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NURSEL İKİNCİ

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Molecular phylogeny and divergence times estimates of *Lilium* section *Liriotypus* (Liliaceae) based on plastid and nuclear ribosomal ITS DNA sequence data

Nursel İKİNCİ

Biology Department, Faculty of Arts and Science, Abant İzzet Baysal University, TR-14280 Bolu - TURKEY

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Abstract: In the present study the phylogeny and the biogeography of the genus *Lilium* L. section *Liriotypus* Asch. et Graebn. were investigated and divergence times for the section *Liriotypus* were calculated. The study group covers *Lilium* species from Europe, the Italian and Balkan peninsulas, Anatolia, and the Caucasus. Plastid DNA sequence data (the *trn*C-*pet*N intergenic spacer and *pet*N gene) and nuclear DNA ITS sequence data were used to infer the phylogenetic history of the section *Liriotypus*. Molecular phylogenetic dating using the molecular clock hypothesis with sequences of nrDNA ITS region was used to calculate the time of diversification within the section *Liriotypus* and with other members of the genus *Lilium*. The phylogenetic reconstruction based on combined analysis of plastid and nrDNA ITS sequence data shows that all of the analysed species of section *Liriotypus* form a well-supported monophyletic clade. However, there are some incongruences between the analysis made by plastid DNA alone and with a combined dataset regarding the positions of *L. monadelphum* M.Bieb., *L. szovitsianum* Fisch. & Avé-Lall., *L. ciliatum* P.H.Davis, and *L. akkusianum* R.Gämperle. Our molecular dating analysis based on nrDNA ITS sequence data showed that members of the section *Liriotypus* were separated from the rest of the genus *Lilium* approximately 9 million years ago and within this section, speciation increased in the last 6 million years.

Key words: *Lilium, Liriotypus*, Liliaceae, phylogeny, plastid DNA, ITS ribosomal DNA

Lilium cinsi *Liriotypus* seksiyonunun (Liliaceae) plastid ve çekirdek ribozomal DNA ITS dizi verilerine dayalı moleküler filogenetiği ve ayrışma tarihlerinin hesaplanması

Özet: Bu çalışmada *Lilium* cinsi L. *Liriotypus* Asch. et Graebn. seksiyonunun filogenetiği ve biyocoğrafyası araştırılmış ve *Liriotypus* seksiyonu için ayrışma tarihleri hesaplanmıştır. Çalışma grubu Avrupa, İtalya ve Balkan yarımadaları, Anadolu ve Kafkas *Lilium* türlerinden oluşmaktadır. *Liriotypus* seksiyonunun filogenetik geçmişini ortaya çıkarmak için plastid DNA dizi verisi (*trn*C-*pet*N intergenic spacer ve *pet*N geni) ve çekirdek DNA'sı ITS (Internal transcribed spacer) dizi verileri kullanılmıştır. *Liriotypus* seksiyonu içerisindeki türlerin birbirlerinden ve diğer *Lilium* türlerinden ayrışma tarihleri çekirdek DNA'sı ITS bölgesi kullanılarak moleküler saat hipotezi gözönünde bulundurularak moleküler filogenik tarihleme ile hesaplanmıştır. Plastid ve çekirdek DNA dizilerinin birleştirilmesi sonucu yapılan filogenetik analiz incelenen *Liriotypus* seksiyonu türlerinin güçlü bir destekle monofiletik bir grup oluşturduklarını göstermiştir. Ancak,

* E-mail: ikinci_n@ibu.edu.tr

plastid DNA'sı ile birleştirilmiş verilerden yola çıkılarak yapılan analizler *L. monadelphum* M.Bieb., *L. szovitsianum* Fisch. & Avé-Lall., *L. ciliatum* P.H.Davis ve *L. akkusianum* R.Gämperle türlerinin pozisyonu konusunda farklı sonuçlar vermiştir. Çekirdek DNA'sı ITS dizi verisine dayalı moleküler tarihleme analizi *Liriotypus* seksiyonu üyelerinin *Lilium* cinsinin diğer türlerinden yaklaşık dokuz milyon yıl önce ayrıldığını ve bu seksiyon içinde türleşmenin son altı milyon yıl içinde arttığını göstermiştir.

Anahtar sözcükler: *Lilium, Liriotypus*, Liliaceae, filogenetik, plastid DNA, ITS ribozomal DNA

Introduction

Lilium L. (*Liliaceae*) is a bulbous, perennial genus of approximately 100 species that are mainly distributed between the 10° to 60° latitudes of the northern hemisphere (McRae, 1998). The center of origin is south-western and Himalayan Asia (Comber, 1949; Woodcock & Stearn, 1950). The genus *Lilium* has been divided into 5-11 sections by different authors (Endlicher 1836-1840; Baker, 1871; Wilson, 1925; Comber, 1949; Baranova, 1988) based mainly on morphological character. The most commonly utilised character are the flower shape and pose. Comber (1949) classified the *Lilium* species into 7 sections according to 15 character. He used germination type, leaf arrangement, shape, bulb colour and habit, seed weight, stem type, flower shape, and nectar type as the main distinguishing character. According to his classification, sect. *Liriotypus* Asch. & Graebn. comprises about 20 species and includes all the European and Caucasian lilies except for *L. martagon* L. Members of sect. *Liriotypus* mainly have delayed epigeal germination, scattered leaves, numerous entire bulb scales and Turk's-cap flowers, except for *L. candidum* L. and *L. bulbiferum* L. Baranova (1988) divided the genus *Lilium* into 11 sections. According to her classification, European and Caucasian lilies are grouped under 4 sections. Monotypic sect. *Lilium* contains *L. candidum*, while *Lilium martagon* is placed in sect. *Martagon* Duby, which includes other lilies with verticillate leaf arrangement. *Lilium bulbiferum* is grouped under sect. *Pseudolirium* Endl., which contains upward-flowered lilies. The rest of the European and Caucasian lilies are grouped under sect. *Eurolirium* Baranova.

All the species in sect. *Liriotypus* have similar linear to lanceolate leaves and wind-dispersed winged seeds. Furthermore, they have very similar bulbs, which are concentric ovoid, with scales that are yellow, white or pinkish in color. The main centers of diversity of the members of sect. *Liriotypus* are the Caucasus together with northeastern Turkey and the Balkan peninsula. *Lilium polyphyllum* D.Don from the western Himalayas was also previously classified under sect. *Liriotypus* (Comber, 1949), but has been transferred to sect. *Nepalensia* Baranova by Baranova (1988). This species has both scattered leaves and whorled leaves at the lower part of the stem. This feature is not found in other members of sect. *Liriotypus* (Woodcock & Stearn, 1950).

Lilium bulbiferum, having erect flowers in a terminal umbel, was also included in sect. *Liriotypus* by Comber (1949). However, this species was included in sect. *Isolirion* Baker by Baker (1871) and in sect. *Pseudolirium* by Wilson (1925), together with other erect flowering lilies with non-revolute perianth segments. Wilson's (1925) sect. *Pseudolirium* is equivalent to sect. *Isolirion* of Baker (1871), comprising the erect-flowering lilies. Baranova (1988) also placed *L. bulbiferum* in sect. *Pseudolirium,* which includes both Asian and American lilies with upward or cup-shaped flowers. There are only 6 *Lilium* species with such a flower shape, distributed through Europe (*L. bulbiferum*), North America (*L. philadelphicum* L. and *L. catesbaei* Walter), and Asia (*L. dauricum* Ker Gawl., *L. concolor* Salisb., and *L. tsingtauense* Gilg) and these are considered the most primitive lilies based on the age and area hypothesis (Stoker, 1939). Among the European and Caucasian lilies, only one variety of *L. bulbiferum* has stem bulbils (McRae, 1998). Recent molecular studies indicated that *L. bulbiferum* is not part of sect. *Liriotypus* (Nishikawa, 2001; İkinci et al., 2006) but rather is placed in sect. *Sinomartagon* H.F.Comber. The results of Nishikawa et al. (2001) indicated that sect. *Sinomartagon* consists of sect. *Daurolirion* H.F.Comber and *L. bulbiferum*. Comber's (1949) sect. *Sinomartagon* contains eastern Asian *Lilium* species with scattered leaves and Turk's-cap flowers.

The available chromosome studies show that the chromosome number is 2n = 24 for members of sect. *Liriotypus* (Stewart, 1947; Kudriashova, 1969; İnceer et al., 1999, 2002; Siljak-Yakovlev et al., 2003; Muratović et al., 2005, 2010). In addition, there are pollen studies for *L. martagon, L. candidum, L. pyrenaicum* Gouan, *L. szovitsianum Fisch. & Avé-Lall.,* and *L. kesselringianum* Miscz. (Baranova, 1985; Kosenko, 1999). Pollen morphology studies of *L. kesselringianum* indicated that its pollen is monosulcate, the surface of its aperture membrane is granular, and its exine surface is macroreticulate.

There have been several molecular phylogenetic studies on the genus *Lilium* that also included species from sect. *Liriotypus*. The most commonly employed nuclear marker is the nuclear ribosomal DNA ITS region (Dubouzet & Shinoda, 1999; Nishikawa et al., 1999, 2001; Rønsted et al., 2005; İkinci et al., 2006; Rešetnik et al., 2007). This region has been used for other monocots to elucidate taxonomic problems of different genera (Dizkırıcı et al., 2010). Hayashi and Kawano (2000) used *rbcL* and *matK* gene sequence data as plastid markers for resolving the phylogenetic relationships of *Lilium* and closely related genera. Rønsted et al. (2005) employed the *matK* gene and the *trnK* intron, *rpl16* plastid gene and the nuclear ribosomal ITS region for 14 *Lilium* species. In addition, the above-mentioned molecular phylogenetic studies showed that *Fritillaria* L., *Nomocharis* Franch., and *Cardiocrinum* (Endl.) Lindl. are the closest genera to *Lilium* (Nishikawa et al., 1999; Hayashi & Kawano, 2000; Patterson & Givnish, 2002; Rønsted et al., 2005).

The first purpose of this study is to present the phylogenetic relationships of sect. *Liriotypus* based on nuclear and plastid DNA markers. The *trn*C-*pet*N intergenic spacer and *pet*N gene that are located in the large single copy (LSC) region of chloroplast DNA (Demesure et al., 1995) were used as plastid markers. Plastid sequence data were obtained for 22 species for the first time for this study. Nuclear DNA ITS sequences were obtained from our previous publications and from GenBank. There has been only one study about age calculations of the genus *Lilium* where phylogenetic dating was performed for major clades of Liliales by utilising plastid *rbcL* sequence data (Vinnersten & Bremer, 2001). Therefore, our second purpose is to measure the divergence times for the members of sect. *Liriotypus* by utilising the nrDNA ITS sequences.

Materials and methods

Plant material

DNA was extracted from leaf tissue dried by silica gel in the field, and for a few taxa, from herbarium specimens. Chloroplast DNA sequences of the *trn*C-petN intergenic spacer and the *pet*N gene were generated for the first time for 22 species in this study. The ingroup consisted of 20 *Lilium* taxa, as outgroups *Fritillaria latifolia* Willd. and *Cardiocrinum giganteum* (Wall.) Makino were used based on the results of Rønsted et al. (2005) and İkinci et al. (2006). Sequence information for the nrDNA ITS region comes from earlier publications (Nishikawa et al., 1999; Rønsted et al., 2005; İkinci et al., 2006; Rešetnik et al., 2007). Voucher information and GenBank accession numbers of all the analysed taxa are given in Table 1.

DNA extraction, PCR amplification and sequencing

Total DNA extractions were performed using a modified CTAB protocol (Doyle & Doyle, 1987). The *trn*C-*pet*N intergenic spacer and *pet*N gene were amplified using the primers *trnC* (5‘- CCA GTT CAA ATC TGG GTG TC-3’) (Demesure et al., 1995) and *petN2R* (5‘- CCA TTA AAG CAG CCC AAG CAA GAC-3’) (Lee & Wen, 2004). PCR amplifications were performed with 0.2 μM dNTP’s, 0.02 μM of each primer, 0.2 U Taq polymerase (Qbiogene) in 10 μL 1× buffer and the following temperature profile: 2 min at 94 °C, then 40 cycles of 30 s at 94 °C, 1 min at 62 °C, 1 min at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were visualised on 1.5% agarose gel electrophoresis. Purification of the PCR products was done by utilising Agencourt® AMPure® magnetic beads (Agencourt Bioscience Corporation, Beverly, MA, USA) according to the manufacturer’s instructions. Cycle sequencing of the purified PCR products was performed by using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter) and the sequences were analysed on a CEQ 8000 automated sequencer (Beckman Coulter).

Table 1. Taxa sampled, voucher information, and GenBank accession numbers for DNA sequences used in this paper.

	nrDNA ITS		Plastid DNA	
Taxon	Voucher	GenBank accession	Voucher	GenBank accession
Lilium L.				
Section *Archelirion* Baker				
L. auratum Lindl.	Nishikawa et al. (1999)	AB020472		
L. japonicum Thunb.	Nishikawa et al. (1999)	AB020451		
Section *Daurolirion* H.F.Comber				
L. dauricum Ker Gawl.	Nishikawa et al. (1999)	AB020473	İkinci 3666 (AIBU)	FN677510
Section *Leucolirion* Wilson				
L. longiflorum Thunb.	Yang & Chen unpubl.	AY684927		
L. philippinense Baker	Nishikawa et al. (1999)	AB020437		
Section *Liriotypus* Asch. & Graeb.				
L. akkusianum R.Gämperle	İkinci 1928 (AIBU)	AM292422	İkinci 1928 (AIBU)	FN677501
L. albanicum Griseb.	Dörfler 432 (WU)	AM292432	İkinci 2269 (AIBU)	FN677499
L. armenum Miscz. ex Grossh.	İkinci 1933 (AIBU)	AM292425	İkinci 1934 (AIBU)	FN677505
L. artvinense Miscz.	İkinci 1960 (AIBU)	AM292427	İkinci 1960 (AIBU)	FN677502
L. bosniacum (G.Beck) Beck ex Fritsch	Zollitsch (M-0056366)	AM292423		
L. bulbiferum L.	Nishikawa et al. (1999)	AB020468		
L. candidum L.	İkinci 1912 (AIBU)	AM292424	İkinci 1912 (AIBU)	FN677507
L. carniolicum Bernh. ex W.Koch	1988 (M-0056392)	AM292419	Korb 1951-3030 (W)	FN677514
L. chalcedonicum L.			İkinci 2255 (AIBU)	FN677498
L. ciliatum P.H.Davis	İkinci 1932 (AIBU)	AM292421	İkinci 2275 (AIBU)	FN677512
L. jankae A.Kern.	J.Cstaó (B)	AM292431	İkinci 3669 (AIBU)	FN677509
L. kesselringianum Miscz.	İkinci 1966 (AIBU)	AM292429	İkinci 1966 (AIBU)	FN677497
L. monadelphum M.Bieb.	29 May 1970 (JE)	AM292418	coll. Ignot. (JE)	FN677511
L. pomponium L.	Nishikawa et al. (2001)	AB035281	İkinci 999 (AIBU)	FN677500
L. ponticum K.Koch	İkinci 1944 (AIBU)	AM292426	İkinci 1944 (AIBU)	FN677504
L. pyrenaicum Gouan	Nishikawa et al. (1999)	AB020428	İkinci 3670 (AIBU)	FN677508
L. rhodopaeum Delip.	Strid et al. 19503 (B)	AM292430	Strid et al. 19503 (B)	FN677517
L. szovitsianum Fisch. & Avé-Lall.	İkinci 1973 (AIBU)	AM292428	İkinci 1973 (AIBU)	FN677503
Section *Martagon* Rchb.				
L. martagon L.	Nishikawa et al. (1999)	AB020455	İkinci 1924 (AIBU)	FN677518
Section *Pseudolirium* Endl.				
L. canadense L.	Nishikawa et al. (1999)	AB020457		
L. philadelphicum L.	Nishikawa et al. (1999)	AB020432		
Section *Sinomartagon* H.F.Comber				
L. nepalense D.Don	Nishikawa et al. (1999)	AB020444		
L. pumilum DC.	Nishikawa et al. (1999)	AB020430		
L. davidii Duch.	Nishikawa et al. (1999)	AB020461	İkinci 3667 (AIBU)	FN677513
L. henryi Baker			İkinci 3672 (AIBU)	FN677516
Nomocharis Franch.				
Nomocharis saluenensis Balf.	Nishikawa et al. (1999)	AB020449		
Outgroups				
Cardiocrinum giganteum (Wall.) Makino	Nishikawa et al. (1999)	AB020466	İkinci 3671 (AIBU)	FN677515
Fritillaria latifolia Willd.	İkinci 1977 (AIBU)	AM292420	İkinci 1977 (AIBU)	FN677506
Notholirion thomsonianum (Royle) Stapf.	Rønsted et al. (2005)	AY616752		

Alignment and phylogenetic analysis

The DNA sequences were aligned using BioEdit (version 5.0.6, Hall, 1999) and then corrected manually. Maximum parsimony analysis was performed with the obtained sequences by using PAUP* (version 4.0b10, Swofford, 2002). Plastid DNA sequences were analysed independently and in combination with the previously published ITS sequences (İkinci et al., 2006). The analyses were conducted using a heuristic search with 1000 replicates of random addition sequences and a maximum of 10 trees retained per replicate. Starting trees were obtained via random stepwise addition with tree-bisection-reconnection (TBR) branch swapping and the MulTrees option in effect. Character states were specified as unordered and unweighted. A bootstrap analysis was performed to measure the support for clades with a bootstrap replicate of 1000.

Divergence times estimation and model selection

Divergence times were estimated by using the sequences of nrDNA ITS region. In order to accomplish a maximum likelihood (ML) analysis, the best fitting model of sequence evolution was determined using a hierarchical likelihood ratio test executed in MODELTEST (version 3.06, Posada & Crandall, 1998). This resulted in acceptance of the Kimura two parameter model. The model selected was trN+G. Using this model, the ML search was performed with Treefinder (version June 2004, Jobb et al., 2004). In order to calibrate the chronogram, data were obtained from the previous dating of monocots (Bremer, 2000; Vinnersten & Bremer, 2001). They used *rbcL* sequences of 44 taxa representing 7 families of Liliales and outgroups and estimated the split between Liliales and their sister group as occurring 112 mya and the age of the basal node within Liliales as 82 ± 10 my. They used a nonparametric rate smoothing approach (Sanderson, 1997) and presented the age of the node separating the *Fritillaria* and *Nomocharis* clade from *Lilium* as 6 ± 2.9 my (confidence interval estimated using the mean branch length (mbl) method) and 7 ± 5.3 my (confidence interval estimated using the nonparametric rate smoothing (nprs) method). We used these dates to calibrate our tree. *Lilium ponticum* K.Koch and *L. artvinense* Miscz. were excluded from the study since they had zero length.

Results

Sequence characteristics of the plastid and nuclear DNA ITS region and a description of consensus trees obtained from these 2 datasets are listed in Table 2.

Table 2. Sequence characteristics of the *trn*C - petN intergenic spacer with the *pet*N gene and the internal transcribed spacer region (ITS, 5.8S rRNA, ITS2) of section *Liriotypus*.

Sequence Characteristics	*trn*C - petN intergenic spacer + *pet*N gene	ITS region	Combined (plastid + ITS)
Total characters (bp)	535	654	1189
Number of taxa	22	21	21
No. of variable positions	32 (6.0%)	178 (27.2%)	210 (17.7%)
No. of parsimony-informative positions	11 (2.1%)	92 (14.1%)	103 (8.7%)
Length	36	288	318
Consistency index	0.88	0.70	0.72
Retention index	0.88	0.70	0.74

Analyses of plastid DNA data

Phylogenetic analysis was performed with 22 taxa, and *Fritillaria* and *Cardiocrinum* were used as the outgroup. The resulting strict consensus tree together with bootstrap percentages is shown in Figure 1. Section *Liriotypus* was supported by 83 bootstrap percentages (BP). The *Lilium jankae* A.Kern and *L. rhodopaeum* Delip. clade received moderate support (64 BP). NE Turkish and Caucasian species (excluding *L. szovitsianum* and *L. monadelphum*) received good support (88 BP). Within this group, the clade formed by *L. kesselringianum*, *L. ciliatum*, *L. ponticum*, and *L. artvinense* received moderate support (67 BP). The last clade was formed by *L. dauricum* Ker Gawl., *L. martagon*, and *L. davidii* Duch. with a support value of 79 BP.

Analyses of combined Plastid DNA and ITS datasets

Our phylogenetic analysis based on nrDNA ITS sequences was published previously (İkinci et al., 2006). Therefore, we present here only the combined analysis of plastid and ITS DNA sequences. The combined dataset included 21 taxa that have sequence data for both nuclear and plastid regions. *Lilium chalcedonicum* L. and *L. henryi* Baker were excluded from the combined analysis since they have only plastid sequence data. *Lilium bulbiferum* and *L. bosniacum* (G.Beck) Beck ex Fritsch have sequence

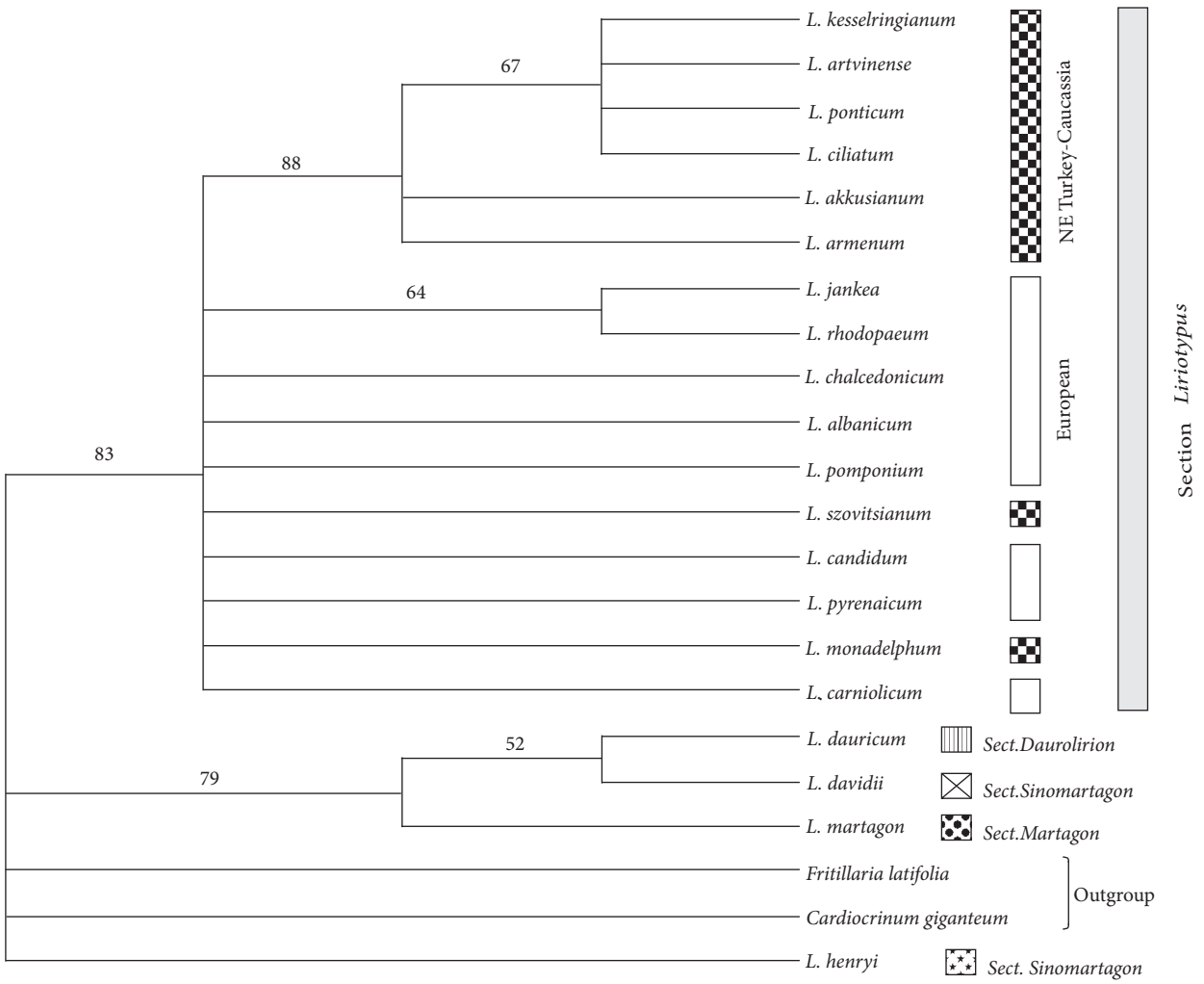

Figure 1. Phylogenetic tree of cpDNA (trnC-petN intergenic spacer + petN gene) data. Bootstrap analysis was performed with 1000 replicates.

data of the DNA ITS region (İkinci et al., 2006) but not of the plastid region. Therefore, these 2 species also could not be included in the combined analysis. The combined dataset contains a much higher number of parsimony informative sites due to variation in the ITS sequences. The strict consensus tree obtained from the combined analysis provides more resolution than the analysis of plastid data (Figure 2). The *Lilium* species form a monophyletic group with a very high support of 100 BP. Monophyly of sect. *Liriotypus* is also supported by a value of 100 BP. There are 2 major groupings in sect. *Liriotypus*. The first clade containing all NE Turkish and Caucasian lilies except *L. ciliatum* and *L. akkusianum* has a bootstrap support of 96%. Phylogenetic relationships within this clade are fully resolved with moderate bootstrap values. *Lilium monadelphum* is a sister to all 5 other species in this clade and they have a support of 62 BP. *Lilium szovitsianum* is a sister to 4 other species in this group. The *L. artvinense*, *L. kesselringianum,* and *L. ponticum* clade has good support (82 BP).

The second clade within sect. *Liriotypus* contains all the European species plus *L. candidum, L. ciliatum,* and *L. akkusianum* and has a support of 89 BP. The *Lilium akkusianum* and *L. ciliatum* clade has strong support (93 BP), while the other clade of European lilies is moderately supported (75 BP). The *Lilium candidum*, *L. jankae,* and *L. rhodopaeum* clade is weakly supported, yet the *L. jankae* and *L. rhodopaeum* clade has moderate support (81 BP). Monophyly of these 2 species was further supported by the analysis of plastid DNA sequences. The *Lilium chalcedonicum*, *L. pyrenaicum,* and *L. pomponium* L. clade has weak support (56 BP). Nevertheless, *L. pomponium* and *L. pyrenaicum* are sisters to each other with a BP of 81. The last clade in this group is

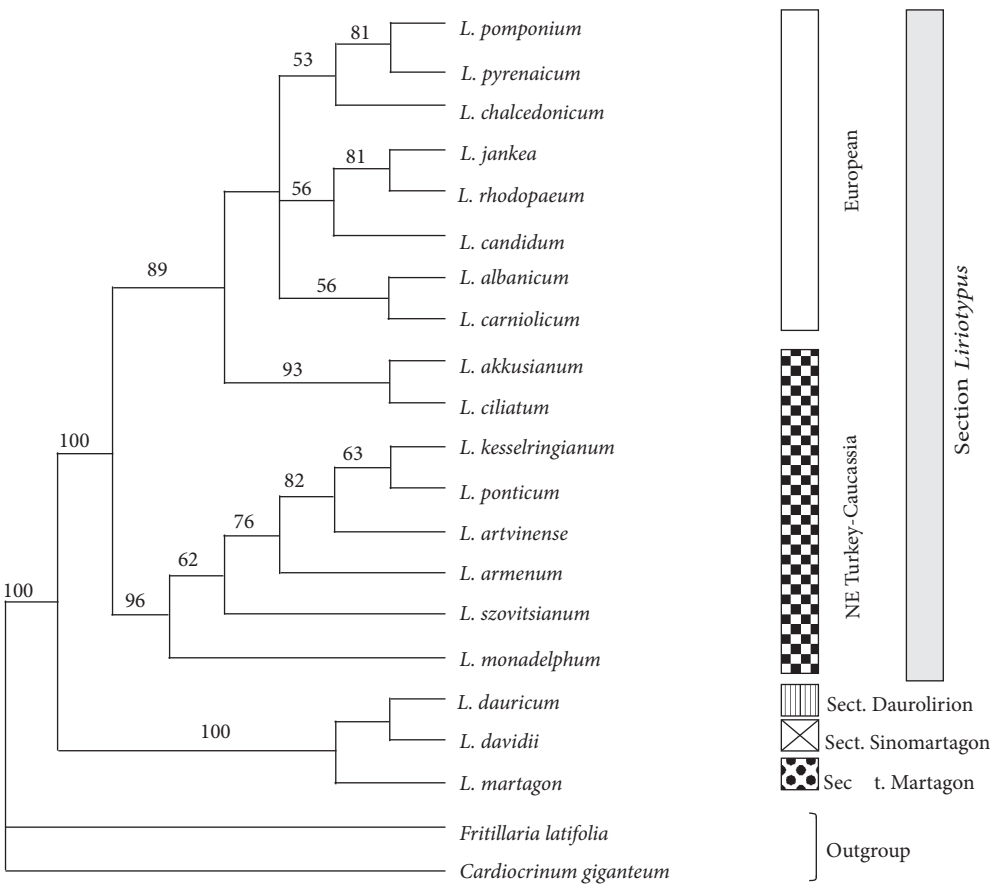

Figure 2. Phylogenetic tree of the combined data set of cpDNA (trnC-petN intergenic spacer + petN gene) and nrITS region (ITS, 5.8S rRNA, ITS2). Bootstrap analysis was performed with 1000 replicates.

formed by *L. albanicum* Griseb. and *L. carniolicum* with low support (56 BP).

Divergence times estimates

Our molecular dating analysis based on nrDNA ITS sequence data showed that the *Lilium* species were separated from closely related genera (*Cardiocrinum, Fritillaria,* and *Notholirion* Wall. ex Voigt) around 12 million years ago (mya) (Figure 3). Members of the section *Liriotypus* were separated from the rest of the genus *Lilium* approximately 9 mya. Within the section *Liriotypus*, the first diversification took place around 8 mya with the separation of the Caucasian lilies *L. kesselringianum*, *L. szovitsianum*, *L. monadelphum,* and *L. armenum* Miscz. ex Grossh. from the other members of the section *Liriotypus*. Two Turkish endemics, *L. akkusianum* and *L. ciliatum* diverged from the rest of the European lilies (including *L. candidum*) less than 6 mya. The clade containing *L. candidum, L. rhodopaeum* and *L. jankae* separated from other European lilies less than 5 mya.

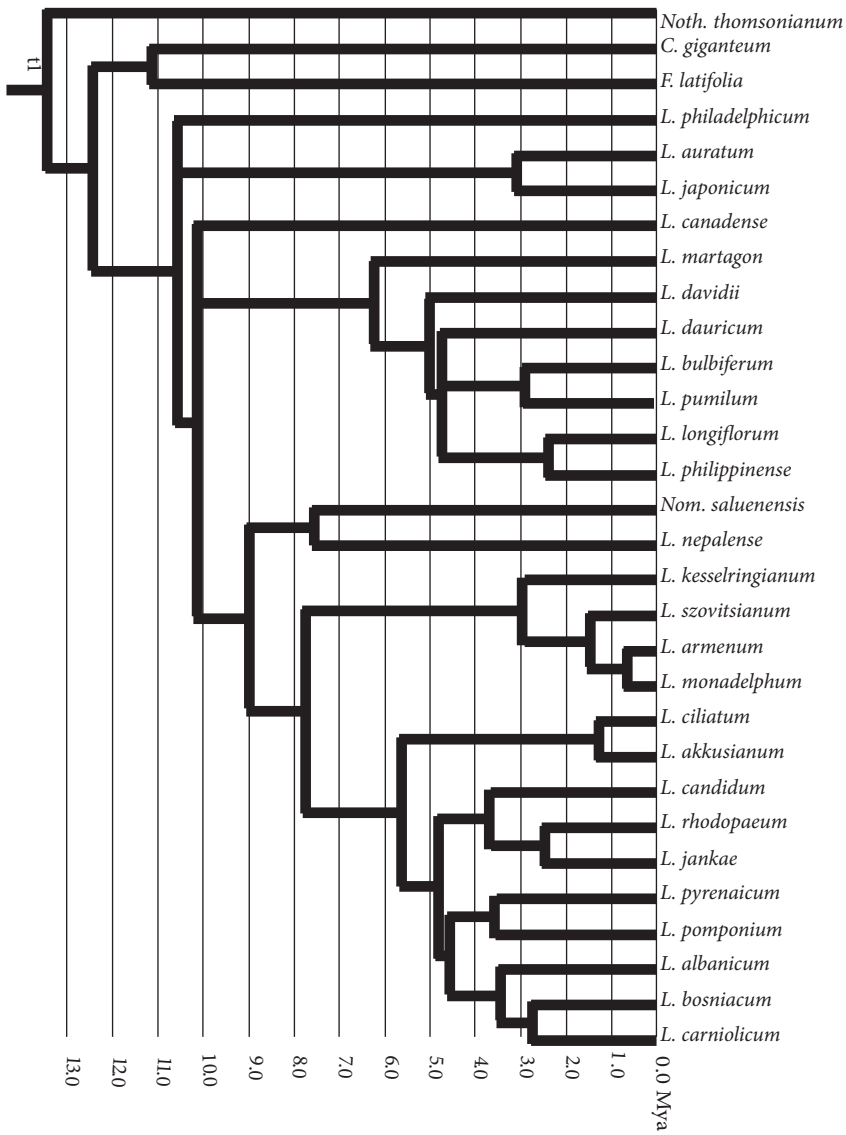

Figure 3. Dated phylogenetic tree from a maximum likelihood (ML) analysis of nrDNA ITS sequence data for Lilium species.

Discussion

The analysis of plastid DNA sequences supports the monophyletic origin of sect. *Liriotypus*. *Lilium henryi* is not grouped with other *Lilium* species. Comber (1949) placed *L. henryi* in sect. *Sinomartagon* but this species has several deviating character from the other members of the section (i.e. delayed epigeal germination, heavy seeds, and different bulb colour). Plastid data does not provide enough variation for further resolution of the relationships within sect. *Liriotypus*; however, there is a grouping within sect. *Liriotypus* that separates NE Turkish and Caucasian lilies from other members of the section. Two Caucasian species (*L. szovitsianum* and *L. monadelphum*) were clustered with European lilies (Figure 1). This result is not congruent with our knowledge about the morphology and geography of these species. Additionally, these 2 species were found to be part of the NE Turkish and Caucasian group for the combined analysis with a high support value of 96 BP (Figure 2). *Lilium monadelphum, L. szovitsianum*, and *L. armenum* are morphologically and geographically closely related taxa. They were treated as only different varieties of *L. monadelphum* (Davis & Henderson, 1984). The major difference between *L. monadelphum* and *L. szovitsianum* is the color of their pollen, which is yellow in the former and bright red or red-brown in the latter. Additionally, the shape of the ovary and the stigma differs in the 2 species. *Lilium monadelphum* occurs in the northern Caucasus but *L. szovitsianum* is distributed through the southern Caucasus and in NE Turkey.

Lilium kesselringianum, L. ciliatum, L. ponticum, and *L. artvinense* formed a clade in plastid DNA analysis (Figure 1). When *L. ciliatum* is excluded we see the same relationship in the combined analysis with even higher support. Among these 4 species, *L. ponticum, L. artvinense,* and *L. ciliatum* have similar flower morphology, i.e. small and strongly recurved perianth segments. In terms of floral morphology, *L. kesselringianum* (with a pale cream to pale yellow color, larger and only slightly recurved perianth segments, and orange pollen) is more similar to *L. akkusianum* and to other species of the *Monadelphum* group. *Lilium kesselringianum* is also part of the so-called *Monadelphum* group and is also thought to be a variety of *L. monadelphum* (Ferns, 1978). Both *L. kesselringianum* and *L. monadelphum* have delayed hypogeal germination (Matthews, 1989). *L. armenum* and *L. akkusianum* have similar flower form but different leaf and bud indumenta. They have a parapatric distribution pattern in NE Turkey. *Lilium ciliatum* and *L. akkusianum* are not grouped together in the plastid DNA analysis, but in the combined analysis (93 BP). Our previous nrDNA ITS results (İkinci et al., 2006) and RAPD results (İkinci & Oberprieler, 2010) clearly indicate a very close relationship between these 2 Turkish endemics. These 2 species have the synapomorphic character of long hairs on leaf margins and on flower buds. A possible explanation for the incongruence between plastid and nuclear DNA analysis can be the limited character evolution in plastid DNA.

L. jankae and *L. rhodopaeum* formed a monophyletic group both in the plastid (64 BP) and in the combined analysis (81 BP). Yellow flowering *L. jankae* has leaf ciliate on margins and on veins (Synge, 1980). This species is traditionally included within the *Carniolicum* group of lilies together with *L. albanicum, L. bosniacum,* and *L. carniolicum* (Matthews, 1980). Inclusion of *L. jankae* within the *Carniolicum* group is not supported by either of our analyses. On the other hand, *L. rhodopaeum,* also with yellow flowers and from the Rhodope Mountains of Bulgaria and Greece, is considered to be a distinct taxon with similar flower morphology to the *Monadelphum* group. Leaf pubescence is alike in both species. These 2 species have 6 secondary constrictions (SCs) in their chromosomes (Muratović et al., 2010). *Lilium candidum* is a sister to the *L. jankae* and *L. rhodopaeum* clade with low support (53 BP) in the combined analysis. In the plastid sequence analysis it is together with other European lilies. *Lilium candidum* has exclusive character among the members of sect. *Liriotypus* (pure white funnel-shaped flowers, overwintering basal leaves, immediate epigeal germination) and a different habitat preference (maquis, rocky deciduous forests, and grasslands ranging from sea level to 1300 m) (Davis & Henderson, 1984) and all other species of the section are found in alpine/subalpine meadows, woodlands, or mountains. Our molecular phylogenetic analyses do not support the classification of Baranova (1988) where *L. candidum* is placed in a monotypic section *Lilium*. In contrast

to our findings, a recent molecular cytogenetic study indicated that *L. candidum* differs from all other European lilies (Muratović et al., 2010).

Lilium albanicum has yellow flowers and its leaves are glabrous on the veins. In contrast, *L. carniolicum* has red or orange flowers and its leaf veins are hairy. These 2 species together with *L. candidum* have 8 SCs in their chromosomes and all other European lilies of sect. *Liriotypus* have only 6 SCs (Muratović et al., 2010). According to Matthews (1989) *L. albanicum*, *L. carniolicum*, *L. jankae*, and *L. ponticum* are varieties of *L. pyrenaicum*. *Lilium ponticum* was also considered to be a subspecies of *L. carniolicum* (Davis & Henderson, 1984). Nevertheless, our combined analysis does not corroborate the classification proposed by Matthews (1980, 1989). Furthermore, Muratović et al. (2010) suggests that members of the *L. carniolicum* complex are distinct species.

Lilium pyrenaicum and *L. pomponium* are monophyletic in the combined analysis. *L. pomponium* has bright red flowers and occurs in the Alpes Maritimes of France and Italy. Yellow or orange-red flowered *L. pyrenaicum* is found in northern Spain and western France. *Lilium chalcedonicum* is a sister to this clade. *Lilium chalcedonicum* is distributed through Greece, Macedonia, and southern Albania. This species has red or orange flowers and scattered leaves, which unlike those of *L. pomponium,* are adpressed to the stem in the upper parts. All 3 species have delayed epigeal germination (Matthews, 1989).

The fossil record for monocotyledons is especially sparse compared to other flowering plant groups (Gandolfo et al., 2000). Therefore, we had to calibrate our tree data based on previous works. Divergence times estimates based on nrDNA ITS sequences indicated that sect. *Liriotypus* separated from the other lilies 8 mya and an increase in speciation events within this section occurred in the last 6 my. As climates cooled in the Pliocene, the climate of southern Europe first became warm-temperate and then transformed into the present Mediterranean climate. At the end of the Dacian period, 4-5 mya, a reduction in water levels and the rise of the Earth's crust in the northern Caucasus led to the separation of the Black Sea and Caspian Sea basins (Olteanu & Jipa, 2006). Additionally, climatic changes occurred after the desiccation of the Mediterranean Sea (Messinian salinity crisis) between 5.96 and 5.33 mya in the Mediterranean basin (Duggen et al., 2003). All of these climatic changes may have triggered speciation in sect. *Liriotypus* of *Lilium*.

In summary, our results once again confirm the monophyly of section *Liriotypus* when *L. bulbiferum* is excluded. The species of sect. *Liriotypus* form a single lineage, including *L. candidum*. At this stage of the research we do not have a hypothesis about the incongruence between the plastid and nuclear ITS DNA sequence analyses. However, a possible explanation could be hybridisation in the ITS nrDNA region or, as stated above, limited character evolution in plastid DNA.

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