farklı DNA kaynağı kullanılarak yapılan maximum likelihood (en büyük olabilirlik kestirimi) yöntemi kullanılarak çizilen filogenetik ağaçlar bazı farklılıklar göstermesine rağmen monokotil petaloid taksonların dikotiledon geofit taksonlarından ayrıldığı görülmüştür. Ayrıca her iki moleküler marker kullanılarak gerçekleştirilen filogenetik analizlerde *Serapias* L.*,* *Anacamptis* Rich.*,* *Cephalanthera* Rich.*,* *Hymanthoglossum* Spreng. ve *Dactylorhiza* Necker ex Nevski cinslerinin *Orchis* L. içerisine dahil olduğu görülmüştür. Bu nedenle, bu cinse ait alt türlerin akrabalık ilişkileri bizim sonuçlarımıza göre yeniden değerlendirilmelidir. Dikotil geofitler, Flora of Turkey'deki klasik sistematik düzene göre moleküler filogeni göstermekte ve bu taksonların tür içi ve türler arası ayrımlarının doğruluğunu ortaya koymaktadır.

Anahtar kelimeler: Geofit, ITS, *trn*L-F, moleküler filogeni.

Introduction

The greatest diversity of bulbs, including petaloid monocotyledons with corms, rhizomes and tubers, can be found in the Flora of Turkey. A classification of plants, proposed by the Danish botanist C. Raunkiaer, based on the position of perennating buds in relation to the soil surface. These are chamaephyte; epiphyte; geophyte; hemicryptophyte; phanerophyte; therophyte. Raunkiaer (1934) has classified plants according to the place where the growth point is located during the less favorable seasons, provided the plant maintains the capability to survive these difficult condition. 'Geophyte' is a Latin word which means 'ground plants or hidden plants' and it is formed by the combination of the words "geo" meaning 'place' and 'phyta' meaning plant. The stems of these plants are metamorphosed in the form of bulb, tuber, corm or rhizome and are located below the soil level (Anonymous, 2020). The geophytes, plants with underground perennating organs like bulbs, corms, tubers or rhizomes lose their aerial parts annually. Perennial belowground elements allow plants to survive periods of severe climate conditions (Dafni et al., 1981; Parsons, 2000; Procheş et al., 2005; Procheş et al., 2006; Kamenetsky, 2013). According to Zencirkıran (2002), although the aboveground organs dry out after completing their development in the growing season, geophytes; are biannual or perennial plants that have organs that can survive under the ground. In geophytes, budding occurs under the ground, while other plants are at or above the ground level. Furthermore, it is known that species of geophytes in different lineages of angiosperms have increased their genome size, facilitating the production of larger cells in the underground perennating organs, which is advantageous for fast growth in seasonal habitats (Vesely et al., 2012).

Geophytes are often conspicuous components of vegetation after burning (Doussi and Thanos, 2002; Verboom et al., 2002; Tyler and Borchert, 2003; Koniak et al., 2009). This life form is more common in monocots, in families like Iridaceae, Orchidaceae, Hyacinthaceae, Amaryllidaceae and Anthericaceae and only occurs in very few dicot taxa (Meerow, 2013). Geophytes have an economic value due to their use in the pharmaceutical industry and remarkable flowers (Güner et al., 1991). Due to their aesthetic properties, fragrances and usability as cut flowers, they are the most preferred plants among ornamental plants (Çığ and Başdoğan, 2015). Geophytes create a strong visual impact due to contribute to the enrichment of landscape both aesthetically and functionally. With the help of their year round blooming feature, they avoid monotony in landscape designs. (Seyidoğlu et al., 2009). Geophytes are used in many areas such as. rock gardens, curbs, grass areas, building entrances, roadsides. In recent years, it has been given possibility of having the impressive design with the participation of Geophytes to planting works (Akdeniz and Zencirkıran, 2016).

Turkey is very rich in native plant diversity which in proportion to its area. The main reasons for this, being at the intersection of different phytogeographical region and different elevation and habitat diversity as well as climatic features. Within the studies about the natural flora in recent years, it is revealed that Turkey's flora consists of about 12500 plant taxa (Özhatay et al., 2003). Geophytes are an important part of this rich flora. According to Davis (1965-1985) geophytes are represented by nearly 600 plant taxa in Turkey and about 40% of them is endemic. This number is 800 according to Güner (2006) and 900 according to Kandemir and Yakupoğlu (2016).

The economic and evolutionary importance of these taxa warrant increased scientific attention. To date, most studies pertaining to geophyte evolution have focused on a handful of taxonomic groups (Patterson and Givnish, 2002; Perret et al., 2003; Wilson, 2006; Oberlander et al., 2009; Evans et al., 2014; Sosa et al., 2016) or geographic regions (Pate and Dixon, 1982; Rundel, 1996; Hoffmann et al., 1998; Parsons, 2000; Parsons and Hopper, 2003; Cuéllar-Martínez and Sosa, 2016; Sosa and Loera, 2017).

Because of minimal distinction of genetic, phenotypic and morphological characteristics, interspecific gene flow and incomplete classification of lineage sorting (Ebersberger et al., 2007) it is inconvenient to find out phylogenetic relationships among groups that have diverged recently and/or rapidly (Mallo and Posada, 2016; Fernández-Mazuecos, et al., 2017). Today, molecular phylogenetic approaches aim to investigate many new genomic regions and create their combinations for reflecting history accurately (i.e., the "species tree"; Ebersberger, et al., 2007).

The aim of this study is to provide first report on the phylogenetic relationship of geophytes grown naturally in Kulp-Diyarbakır. In this study, we used molecular data from entire nrDNA ITS region and we further included sequence information from the cpDNA intergenic spacer between the *trnL* (UAA) 3' exon and *trnF* (GAA) to provide a more comprehensive taxonomic and phylogenetic results and a more stable classification.

Material and Methods

Plant material: Plant material was obtained from silica-gel dried leaved of collected specimens in the wild. from natural habitats in Kulp-Diyarbakır, 2020. The plant materials were identified by Dr. A. Koçak according to Flora of Turkey and East Aegean Islands (Davis, P.H. 1965-1985). Voucher specimens were deposited at the Molecular Biology and Genetics Laboratory of Bingol University. Plant taxa used in this study; *Orchis coriophora* L., *O. mascula* (L.) L. subsp. *pinetorum* (Boiss & Kotschy) G. Camus, *O. punctulata* Steven ex Lindley, *O. laxiflora* Lam., *O. anatolica* Boiss., *O. simia* Lam., *O. papilionacea* L. subsp. *papilionacea, O. tridentata* Scop, *Allium* *scorodoprasum* L. subsp. *rotundum* (L.) Stearn, *A. pallens* L. subsp. *pallens*, *A. cardiostemon* Fisch. & C.A. Mey, *A. wiedemannianum* Regel, *Eminium rauwolffii* (Blume) Schott var. *rauwolffii*, *Scilla siberica* Haw. subsp. *armena* (Grossh.) Mordak, *Ixiolirion tataricum* (Pall.) Schult. & Schult.f. var. *tataricum*, *Gynandriris sisyrinchium* (L.) Parl., *Fritillaria imperialis* L., *F. minuta* Boiss. & Noë., *Anacamptis pyramidalis* (L.) Rich., *Himantoglossum affine* (Boiss.) Schltr., *H. comperianum* (Steven) P.Delforge, *Ranunculus millefolius* Sol., subsp. *millefolius, R. kotchii* Ledeb., *R. asiaticus* L., *R. cuneatus* Boiss., *Muscari comosum* (L.) Mill., *Corydalis caucasica* DC. subsp*. caucasica, Anemone coronaria* L., *Gagea luteoides* Stapf., *G. villosa* (M.Bieb.) Sweet var. *villosa*, *G. commutata* K.Koch. (Güner, 2018), *Gladiolus italicus* Mill., *Bunium paucifolium* DC., *Tulipa armena* Boiss var. *armena*, *Ornithogalum umbellatum* L., *Serapias vomeracea* (Burm.f.) Briq, *Colchicum szovitsii* Fsich. & C.A. Mey. subsp. *szovitsii*, *Dactylorhiza romana* (Seb.) Soó subsp. *romana*, *Iris reticulata* M.Bieb. var. *reticulata*, *I. persica* L., *Crocus biflorus* Mill. subsp. *tauri* (Baw) B.Mathew, *Cephalanthera longifolia* (L.) Fritsch, *Geranium libanoticum* Schenk.

DNA extraction, amplification, and sequencing: Total genomic DNA was extracted by Plant DNA isolation kit supplied by Macherey-Nagel. Polymerase chain reaction (PCR) of the whole region of nrDNA ITS were performed using the ITS AB101 and ITS AB102 primers (Douzery et al., 1999). PCR amplifications were conducted according to the protocols described in Sonboli et al. (2010). Amplification of intergenic spacer between the *trn*L (UAA) 3' exon and *trn*F (GAA) (B49873 and A50272 primers) was performed according to the protocols of Taberlet et al. (1991). Sequencing reactions were performed using ABI 3730 XL (Applied Biosystems).

Alignment and phylogenetic analyses: Phylogenetic analysis were undertaken using ITS1 and ITS2 and *trn*L-F data sets of samples and each included the sequences from the GenBank database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) were aligned using ClustalW (Thompson et al., 1994) software and subsequently checked visually (Table 1). Variable sites, number of parsimony-informative sites, transition, transversion, genetic distance, nucleotide diversity, and divergence within species were computed as molecular diversity statistics for each dataset using Molecular Evolutionary Genetics Analysis software (MEGA 11.0; Tamura et al., 2021). Ultimately, phylogenetic trees were constructed by Maximum Likelihood Method with 1000 bootstrap replicates.

Results and Discussion

The aligned data set of entire ITS and *trn*L-F included a total of 78 (41), and 60 (18) taxa and taxa number taken from GenBank is specified in parenthesis. ITS and *trn*L-F sequences length, GC% content, conserved sites, parsimony informative and variable sites statistics are showed in Table 2.

In total 48 plant taxa among to geophytes were collected from their natural grown area in Kulp-Diyarbakır. Two different primer sites ITS5-ITS4 and A101-A102 were used in PCR reactions for multiplication of nrDNA ITS1, 5.8S and ITS2 regions all of them gave bands around 850-900 bp. Together with 37 taxa nrDNA ITS1, 5.8S and ITS2 regions DNA sequences 41 taxa sequences found from GenBank and evaluated for phylogenetic tree construction. Two sets of primers were used for polymerization of spacer between the *trn*L (UAA) 3ˈ exon and *trn*F (GAA) from chloroplast DNA (cpDNA). All of the samples were amplified with the primer set used for polymerization of intergenic spacer between the *trn*L (UAA) 3ˈ exon and *trn*F (GAA) cpDNA. All gave bands between 300-350 bp. 18 different sequences obtained from GenBank and were added during phylogenetic tree evaluation.

According to our nrDNA analysis results, it is clearly seen that monocotyl petaloid taxa differ from dicotyledonae geophyte ones (Figure 1). Also Orchidaceae family members were completely separated from dicotyledonae geophytes and other monocotyl petaloid families that studied. According to our chloroplast DNA analysis results, all taxa members of monocotyl petaloid came together and completely separated from dicotyl geophyt taxa (Figure 2). Both nrDNA and cpDNA results show that the description of *Orchis, Serapias, Anacamptis, Cephalanthera, Hymanthoglossum* and *Dactylorhiza* genera should be done very carefully. The infraspecific relationships of these genera should be re-evaluated according to our molecular phylogenetic study results. Any phylogenetic problems are not observed in the description of other monocotyl petaloid genera. The studied dicotyl geophyts show molecular phylogeny in accordance with the classical systematic order in Flora of Turkey and reveal the accuracy of interspecific and infraspecific distinctions of these taxa. When compared at the family classification in Flora of Turkey, all genus were gathered together according to the ITS sequence analysis. Despite the enormity of the nuclear genome derived ITS sequences, Iridaceae and Liliaceae are not coherent to this classification according to the *trn*L-F data.

Previous studies indicate that molecular data strongly verify the monophyly of Liliaceae (Thomas et al., 2002). Fay et al., (2006) separated the Liliaceae some clades namely, clade A, B, C, D, and E depends on the results of plastid *rbcL, trnL* intron, *trnL-F* intergenic spacer, *rnatK*, and *ndhF*, and mitochondrial *atp*1 DNA sequences. Clade A includes *Amana* and *Tulipa*, clade B includes *Cardiocrinum*, *Fritillaria* and *Lilium*, clade C includes, *Clintonia* and *Medeola*, clade D includes, clade A, clade B, clade C and *Gagea* and clade E includes *Streptopus*, *Prosartes* and *Scoliopus.* We analyzed *Fritillaria, Gagea* and *Tulipa* in our research. In both trees *Fritillaria, Gagea* and *Tulipa* is sister and show strong relationship.

In recent years, phylogenetic relationships among members of genus *Orchis* and allied genera *Aceras, Barlia, Neotinea, Ophyrs, Anacamptis, Dactylorhiza, Cephalanthera, Himantoglossum* and *Serapias* were inferred from nucleotide sequence variation in the internal transcribed spacer (ITS) regions of nrDNA, cpDNA and RFLP's. The results of these studies showed that, some molecular data give close concordance with floral morphology but some others conflict. Previous authors separated *Orchis* some sections based on karyology, molecular data, morphology and enzymatic characters (Vermeulen's, 1972; Cauwet-Marc and Balayer, 1984; Rossi et al., 1994). Phylogenetic relations of *Orchis* and some related genera also studied by some researchers (Cozzolino et al., 1997; Acto et al., 1998). Molecular analysis based on ITS region data set and a chloroplast DNA restriction fragment length polymorphism carried out Cozzolino et al. (1998) and Aceto et al. (1998) showed that *Orchis* is parahyletic. This consequence depends on the fact that *Orchis* also contains *Acreas* and *Dactylorhiza.* In this relevant study except *Acreas, Orchis* and allied genera *Dactlylorhiza, Serapias, Cephalanthera, Anacamptis* and *Himantoglossum* phylogenetic relationships were investigated. The phylogenetic hypothesis represented here show contrasts with the previous knowledge based on morphology of Vermeulen (1972). Phylogenetic analysis depends on ITS show congruent with the results of *trn*L-F. One of the relevant differences between the two analyses is in the position of *Serapias and Cephalanthera* which are external group to *Orchis* in phylogenetic tree based on ITS sequences and sister group each other in phylogenetic tree constructed by *trn*L-F data. The other difference is the position of the *Anacamptis*, which is nested in our ingroup in *trn*L-F analysis whereas spread in *Orchis* species considering the ITS sequences. According to the results of Aceto et al. (1998) consensus tree for ITS shows *Himantoglossum* at the base of the tree in a sister group relationship of the rest of the ingroup. On the contrary of this results, our both ITS and *trn*L-F analysis results shows that *Dactlylorhiza* and *Himantoglossum* are in close relationship with *Orchis*, *Allium* belongs to Amarylidaceae family of the monocot Asparagales order as Orchidaceae family. Phylogenetic results based on nuclear and chloroplast derived sequences are compatible with the expected close relationship between *Orchis* and *Allium.*

Ixioideae, the largest subfamily of Iridaceae and diverse from other members by having specialized pollen grains with tectate-perforate and scabrate sculpturing (Goldblatt et al., 1991). Crocoideae is the synonyms of the Ixioideae and represented by three genera in the flora of Turkey: *Crocus, Romulea* and *Gladiolus* (Güner et al., 2000). Based on their connate tepals, sessile flowers, operculate pollen with porous sculpturing exine, closed leaf sheath, and having corm they are count in monophyletic (Rashed-Mohassel, 2006). *Crocus* and *Gladiolus* are dissimilar to the other members of the subfamily by the terms of corm structure (Erol et al., 2008). According to our results of ITS and *trn*L-F data sequences *Crocus* and *Gladiolus* show very close relationship. Also, *Iris* belongs to Iridaceae subfamily of Iridoideae and tribe Irideae (Goldblatt, 2000). *Gynandriris* is a genus in Irideae tribe and *Moraea* Mill. *Moreae* and *Iris* constitute the major genera of Iridaea. *Moraea* have *Iris*-like flowers (Allen, 1975), however, *Iris* shows vegetative diversity whereas *Moreae* is characterized by vegetative uniformity and floral divergence (Goldbatt, 2000). *Gynandriris sisyinchium* (L.) Parl., *Morea austris* is nested in *Iris* in phylogenetic tree constructed by *trn*L-F data. Irideceae and allied family Asian Ixioliriaceae not indicated by external morphology. According to Goldbatt (2000) these two families is differentiated very long time ago and their relationship have been obscured at the morphological level and Iridaceae appear phylogenetically isolated (Goldbatt, 2000). Chase et al. (1995) studied molecular phylogeny by plastid gene *rbc*L and indicated that Iridaceae and Ixioliriaceae specify strong closer relationship among other members of Asparagales (Chase et al., 1995). Clearly considering the ITS and *trn*L-F derived trees Iridaceae and Ixioliriaceae are phylogenetically separated.

*Muscar*i and *Scilla* are belonged to the Asparagaceae, subfamily of Scilloideae. Both sequences data indicate close relationship of *Muscari* and *Scilla.* Previous data also revealed *Muscari* closely related to *Scilla* (Pfosser and Speta, 1999).

The *Ranunculus* and *Anemone* are members of Ranunculaceae. Cladistic molecular analysis of Ranunculaceae was done by some researchers as Johansson and Jansen (1993), Johansson (1995), and Hoot (1995) and they closely allied *Ranunculus* to *Anemone* L. We found that *Ranunculus* and *Anemone* show close relationship and relevant data support the preceding researches.

In conclusion, molecular systematics of all species belonging to the genera should take into account in order to clearly identify the genera of the studied geophytic taxa and to reveal the intraspecific and interspecific relationships clearly.

Table 1. Accession number from the NCBI database

Specimens	**Internal transcribed spacer (ITS)**	***trnL-F***
Fritillaria imperialis	AY616725.1	
Fritillaria chitralensis	AY616716.1	
Fritillaria minuta	AY616733.1	
Fritillaria crassifolia	AY616717.1	
Fritillaria thunbergii		KF851029.1
Tulipa armena var. *armena*	Q776500.1	
Tulipa julia	HF952964.1	
Tulipa borszczowii	HF952959.1	
Tulipa agenensis	JQ280384.1	
Iris minutoaurea	KT119547.1	
Iris odaesanensis	KT595384.1	
Iris lactea	DQ277639.1	
Iris koreana	KT634245.1	
Iris caucasica subsp. *turcica*		KY319464.1
Iris histrio		JQ413996.1
Crocus biflorus subsp. *adamii*	HE663958.1	
Crocus almehensis	HE801162.1	
Crocus roopiae	LN864717.1	
Crocus neglectus		KT357298.1
Ornithogalum refractum	HQ615075.1	
Gagea fragifera	EU912046.1	AM283102.1
Gagea villosa var. *hermonis*		KU232888.1
Ixiolirion tataricum		KF261069.1
Ranunculus linearilobus	MW737445.1	
Ranunculus leptorrhynchus	MW737444.1	
Ranunculus oxyspermus	MT271834.1	
Ranunculus kochii	AY680193.1	
Anemone edwardsiana	FJ639880.1	
Corydalis pumila	MN662999.1	
Corydalis paczoskii		HE603350.1
Bunium elegans	KF974538.1	
Bunium allioides	JX312805.1	
Allium pallens	KP221824.1	
Allium paniculatum	AJ411949.1	
Allium longipapillatum	MK776898.1	
Allium chrysantherum	MG944302.1	
Allium cardiostemon	FM177277.1	
Allium rothii	FM177400.1	
Allium oleraceum		FJ628602.1
Allium sativum		EU626261.1
Allium latifolium		MT130438.1
Serapias orientalis	KY512512.1	
Serapias nurrica		EF690287.1
Anacamptis papilionacea	KY512514.1	
Anacamptis coriophora	MF944259.1	KU931746.1
Anacamptis palustris	KU931742.1	
Orchis purpurea	MT179742.1	
Orchis adenocheila	KU931695.1	
Cephalanthera humilis		JN706694.1
Dactylorhiza umbrosa		KU931765.1
Ornithogalum refractum		HQ645873.1
Gladiolus illyricus		KM887320.1
Ixiolirion tataricum		AJ290314.1
Eminium spiculatum		AM933357.1
Geranium tuberosum		KY606615.1

Table 2. Numeric information of ITS and *trn*L-F

	ITS	*trn*L-F
Length of the aligned sequence	885	331
GC% content	56	33.2
Conserved sites	3	1
Parsinomy informative sites	813	305
Variable sites	856	307

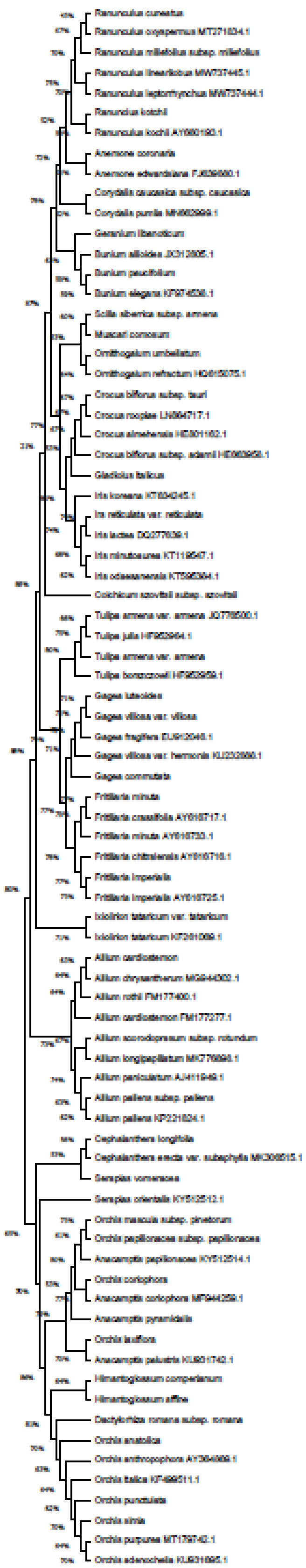

Figure 1. Maximum Likelihood tree based upon the Tamura-Nei model of nrDNA ITS region with 1000 bootstrap replicates.

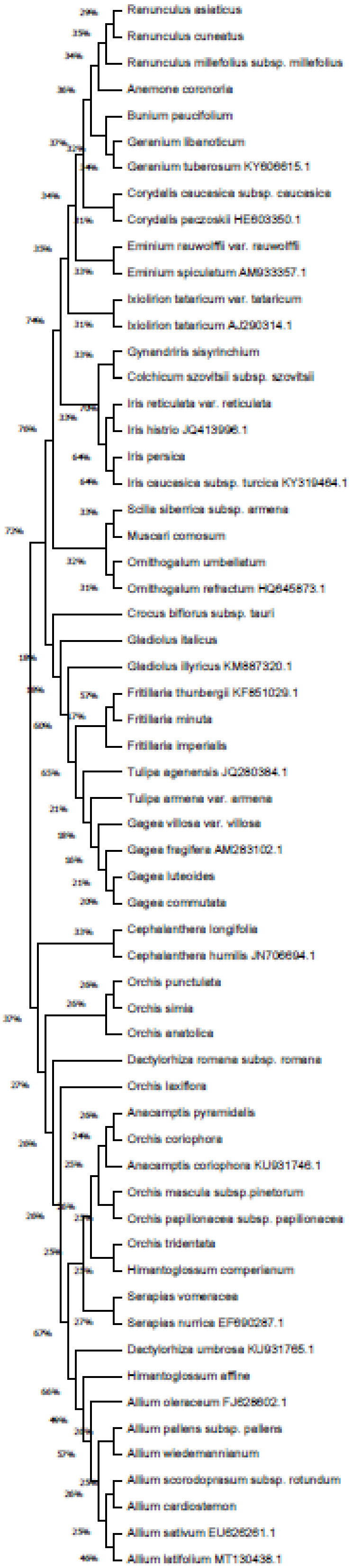

Figure 2. Maximum Likelihood cpDNA tree based on the data obtained from the sequences of the *trn*L-F

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