

Molecular phylogenetic evidence for the monophyly of *Fritillaria* and *Lilium* (Liliaceae; Liliales) and the infrageneric classification of *Fritillaria*

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Abstract

We present phylogenetic analyses of 37 taxa of *Fritillaria* (Liliaceae), 15 species of *Lilium*, and several outgroup taxa from Liliaceae s.s. to investigate the generic delimitation of *Fritillaria* in relation to *Lilium* as well as infrageneric relationships within *Fritillaria*. We used DNA sequences from the maturase-coding plastid *matK* gene and the *trnK* intron, the intron of the ribosomal protein-coding *rpl16* plastid gene, and the nuclear ribosomal internal transcribed spacers (ITS). Phylogenetic analysis using maximum parsimony defined *Fritillaria* and *Lilium* (the latter including *Nomocharis*) as sister taxa. *Fritillaria* sections *Fritillaria* and *Liliorhiza* are supported in part, and some of the most enigmatic species usually included in *Fritillaria* (sections *Petilium* and *Theresia* and the monotypic genus *Korolkowia*) are closely related. The results support the new classification of *Fritillaria* proposed by Rix. We postulate independent origins of the underground bulbils found in *Fritillaria davidii* and the remainder of subgenus *Liliorhiza*.

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Keywords: *Fritillaria*; ITS; *Korolkowia*; *Lilium*; *matK*; *Nomocharis*; *rpl16*

1. Introduction

Fritillaria L. (Liliaceae) comprises about 100 species of geophytic perennials occurring in most temperate regions of the Northern Hemisphere, from North America, through Europe, the Mediterranean region and Central Asia, to China and Japan. *Fritillaria* is putatively closely related to *Lilium* L., which likewise consists of about 100 species widespread in the Northern Hemisphere, but with a pronounced center of diversity in southwestern and Himalayan Asia. The typical bulb of *Fritillaria* species consists of a few fleshy, tightly packed

scales and a thin, translucent tunic, which usually disappears as the bulb increases in size. Some *Fritillaria* species have naked bulbs consisting of many scales, which resemble those of *Lilium*, and these species also have numerous loosely attached bulbils. The flowers are usually nodding and solitary, but some species can also form umbels or many-flowered racemes. The perianth is campanulate, and the segments are often marked with alternating squares of light and dark colors (tessellation; Rix, 1984). *Fritillaria* species are becoming increasingly popular in horticulture, and many species are commercially available. The bulbs of some species of *Fritillaria* are known to contain pharmacologically active steroidal alkaloids and have been prescribed in Chinese medicine for cough and fever under the name *bei-mu* since ancient times (The State Pharmacopoeia of the People's Republic of China, 2000).

Following the description of *Fritillaria* by Linnaeus (1753), there have been several attempts at subdividing

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Table 1
Overview of classifications of *Fritillaria*

Baker (1874)	Bentham and Hooker (1883)	Boissier (1882)	Turrill and Sealy (1980)	Rix (2001)
<i>Fritillaria</i>	<i>Fritillaria</i>	<i>Fritillaria</i>	<i>Fritillaria</i>	<i>Fritillaria</i>
Subgenera	Sections	Sections	Sections	Subgenera
<i>Eufritillaria</i>	<i>Eufritillaria</i> (incl. <i>Monocodon</i> and <i>Goniocarpa</i>)	<i>Eufritillaria</i> (incl. <i>Amblirion</i>)	<i>Fritillaria</i>	<i>Fritillaria</i>
<i>Monocodon</i>		Subsections	Subsections	Section
<i>Goniocarpa</i>		<i>Trichostyleae</i>	<i>Fritillaria</i>	<i>Olostyleae</i> (6 series)
<i>Amblirion</i>	<i>Amblirion</i>	<i>Olostyleae</i> (incl. <i>Rhinopetalum</i>)	<i>Olostyleae</i>	<i>Fritillaria</i> (10 series)
<i>Rhinopetalum</i>				<i>Rhinopetalum</i>
				<i>Japonica</i>
<i>Theresia</i>	<i>Theresia</i> (incl. <i>Rhinopetalum</i>)	<i>Theresia</i>	<i>Theresia</i>	<i>Theresia</i>
<i>Petilium</i>	<i>Petilium</i>	<i>Petilium</i>	<i>Petilium</i>	<i>Petilium</i>
<i>Liliorhiza</i>	<i>Liliorhiza</i> (incl. <i>Korolkowia</i>)	<i>Liliorhiza</i>	<i>Liliorhiza</i>	<i>Liliorhiza</i> (3 series)
				<i>Davidii</i>
<i>Korolkowia</i>			Genus <i>Korolkowia</i>	<i>Korolkowia</i>
<i>Notholirion</i>	Genus <i>Notholirion</i>			

See text for details.

the genus (Table 1). In a revision of the genus, Baker (1874) subdivided the species into 10 subgenera based on characters of the bulb, style, form of the nectary, and capsule-valves. Bentham and Hooker (1883) reduced these to five sections and transferred Baker's subgenus *Notholirion* to *Lilium*. For *Flora Orientalis*, Boissier (1882) subdivided section *Fritillaria* into two subsections defined by an entire or trifold style: subsections *Trichostyleae* (including Baker's subgenera *Fritillaria* and *Monocodon*) and *Olostyleae* (including Baker's subgenera *Rhinopetalum* and *Amblirion* except *F. pudica*). Subgenera *Liliorhiza* and *Goniocarpa* did not come into consideration since these species do not occur in the area covered by *Flora Orientalis*. Komarov (1935) considered Baker's (1874) subgenera *Korolkowia* and *Rhinopetalum* to be genera in their own right. Turrill and Sealy (1980) followed Boissier (1882) in their revision of the genus and divided *Fritillaria* (excluding *Korolkowia*) into four sections, referring to Boissier's subsection *Trichostyleae* as subsection *Fritillaria*. All American species were united in section *Liliorhiza*.

Fritillaria is currently being revised by Rix (2001; unpublished), who recognizes eight subgenera, *Davidii* (including only *F. davidii*), *Liliorhiza* (with a number of series), *Japonica* (including *F. japonica*, *F. amabilis*, and three closely related species), *Fritillaria* (including two sections, *Olostyleae* and *Fritillaria*, and a number of series), *Rhinopetalum*, *Petilium* and the monotypic *Theresia* and *Korolkowia*. According to Rix (1977), the center of diversity may be found in Iran, where groups from central Asia, the Mediterranean, and the Caucasus meet.

Nectaries in *Fritillaria* are large and well developed and have been used as basis for subgeneric classifications. Bakshi-Khaniki and Persson (1997) studied nectary morphology in 31 southwestern Asian *Fritillaria* species. In most species studied, the nectaries are linear to lanceolate or ovate and weakly impressed upon the tepals. Species belonging to subgenus *Rhinopetalum*

Baker have deeply depressed nectaries with a slit-like orifice bordered by two lobes, which are densely hairy on the lower part. Bakshi-Khaniki and Persson (1997) considered the particular structure of the nectaries in the *Rhinopetalum* group to warrant separation into a separate genus, *Rhinopetalum* Fisch. ex. Alexand., as previously suggested by Komarov (1935).

Fritillaria species have among the largest reported genomes of all angiosperms, with a 1C value (DNA content of the unreplicated haploid chromosome complement) as high as 127.4 pg for tetraploid *F. assyriaca* Baker (Bennett and Smith, 1976). A number of karyological studies have revealed variation among and within species, but no taxonomic conclusions have been reached (i.e., LaCour, 1978; Zaharof, 1989). However, Old World groups tend to have little or no heterochromatin, whereas New World groups tend to have abundant heterochromatin (Darlington and Wylie, 1955). Chromosome numbers have been reported for more than 50 species of *Fritillaria*, and most species have a basic chromosome number, $x = 12$, although $x = 9$, 11, and 13 also have been reported in a few species, but no particular pattern is evident (Fedorov, 1969).

In a preliminary study based on molecular data including species of *Fritillaria* and *Lilium* (Fay and Chase, 2000), the *Fritillaria* species fell in two strongly supported clades, one including the North American species and the other Eurasian species. *Lilium* was monophyletic, but the exact relationships of the two clades of *Fritillaria* and *Lilium* were unclear. Liliaceae *sensu* Chase et al. (2000) and APG II (2003) include Calochortaceae and Tricyrtidaceae, and Liliaceae are now treated as a family of 16 genera: *Amana* Honda, *Calochortus* Pursh., *Cardiocrinum* (Endl.) Lindl., *Clintonia* Raf., *Erythronium* L., *Fritillaria* (including *Korolkowia* Regel), *Gagea* Salisb., *Lilium* (including *Nomocharis* Franch.), *Lloydia* Salisb. ex Rchb., *Medeola* L., *Notholirion* Wall. ex Boiss., *Prosartes* D. Don., *Scoliopus* Torr., *Streptopus* Michx., *Tricyrtis* Wall., and *Tulipa* L. Other

recent treatments (i.e., Tamura, 1998) segregated *Calochortus*, *Prosartes*, *Scoliopus*, *Streptopus*, and *Tricyrtis* into Calochortaceae s.l., largely based on karyological differences. However, there are karyological similarities of *Tricyrtis* to *Tulipa* and *Scoliopus* to *Clintonia*/*Medeola*, so although the other genera of Calochortaceae are different in some characters from those listed above, the existence of intermediates makes a narrower circumscription of Liliaceae (excluding Calochortaceae sensu Tamura, 1998) unnecessary.

The purpose of this study was to determine the circumscription of *Fritillaria* and *Lilium*, as well as infra-generic relationships in the former. To evaluate phylogenetic relationships, we employ the maturase-coding plastid *matK* gene and the flanking *trnK* intron sequence, hereafter the *matK* region, the intron of the ribosomal protein-coding plastid *rpl16* gene and the nuclear ribosomal internal transcribed spacers (ITS). We also discuss biogeography and the evolution of bulb structure based upon the phylogenetic patterns.

2. Methods and materials

2.1. Plant material

Sequences of the *matK* region, *rpl16* intron and ITS for 37 species of *Fritillaria* representing all subgenera and sections sensu Rix (2001), 15 species of *Lilium* (including *Nomocharis*), as well as representatives of *Cardiocrinum* and *Notholirion*, were included in this study (Table 2). For the analysis of only the *matK* region, additional representatives of the remainder of the genera of Liliaceae sensu Chase et al. (2000) and the related families, Melanthiaceae, Colchicaceae, Philesiaceae, and Smilacaceae were included (Table 2). The genus *Amana* was represented by *Tulipa erythronioides* Baker, which should be transferred to *Amana*, as it has been shown to be member of *Amana* and sister to *Amana edulis* (Miq.) Honda in molecular studies (Fay et al., in press). Vouchers were deposited in the Herbarium at the Royal Botanic Gardens, Kew (K), except *Alstroemeria*, which was deposited in the Herbarium of the University of Michigan (MICH).

2.2. DNA extraction, amplification, and sequencing

DNA extractions were performed using 0.2–0.3 g silica dried leaves and a modified version of the 2× CTAB method of Doyle and Doyle (1987). Before precipitation, an aliquot was purified using QIAGEN PCR purification kit (QIAGEN, Crawley, West Sussex, UK) following the manufacturer's protocols. The remainder of the DNA was purified using a cesium chloride/ethidium bromide gradient (1.55 g ml⁻¹ density) followed by a dialysis and is deposited in the DNA Bank at the Royal Botanic Gar-

dens, Kew (aliquots of which are available upon request and payment of a small handling charge; <http://www.kew.org/data/dnaBank/homepage.htm>).

The *matK* region could in most cases be amplified in one piece using the primers –19F (Molvray et al., 2000) and *trnK*-2R (Johnson and Soltis, 1994). The *matK* region constitutes approximately 1500 bp, and for sequencing we used various strategies, the most successful being a combination of the –19F and 2R primers with additional use of the internal primers 390F and 1326R of Cuénoud et al. (2002). The *rpl16* intron was amplified using the primers 1661R and 71F of Jordan et al. (1996). In many cases the internal primer 158F, designed originally for palms (AAGAAACAGTCA CTATATGA; Asmussen, unpub.), was used to avoid a long region of T/A sequence, which interfered with sequencing at the beginning of the *rpl16* intron. Amplification of ITS was carried out using the 17SE and 26SE primers of Sun et al. (1994). DMSO (2%; dimethylsulfoxide) was added to reduce secondary structure problems common in ITS (Baldwin et al., 1995; Chase et al., 2003; Winship, 1989). Amplified products were purified with the QIAGEN PCR purification kit (QIAGEN, Crawley, West Sussex, UK) following the manufacturer's protocols.

Cycle sequencing reactions were carried out using the BigDye Terminator Mix (Applied Biosystems, ABI, Warrington, Cheshire, UK). Products were run on an ABI 3100 Genetic Analyzer or an ABI 377 automated sequencer according to the manufacturer's protocols. Both strands were sequenced for each region for all but four taxa (due to sequencing failures for the *rpl16* intron, *F. gibbosa* and *L. speciosum* were only sequenced with the primer 158F and *F. japonica* and *F. alburyana* only with the primer 1661R). For this study, 155 sequences were generated and 24 sequences were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov>). GenBank accession numbers are listed in Table 2.

2.3. Alignment and phylogenetic analysis

New sequences were edited and assembled using Sequencer 4.1.2 software (Gene Codes, Ann Arbor, MI, USA). All sequences were easily aligned by eye in PAUP v. 4.0b10 for Macintosh (Swofford, 2002), following the guidelines of Kelchner (2000). Matrices can be obtained as NEXUS files from the last author (m.chase@kew.org). Cladistic analyses were conducted using PAUP v. 4.0b10 (Swofford, 2002). All changes were assessed as unordered and equally weighted (Fitch parsimony; Fitch, 1971). Indels were coded as missing data and not included in the analyses because all groups marked by indel patterns were already well supported by the bootstrap procedure (see below); coding them would not have produced any additional support. Data were analyzed in three steps. First (analysis I), we analyzed the *matK* region including representatives of all genera of

Table 2
Details of material included in this study

Taxa	Voucher information	<i>ITS</i>	<i>matK</i>	<i>rpl16</i>
<i>Fritillaria</i> L.				
Subgenus <i>Davidii</i> Rix				
<i>F. davidii</i> Franchet	<i>B. Mathew</i> 2000. Bulblets. No voucher	AY616718	AY624435	AY624381
Subgenus <i>Fritillaria</i>				
Section <i>Fritillaria</i>				
<i>F. acmopetala</i> Boiss.	<i>Chase 2565</i> (K), Kew 1959-59401	AY616709	AY624426	AY624372
<i>F. aurea</i> Schott	<i>Chase 3487</i> (K), Kew 1973-21448	AY616713	AY624430	AY624376
<i>F. crassifolia</i> Boiss & Huet. subsp. <i>kurdica</i> (Boiss & Noë) Rix	<i>Chase 2559</i> (K), Kew 1985-926	AY616717	AY624434	AY624380
<i>F. hermonis</i> Fenzl subsp. <i>amana</i> Rix	<i>Chase 2563</i> (K), Kew 1974-2043	AY616724	AY624440	AY624387
<i>F. lusitanica</i> Wikstr.	<i>Chase 2603</i> (K), Kew 1980-3020	AY616728	AY624443	AY624391
<i>F. meleagris</i> L.	<i>Chase 2566</i> (K), Kew 1990-3088	AY616730	AY624445	AY624393
<i>F. michailowskyi</i> Fomin	<i>Chase 2583</i> (K), Kew 1981-3060	AY616731	AY624446	AY624394
<i>F. olivieri</i> Baker	<i>Chase 2569</i> (K), Kew 1963-51207	AY616734	AY624449	AY624397
<i>F. pallidiflora</i> Schrenk	<i>Chase 2567</i> (K), Kew 1959-1103	AY616735	AY624450	AY624398
<i>F. reuteri</i> Boiss.	<i>Chase 2568</i> (K), Kew 1969-6106	AY616741	AY624456	AY624404
<i>F. tenella</i> M.Bieb.	<i>Chase 2561</i> (K), Kew 1955-12701	AY616744	AY624458	AY624407
<i>F. tubiformis</i> Gren. & Godr.	<i>Chase 2558</i> (K), Kew 1966-109	AY616745	AY624459	AY624408
Section <i>Olostylea</i> Boiss.				
<i>F. alburyana</i> Rix	<i>Chase 3470</i> (K), Kew 1994-3139	AY616712	AY624429	AY624375
<i>F. caucasica</i> Adam	<i>Chase 3488</i> (K), Kew 1989-1112	AY616715	AY624432	AY624378
<i>F. minuta</i> Boiss. & Nöe	<i>Chase 2562</i> (K), Kew 1978-3492	AY616733	AY624448	AY624396
Subgenus <i>Japonica</i> Rix				
<i>F. japonica</i> Miq. var. <i>koidzumiana</i> (Ohwi) H.Hara & Kanai	<i>Chase 749</i> (K), Kew 1981-5579	AY616726	AY624442	AY624389
Subgenus <i>Korolkowia</i> Rix				
<i>F. serwerzowi</i> Regel	<i>Chase 743</i> (K), Kew 1933-Hoog	AY616742	AY624457	AY624405
Subgenus <i>Liliorhiza</i> (Kellogg) Benth. & Hook. f.				
<i>F. affinis</i> (Schult.) Sealy	<i>Chase 446</i> (K), Kew 1989-2020	AY616710	AY624427	AY624373
<i>F. agrestis</i> Greene	<i>D. King.</i> (K), 01.04.01. Parkfield Grade, Fresno Co. CA	AY616711	AY624428	AY624374
<i>F. camtschatcensis</i> (L.). Ker-Gawl.	<i>Chase 3580</i> (K), photo, ex. Cult. B. Mathew	AY616714	AY624431	AY624377
<i>F. eastwoodae</i> Macfarl.	<i>Chase 8980</i> (K), Kew 1986-6357	AY616719	—	AY624382
<i>F. falcata</i> (Jepson) Beetle	<i>Chase 3478</i> (K), Kew 1989-3270	AY616720	AY624436	AY624383
<i>F. gentneri</i> Gilkey	<i>D. King.</i> (K), 8.4.01. Brett Woods, Jacksonville, Jackson Co. OR	AY616721	AY624437	AY624384
<i>F. glauca</i> Greene	<i>D. King.</i> (K), 7.04.01. R&Ready Botanical Wayside, Cave Junction, Josephine Col, OR	AY616723	AY624439	AY624386
<i>F. maximowiczii</i> Freyn.	<i>Chase 8195</i> (K), Kew 1993-3390	AY616729	AY624444	AY624392
<i>F. micrantha</i> Heller	<i>D. King.</i> (K), 29.03.01 Italian Bar, Columbia, Tolumne Co. CA	AY616732	AY624447	AY624395
<i>F. phaeanthera</i> Purdy	<i>Chase 8981</i> (K), Kew 1988-5442	AY616737	AY624452	AY624400
<i>F. pudica</i> (Pursh) Spreng.	<i>Chase 754</i> (K), Kew 1986-6110	AY616738	AY624453	AY624401
<i>F. recurva</i> Benth.	<i>Chase 2560</i> (K), Kew 1989-122	AY616740	AY624455	AY624403
<i>F. striata</i> Eastw.	<i>D. King.</i> (K), 1999.	AY616743	—	AY624406

Subgenus <i>Petilium</i> (L.) Endl.				
<i>F. chitralensis</i> B. Mathew	<i>Chase 3472</i> (K), Kew 1970-4109	AY616716	AY624433	AY624379
<i>F. imperialis</i> L.	<i>Chase 2557</i> (K), Kew 1970-3943	AY616725	AY624441	AY624388
<i>F. raddeana</i> Regel	<i>Chase 745</i> (K), Kew 1973-54	AY616739	AY624454	AY624402
Subgenus <i>Rhinopetalum</i> Fisch.				
<i>F. gibbosa</i> Boiss.	photo M.W. Chase	AY616722	AY624438	AY624385
<i>F. karelini</i> (Fisher ex D.Don) Baker	<i>Chase 3471</i> (K), Kew 1994-218	AY616727	—	AY624390
Subgenus <i>Theresia</i> Koch				
<i>F. persica</i> L.	<i>Chase 3496</i> (K), Kew 1923-41201	AY616736	AY624451	AY624399
<i>Lilium</i> L.				
Section <i>Archelirion</i> Baker				
<i>L. humboldtii</i> var. <i>bloomerianum</i> Purdy	<i>Chase 3723</i> (K), Kew 1994-561	AY616746	AY624461	AY624412
<i>L. maritimum</i> Kellogg	<i>Chase 931</i> (K), Kew 1977-1830	AY616748	AY624463	AY624414
<i>L. rubescens</i> S. Watson	<i>Chase 932</i> (K), Kew 1989-197	AY616749	AY624465	AY624418
<i>L. speciosum</i> var. <i>clivorum</i> S.Abe & Tamura	<i>Chase 3724</i> (K), Kew 1993-332	Nishikawa et al. (1999) (AB020432)	AY624467	AY624420
Section <i>Leucolirion</i> Wilson				
<i>L. regale</i> E.H. Wilson	<i>Chase 3696</i> (K), Kew 1979-879	Nishikawa et al. (1999) (AB020434)	AY624464	AY624417
Section <i>Liriotypis</i> Asch. et Graeb.				
<i>L. candidum</i> L.	<i>Chase 3717</i> (K), Ex. cult.	Dubouzet and Shinoda (1999) (AF092522)	Hayashi et al. (1998) (AB024545)	AY624409
<i>L. pyreniacum</i> Gouan.	<i>Chase 8639</i> (K), Kew 1995-1667	Dubouzet and Shinoda (1999) (AF092517)	—	AY624416
Section <i>Lophophorum</i> (Bur. & Franch.) F.T.Wang & T.Tang				
<i>L. lophophorum</i> Franch.	Kew 1991-1997 photo	AY616747	AY624462	AY624413
Section <i>Martagon</i> Reichenbach.				
<i>L. martagon</i> L.	<i>Chase 3698</i> (K), Kew 1978-2452	Nishikawa et al. (1999) (AB020455)	Hayashi and Kawano (2000) (AB030872)	AY624415
Section <i>Pseudolirium</i> Endl.				
<i>L. sachalinense</i> Vrishcz.	<i>Chase 933</i> (K), Kew 1987-2468	AY616750	AY624466	AY624419
<i>L. superbum</i> L.	<i>Chase 112</i> (NCU)	Nishikawa et al. (1999) (AB020420)	AY624468	AY624421
<i>L. washingtonianum</i> Kellogg	<i>Chase 3688</i> (K), Kew 1988-406	Nishikawa et al. (1999) (AB020438)	Hayashi and Kawano (2000) (AB030848)	AY624422
Section <i>Sinomartagon</i> H.F. Comber				
<i>L. davidi</i> Duch.	<i>Chase 3697</i> (K), Kew 1979-867	Nishikawa et al. (1999) (AB020461)	AY624460	AY624410
<i>L. henryi</i> Baker	<i>Chase 8640</i> (K), Kew 1979-870	Nishikawa et al. (1999) (AB020467)	Nishikawa et al. (2000) (AB049505)	AY624411
<i>Nomocharis</i> Franch.				
<i>Nomocharis pardanthina</i> Franch.	<i>Chase 934</i> (K), Kew 1990-3521	AY616751	Hayashi and Kawano (2000) (AB030842)	AY624423
Outgroups				
<i>Alstroemeria</i> sp.	<i>Anderson 13653</i> (MICH)	—	AY624481	—
<i>Amana</i> sp. = <i>Tulipa erythronioides</i> Baker	<i>Chase 742</i> (K), Kew 1980-2461	—	AY624472	—
<i>Cardiocrinum giganteum</i> (Wall.) Makino	<i>Chase 3689</i> (K), Kew 1988-4907	Nishikawa et al. (1999) (AB020466)	AY624469	AY624425

(continued on next page)

Table 2 (continued)

Taxa	Voucher information	ITS	matK	rpl16
<i>Calochortus uniflorus</i> Hook & Arn.	Chase 13354 (K), Kew 1991-198	—	AY624478	—
<i>Chamaelirium luteum</i> A. Gray		—	Fuse and Tamura (2000) (AB040196)	—
<i>Clintonia borealis</i> Rafn.	Chase 498 (K), Kew 1981-6330	—	Hayashi et al. (1998) (AB024542)	—
<i>Colchicum speciosum</i> Stev.		—	Fuse and Tamura (2000) (AB040181)	—
<i>Eythronium japonicum</i> Decne	Chase 780 (K), Kew 1979-5130	—	Hayashi et al. (1998) (AB024387)	—
<i>Gagea wilczekii</i> Braun-Blanq. & Maire	Chase 748 (K), Kew 1984-1453	—	AY624470	—
<i>Lapageria rosea</i> Ruiz & Pav.	Chase 181 (NCU)	—	AY624480	—
<i>Lloydia serotina</i> Sweet	Jones s.n. (K), Niwat Ridge, Colorado, USA	—	AY624471	—
<i>Medeola virginiana</i> L.		—	Hayashi et al. (1998) (AB024547)	—
<i>Notholirion thomsonianum</i> (Royle) Stapf.	Chase 448 (K), Kew 1974-025	AY616752	Hayashi et al. (1998) (AB024393)	AY624424
<i>Philesia buxifolia</i> Lam. ex. Poir.	Chase 545 (K), Kew 1965-68407	—	AY624479	—
<i>Prosartes smithii</i> (Hook.) Utech, Shinwari & Kawano	Chase 480 (K), Kew 1986-8059	—	AY624474	—
<i>Scoliopus bigelovii</i> Torr.	Chase 441 (K), Kew 1976-1909.	—	AY624477	—
<i>Smilax china</i> L.		—	Fuse and Tamura (2000) (AB040204)	—
<i>Streptopus parviflorus</i> Franch.		—	Fuse and Tamura (2000) (AB040203)	—
<i>Tricyrtis latifolia</i> Maxim.	Chase 2780 (K), Kew 1981-1770	—	AY624475	—
<i>Tricyrtis perfoliata</i> Masamune	Chase 2783 (K), Kew 1989-3971	—	AY624476	—
<i>Trillium grandiflorum</i> Salisb.		—	Kazempour Osaloo et al. (1999) (AB017392)	—
<i>Tulipa systola</i> Stapf.	Chase 6120 (K), Kew 1977-4287	—	AY624473	—
<i>Uvularia perfoliata</i> L.	Chase 494 (K), Kew 1956-55702	—	AY624482	—
<i>Veratrum stamineum</i> Maxim.		—	Fuse and Tamura (2000) (AB040184)	—

Classification of *Fritillaria* according to Rix (2001). Classification of *Lilium* according to Comber (1949), and Wilson (1925). For voucher information on previously published sequences, see reference cited with sequence.

Liliaceae *sensu* Chase et al. (2000) to investigate generic relationships within Liliaceae and assess monophyly of *Fritillaria* and *Lilium*. For this analysis, sequences of *Lapageria*, *Philesia*, *Smilax*, *Veratrum*, *Chamaelirium*, *Trillium*, *Alstroemeria*, *Uvularia*, and *Colchicum* were included and collectively designated as the outgroup based on a number of previous studies of Liliales (Chase et al., 1995a,b, 2000; Fay et al., in press; Rudall et al., 2000).

In analysis II, data were analyzed as separate plastid and ITS matrices. Because the plastid genome is non-recombining and inherited as a unit, we did not perform separate analyses of the two plastid regions. In all these analyses, the number of outgroup taxa had to be substantially reduced due to alignment problems for ITS and the *rpl16* intron. The initial analysis of the *matK* matrix (analysis I) showed *Cardiocrinum* and *Notholirion* as the closest relatives of *Fritillaria* and *Lilium*, which is in accordance with recent findings (Fay and Chase, 2000; Fay et al., in press; Patterson and Givnish, 2002). Therefore we need only *Cardiocrinum* and *Notholirion* as outgroups for the analyses of the separate plastid and ITS matrices focused on *Lilium* and *Fritillaria*.

The analysis of ITS was conducted using the heuristic search option and 1000 replicates of random taxon addition with tree bisection-reconnection branch swapping (TBR). All other analyses were performed in the same manner, but a limit was used due to the large number of trees produced. Most parsimonious trees were thus obtained by a strategy similar to Chase et al. (2002): (i) 1000 replicates of random taxon addition performed using subtree-pruning-regrafting (SPR) branch swapping with only 25 trees held at each step to save time by avoiding swapping on large numbers of trees; (ii) the trees collectively found in these 1000 replicates were then used as starting trees for a second search using SPR branch swapping until all trees were found or a pre-set maximum of 15,000 trees at this length were found. Relative levels of homoplasy in all the data sets were assessed using the consistency index (CI) and the retention index (RI) including all characters as implemented in PAUP v. 4.0.b10 (Swofford, 2002). In all cases RI was at least 0.75, which indicates that only one island of trees was likely to exist (Maddison, 1991).

In analysis III, both data sets were combined. To assess congruence between the combined plastid and the ITS data sets we carried out 100 replicates of the partition homogeneity test (Farris et al., 1995) as implemented in PAUP v. 4.0.b10 (Swofford, 2002) with heuristic searches, random taxon addition, and a limit of 10 trees per replicate.

Robustness was assessed with the bootstrap (Felsenstein, 1985) using 500 replicates of simple addition, TBR swapping, equal weights, and a limit of 10 trees for each replicate. We defined scores between 50 and 74 bootstrap percentage (BP) as weak support, scores between 75 and

89 BP as moderate support, and scores of greater than 90 BP as strong support. We consider percentages <50% to be unsupported because such groups are often not even present in all shortest Fitch trees. We also report scores greater than 50 BP that are consistent with the strict consensus tree. For analysis II we show only the strict consensus trees with bootstrap percentages indicated to establish that in no cases did conflicting groups receive high bootstrap support in the separate analyses so that it was appropriate to directly combine all data.

3. Results

3.1. Analysis of the large *matK* matrix

The full *matK* matrix included 34 species of *Fritillaria*, 14 of *Lilium* (including *Nomocharis*), and 24 closely related taxa. One of the most parsimonious trees is shown (Fig. 1). Groups not present in all shortest trees are marked with an arrowhead. Bootstrap percentages consistent with the strict consensus tree are indicated below the branches.

In this analysis, the ingroup corresponding to Liliaceae *sensu* Chase et al. (2000) is monophyletic (100 BP). Within Liliaceae, tribes Lilieae (*Fritillaria*, *Lilium*, *Nomocharis*, *Cardiocrinum*, and *Notholirion*; 100 BP), Tulipeae (*Amana*, *Erythronium*, *Tulipa*, *Gagea*, and *Lloydia*; 92 BP), and Medeoleae (*Clintonia* and *Medeola*; 97 BP) are all monophyletic and well supported. We obtained a weakly supported clade including *Prosartes*, *Scoliopus*, *Streptopus*, and *Calochortus* (67 BP) sister to all other Liliaceae including both taxa of *Tricyrtis* (91 BP), indicating the paraphyly of Calochortaceae *sensu* Tamura (1998). A tree including *Tricyrtis* as sister to the remainder of Calochortaceae would be five steps longer (results not shown) than the most parsimonious unconstrained tree.

Subfamily Lilioideae (tribes Tulipeae and Lilieae) are also monophyletic and moderately supported (82 BP). In tribe Lilieae, *Fritillaria* and *Lilium* (84 BP, including *Nomocharis*) are members of a clade (100 BP), but *Fritillaria* is split into two clades that are unresolved with respect to *Lilium*. *Cardiocrinum* is sister (65 BP) to the *Fritillaria*–*Lilium* clade, and *Notholirion* is sister to this clade (100 BP). Tribe Tulipeae are sister (82 BP) to tribe Lilieae, and subfamily Medeoloideae are sister (100 BP) to Lilioideae.

3.2. Analysis of the plastid regions

The reduced *matK* matrix includes the same 34 species of *Fritillaria* and 14 of *Lilium*, but only *Cardiocrinum* and *Notholirion* are included as outgroup. The *rpl16* intron matrix included 37 species of *Fritillaria*, 15 of *Lilium* (including *Nomocharis*), and the outgroup. On

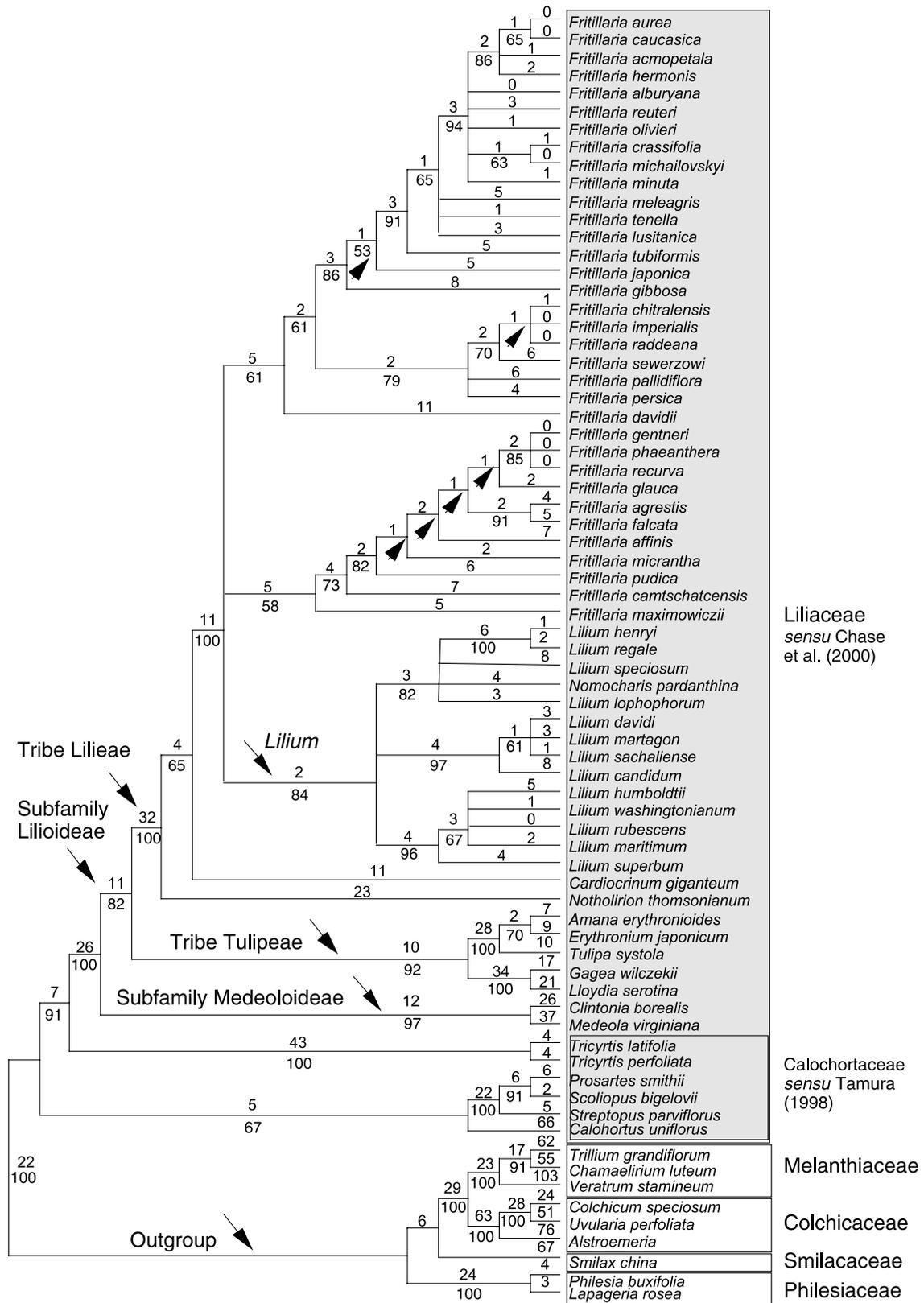


Fig. 1. One of the more than 15,000 most parsimonious trees obtained from analysis of *matK* sequences. Tree length = 1366 steps, CI = 0.67, and RI = 0.83. Branch length and bootstrap percentages (>50%) are shown above and below the branches, respectively. Clades not in the strict consensus tree of all 15,000 trees are marked with an arrowhead. Melanthiaceae, Colchicaceae, Philesiaceae, and Smilacaceae were collectively designated as the outgroup.

average, 955 bp of the *rpl16* intron (the primers were close to the 5' end of the intron) were sequenced. The aligned *rpl16* intron matrix included 1114 positions and contained 23 indels of 1 bp and 14 indels of 2–10 bp. The *rpl16* intron sequences of *F. persica* and *F. hermonis* had deletions of 68 and 17 bp, respectively. *Lilium davidi*, *L. martagon*, and *L. sachalinense* shared an insertion of 36 bp. *Fritillaria davidii*, *F. karelinii*, *F. reuteri*, and *Notholirion thomsonianum* all had apparently independent and overlapping insertions of approximately 20 bp. This part of the matrix could not be aligned and was excluded from the analysis. The combined plastid matrix included all taxa present in at least one of the two matrices. The strict consensus tree is shown in Fig. 2.

The ingroup is well supported (99 BP) and sister to *Cardiocrinum* (<50 BP). *Lilium* (including *Nomocharis*) is monophyletic (79 BP). Species of *Fritillaria* fall in two strongly supported clades, one including all the North American species and *F. maximowiczii* (A; 97 BP) and the other the remaining Eurasian species (B; 91 BP). In the strict consensus tree, *Fritillaria* is paraphyletic, with the Eurasian clade (B) more closely related to *Lilium* than to the North American clade of *Fritillaria* (A). However, this clade is only weakly supported and has less than 50% BP in the separate analyses. In the *matK* analysis, the two clades of *Fritillaria* form a polytomy with *Lilium* (not shown). In the *rpl16* intron analysis, there is little resolution, and neither *Lilium* nor *Fritillaria* form monophyletic groups.

The North American clade (A) in the plastid analysis corresponds to subgenus *Liliorhiza sensu Rix (2001)*, with the exception of *F. davidii* from western China. The southeastern Siberian *F. maximowiczii* and *F. camtschatcensis*, distributed from North America to Asia, appear as successive sister taxa (96 BP each) to the North American species. *F. davidii* is sister (91 BP) to the remaining Eurasian species (80 BP), which split into two well-supported clades. Clade B1 (91 BP) includes the monotypic subgenera *Theresia* (C. Koch) Benth. & Hook.f. (*F. persica*) and *Korolkowia* Regel (*F. sewerzowii*), subgenus *Petilium* L. (*F. pallidiflora* (sect. *Fritillaria*)). Subgenera *Korolkowia* and *Petilium* are sisters (92 BP). Clade B2 (91 BP) corresponds to subgenus *Fritillaria* including genus *Rhinopetalum sensu Komarov*, represented by *F. gibbosa* and *F. karelinii*. Within clade B2, a core clade (B3) comprising subgenus *Fritillaria sensu Rix (2001; excluding subgenus Rhinopetalum and F. japonica)* is strongly supported (95 BP). *Lilium* is split into three clades. The North American species form a strongly supported clade (C1, 100 BP) corresponding to section *Pseudolirium* Endlicher (Comber, 1949) excluding the Russian *L. sachalinense*. The second strongly supported clade included Asian and European species (C2, 96 BP). The North American clade (C1) is sister to (71 BP) the third, weakly supported (57 BP) clade comprising the remaining Asian species, including genus *Nomocharis* (C3).

3.3. Analysis of ITS

The ITS matrix includes 37 species of *Fritillaria*, 15 of *Lilium* (including *Nomocharis*), and 2 outgroup taxa (*Cardiocrinum* and *Notholirion*). On average 650 base pairs (bp) of the ITS region (ITS1 ranged from 221 to 255 bp, 5.8S from 162 to 164 bp, and ITS2 from 231 to 252 bp) were sequenced from each accession. The alignment of the ITS matrix required 21 indels of 1–4 bp each. *Fritillaria maximowiczii* had a larger gap of 21 bp in the ITS1 region. Gaps were placed manually following the criterion of Kelchner (2000). The strict consensus tree is shown in Fig. 3. Most of the clades found in the plastid analysis are also recovered in the ITS analysis.

Lilium (<50 BP) and *Fritillaria* (54 BP) are sisters, although this topology is only weakly supported. *Fritillaria maximowiczii* is sister to all other *Fritillaria* species (<50 BP), whereas it was part of the *Liliorhiza* clade in the plastid analysis (clade A, Fig. 2; 97 BP). The remaining species of subgenus *Liliorhiza* (clade A, Fig. 3) are strongly supported (95 BP), and *F. camtschatcensis* is again sister to the remaining North American species. The other *Fritillaria* subclade (B) with the remaining Eurasian species has less than 50 BP. *F. davidii* is again (>50 BP) sister to the remaining Eurasian species in clade B. Clades B1 (60 BP) and B2 (<50 BP) are also sisters in this analysis. The sister relationship of subgenera *Petilium* and *Korolkowia* is once again strongly supported (97 BP). In this analysis, *F. japonica* is sister to the rest of clade B1, although this is only weakly supported (B1, 60 BP), whereas it was part of the strongly supported (92 BP) clade B2 in the plastid analysis (Fig. 2). The inclusion of subgenus *Rhinopetalum* (*F. gibbosa* and *F. karelinii*) in clade B2 receives less than 50 BP support in this analysis, and clade B3 is moderately supported (77 BP). Relationships within *Lilium* based on ITS differ from the ones found in the plastid analysis (Fig. 2). The North American species form a strongly supported clade (C1, 94 BP) with *L. lophophorum* as sister (<50 BP). Clades C2 and C3 from the plastid analysis (Fig. 2) are not recovered in the ITS analysis. *Lilium davidi*, *L. sachalinense*, and *L. martagon* form a clade (96 BP), but the two European species *L. candidum* and *L. pyrenaicum*, which were also part of clade C2, are now part of clade with the remaining Asian species included in this analysis (>50 BP). None of the differences in topology received >71 BP.

3.4. Combined matrix

Statistical data (# of characters, # trees, length of trees, CI, RI, etc.) for all analyses are presented in Table 3. The partition homogeneity test indicated that the ITS and the combined plastid data sets are highly incongruent (*p* value of 0.01). However, such methods for testing incongruence have been shown to be unreliable (Reeves et al.,

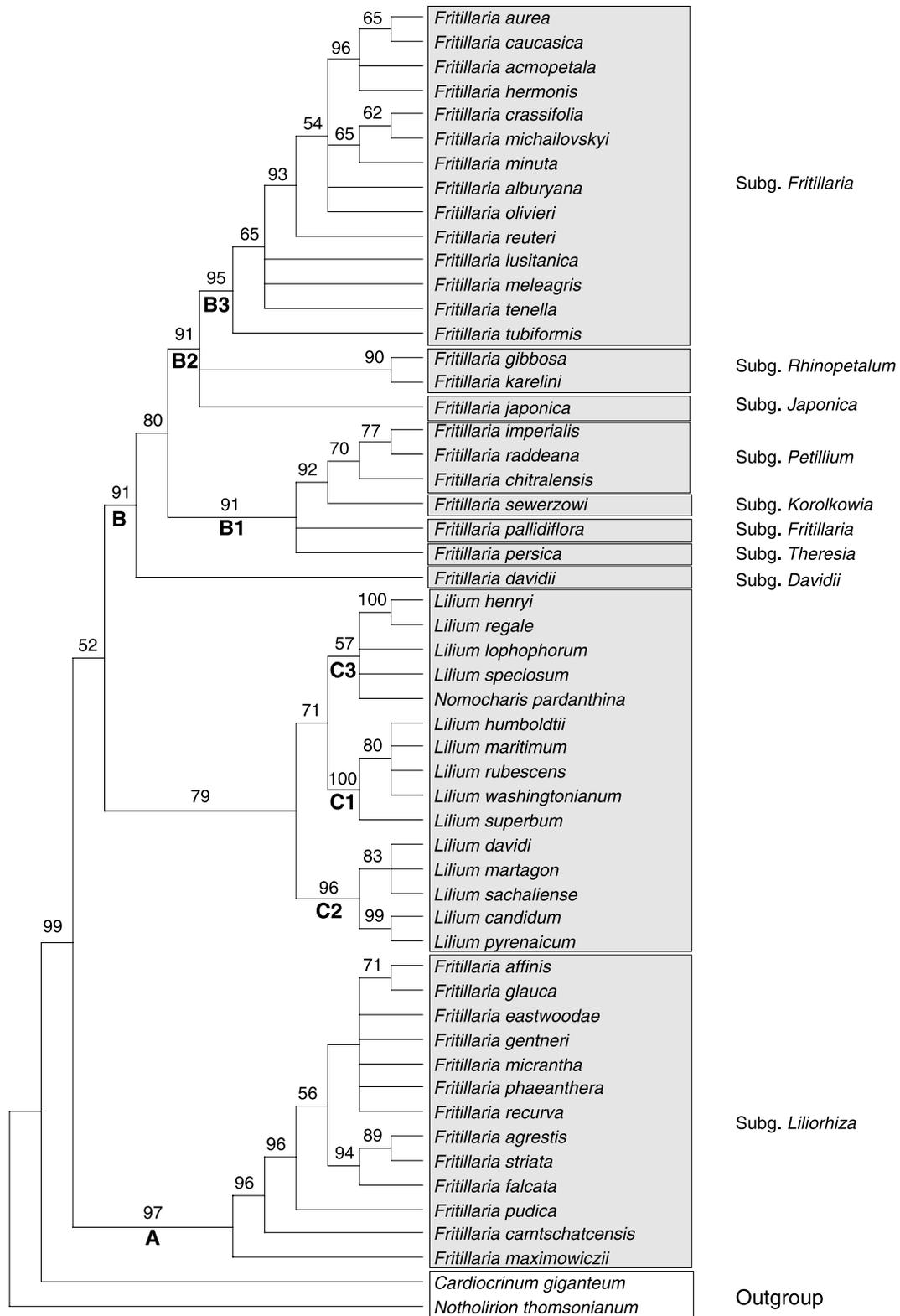


Fig. 2. Strict consensus tree of more than 15,000 most parsimonious trees from analysis of the combined plastid *rpl16* and *matK* sequences. Tree length = 445 steps, CI = 0.82, and RI = 0.90. Bootstrap percentages (>50%) are indicated above the branches. *Cardiocrinum giganteum* and *Notholirion thomsonianum* were designated as the outgroups. The subgenera of *Fritillaria* recognized by Rix (2001) are indicated.

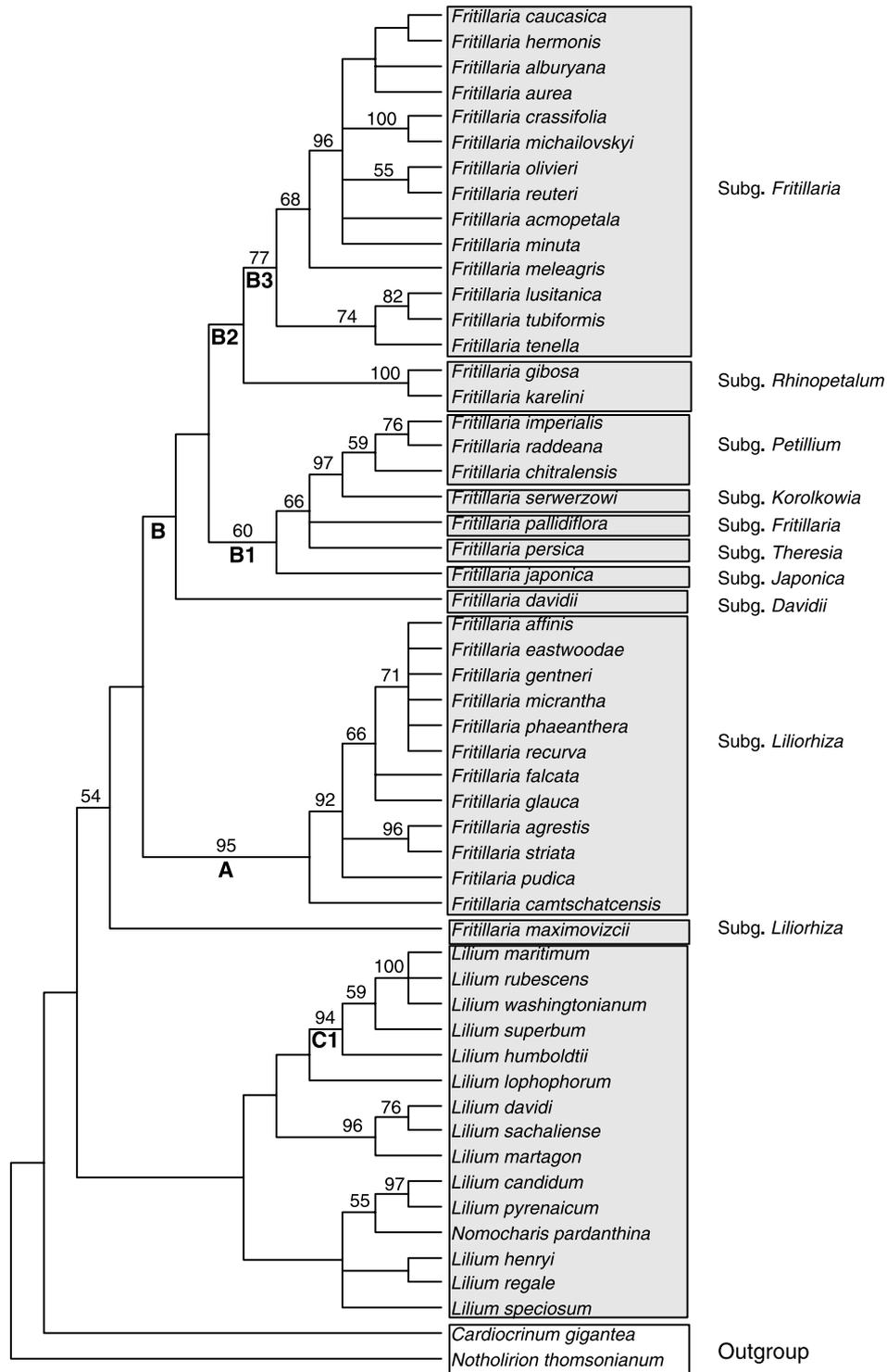


Fig. 3. Strict consensus tree of 972 most parsimonious trees from analysis of ITS sequences. Tree length = 673 steps, CI = 0.56, and RI = 0.75. Bootstrap percentages (>50%) are indicated above the branches. *Cardiocrinum giganteum* and *Notholirion thomsonianum* were designated as the outgroups. The subgenera of *Fritillaria* recognized by Rix (2001) are indicated.

2001; Yoder et al., 2001). Although incongruence does sometimes occur, it is usually focused on specific taxa/clades and not across all taxa, so performing a combined analysis is always desirable, regardless of results from the various measures for determining incongruence.

One of the most parsimonious trees, randomly selected, is shown (Fig. 4); estimated branch lengths on these trees are parsimony optimizations (DELTRAN optimization); clades not present in all shortest trees are marked with an arrowhead, and bootstrap percentages

Table 3
Statistical information related to the various datasets and analyses

Analysis	# positions	# variable positions	# parsimony informative positions	# trees	Length	CI	RI
Large <i>matK</i>	1651	727 (44%)	437 (26%)	15,000	1366	0.67	0.83
Reduced <i>matK</i>	1589	202 (13%)	81 (5%)				
<i>rpl16</i> intron	1094	84 (8%)	58 (5%)				
Combined plastid	2683			15,000	445	0.82	0.90
ITS	660	299 (45%)	178 (27%)	972	673	0.56	0.75
Combined	3343			11,664		0.65	0.79

consistent with the strict consensus tree are provided below the branches.

The ingroup as defined is strongly supported (100 BP) as in the plastid analysis (99 BP). *Lilium* (92 BP instead of <50–79 in the separate analyses) and *Fritillaria* (93 instead <50–54 BP) are now strongly supported as sisters. The North American clade (A) of *Fritillaria* species includes *F. maximowiczii* as in the plastid analysis (Fig. 2) but is instead supported by only 77 BP rather than 97 BP as in the plastid analysis. The Eurasian clade (B) has 76 BP now compared to 91 BP in the plastid (Fig. 2) and <50 BP in the ITS analyses (Fig. 3). The support of clade B1 (which does not include *F. japonica* in this topology) is now 90 BP compared to 91 BP and 60 BP in the plastid and the ITS analyses, respectively. Whereas clade B2 was strongly supported in the plastid analysis (91 BP), this clade received less than 50 BP in the ITS analysis, and likewise clade B2 is only weakly supported (57 BP) in the combined analysis. Within clade B2, we find a well-supported core (B3, 97 BP) with which *F. gibbosa*, *F. karelinii* (subgenus *Rhinopetalum*), and *F. japonica* are only weakly associated. Clade B3 is also present in the separate analyses (77–95 BP), whereas the inclusion of subgenus *Rhinopetalum* and *F. japonica* appears to be more uncertain (see Section 4).

Within *Lilium*, the North American clade (C1) is strongly supported as in the separate analyses (94–100 BP). Clade C2 from the plastid analysis (Fig. 2) is also present in this analysis, but the support has dropped from 96 BP to less than 50 BP. The clade with the remaining Asian species (C3) is not present in the strict consensus tree. However, there is strong support for the sister relationship of *L. henryi* and *L. regale* (99 BP, <50–99 BP in the ITS and the plastid analyses, respectively), and *L. speciosum* is sister to this pair with weak support (61 BP).

4. Discussion

4.1. Congruence of plastid and ITS matrices

Improved resolution and higher BP for some groups when data are combined generally indicate that differences in tree topologies are mostly likely due to sampling

error (too few data) and not from conflicting phylogenetic signals (Hulsbeck et al., 1996; Whitten et al., 2000). Results from the two separate matrices produced highly similar topologies, and there were no differing topologies with bootstrap support >80 BP in the separate analyses. However, even though the differences in topology we observed did not have high bootstrap support, we observed in the combined results a decrease in bootstrap support and resolution in a few cases, indicating contradictory patterns for the major clades of *Lilium* and *Fritillaria*, although no clear reasons for incongruence were obvious. It is noteworthy that *F. japonica* is part of the clade including subgenus *Rhinopetalum* and subgenus *Fritillaria* with strong support in the plastid analysis (Fig. 2, clade B2, 91 BP), whereas it was sister to the *Petilium*, *Korolkowia*, and *Theresia* group in the ITS analysis (Fig. 3, clade B1, 60 BP).

The partition homogeneity test (Farris et al., 1995) indicated that the ITS and the combined plastid data sets are highly incongruent (*p* value of 0.01), but such methods have been shown to be unreliable for establishing data combinability in some cases (Reeves et al., 2001; Yoder et al., 2001). Congruence is better assessed by comparing the combined results on a node-by-node basis with the separate analyses with respect to levels of resolution and bootstrap support (Weins, 1998). Although no incongruent groups of taxa had more than 80 BP in the separate analyses, the decrease in bootstrap support for several clades, and, in one case, loss of resolution (*Lilium*, C3) indicate a degree of contradictory pattern for the major clades of *Lilium* and *Fritillaria*. This extends even to cases in which both plastid and ITS results show the same patterns (i.e., for the inclusion of *F. davidii* in clade B, which went from 91 BP in the plastid analysis to 76 BP in the combined analysis).

Incongruence can be generated by introgression (hybridization), lineage sorting, and gene duplication (paralogy) (Baldwin et al., 1995; Doyle, 1992). Geographical patterns can be neatly mapped onto the plastid trees, but not so on the nuclear tree. The apparent incongruence is unlikely to result from recent hybridization as the taxa involved are geographically isolated. However, this could be the result of older events that took place when the lineages were differently distributed than they are today, as reported by Wendel et al. (1995)

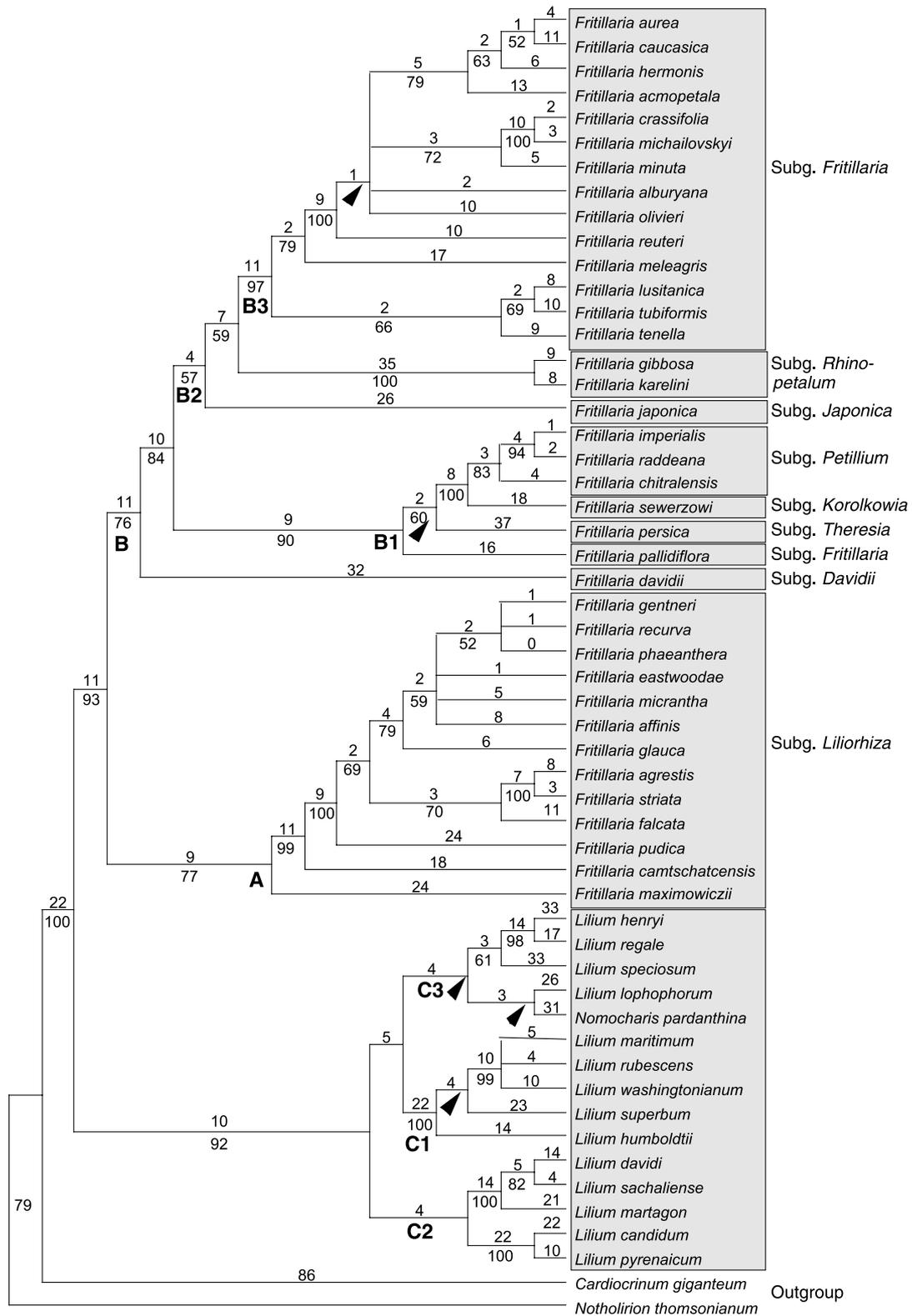


Fig. 4. One of the 11,664 most parsimonious trees obtained from the combined analysis of ITS, *matK* and *rpl16* sequences. Tree length = 1143 steps, CI = 0.65, and RI = 0.79. Branch length and bootstrap percentages (>50%) are shown above and below the branches, respectively. Clades not in the strict consensus tree of all 11,664 trees are marked with an arrowhead. *Cardiocrinum giganteum* and *Notholirion thomsonianum* were designated as the outgroups. The subgenera of *Fritillaria* recognized by Rix (2001) are indicated.

in *Gossypium*. There is no clear pattern that we can associate with paralogy, which would normally involve lineages rather than individual taxa scattered across the clades and should be accompanied by long branches (we did not specifically look for multiple ITS types through cloning, but no heterogeneity typical of multiple copies was observed in the electropherograms).

All cases here for which we observe a decrease in bootstrap support or loss of resolution involve short branches, for which estimates of bootstrap support are less reliable as a means of distinguishing levels of support and should therefore only be compared with caution. The branch supporting the inclusion of *F. davidii* in clade B in the plastid analysis is only two steps long, and the branch supporting the *Lilium* C3 clade in the plastid analysis is only three steps long. There is no strong case for explaining the observed pattern, and as stated by Hulsenbeck et al. (1996) the major problem in assessing incongruence is separating the effects of sampling error from true incongruence. It could be that more taxon sampling and/or additional data from other genes/regions in both the nuclear and plastid genomes will help to assess better the results that we have obtained here. For the time being, it is best to acknowledge a potential problem and hope that future research will shed more light on these patterns.

4.2. Monophyly of *Fritillaria* and *Lilium*

Our results support the circumscription of Liliaceae suggested by Chase et al. (2000), Fay and Chase (2000), and Fay et al. (in press). Liliaceae *sensu* Chase et al. (2000) include Calochortaceae *sensu* Tamura (1998), which are not monophyletic in our analyses. *Tricyrtis* is more closely related to Liliaceae *sensu* Tamura (1998) than to the remainder of Calochortaceae (*Streptopus*, *Scoliopus*, *Prosartes*, and *Calochortus*). A close affinity of *Tricyrtis* to the *Lilium* group was previously suggested by Tamura (1995) and is supported by karyological characters, seed morphology, dark-spotted foliage leaves, and purple-spotted tepals (Tamura, 1998). Generic relationships within Liliaceae based on *matK* sequence data from this study are in accordance with other studies (Patterson and Givnish, 2002; Rudall et al., 2000; Vinnersten and Bremer, 2001). We included representatives of all 16 genera currently treated as part of Liliaceae, and our results show that *Fritillaria* and *Lilium* are evidently sisters (Fig. 1, 100 BP), the closest relatives being *Cardiocrinum* and *Notholirion* in a monophyletic tribe Lilieae (100 BP). We will not address the infrageneric classification of *Lilium* further as only a few clades within *Lilium* are well supported by the bootstrap, and our sampling is more limited than previous studies (Hayashi and Kawano, 2000; Nishikawa et al., 1999).

4.3. Subgeneric classification of *Fritillaria*

Our findings generally support the recent classification of *Fritillaria* by Rix (2001) based on morphological characters, as discussed in the following sections.

4.3.1. *Fritillaria* subgenus *Liliorhiza* and subgenus *Davidii*

The over 20 species (Beetle, 1944; Turrill and Sealy, 1980) in subgenus *Liliorhiza* are predominantly North American (centered in California), but *F. camtschatcensis* ranges into eastern Asia (Japan and Siberia), *F. maximowiczii* and *F. dagana* Turcz. are located in southeastern Russia, and *F. davidii* is isolated in western China. The Asian species form a grade to the strictly American species in the combined analysis (Fig. 4) reflecting their probable origin in Asia, followed by a later dispersal to North America. Species of subgenus *Liliorhiza* have distinctive bulbs formed of several imbricate scales, much resembling *Lilium* bulbs and hence the name. In most species, the bulbs have numerous loosely attached bulbils, the so called “rice-grain bulbils” due to their size and shape. Rix (2001) recognized three series in this subgenus, but in our combined analysis the species in his series are mixed throughout the clade. There is no support for such a subdivision of *Liliorhiza*. *F. davidii* has previously been included in subgenus *Liliorhiza* due to the presence of rice-grain bulbils, but according to our results this isolated Chinese species is more closely related to the Eurasian clade (B). It is possible that rice-grain bulbils have evolved independently in *F. davidii* and subgenus *Liliorhiza* or in the common ancestor of *Fritillaria*, followed by a loss in the clade to which *F. davidii* is sister (Figs. 1–4), and this may possibly be resolved by a comparative study of the ontogeny of *F. davidii* and subgenus *Liliorhiza* rice-grain bulbils. *F. davidii* also differs from the species in subgenus *Liliorhiza* by only having basal leaves and no stem leaves, whereas *Liliorhiza* species have whorled stem leaves. Rix (2001) placed *F. davidii* in its own subgenus, *Davidii*, on the basis of the unusual leaves in this species, which is compatible with our topology.

4.3.2. *Fritillaria* subgenera *Petillum*, *Theresia*, and *Korolkowia*

Subgenus *Petillum* comprises a small group of larger (up to about 100 cm) and sturdier species distributed in Turkey, Iraq, Turkestan, Iran, Baluchistan, Afghanistan, and the western Himalayas. The bulbs are much larger (to a diameter of 8 cm) than those of most *Fritillaria* species and consist of a few, large, erect, imbricate, fleshy scales. Wild types have three to five flowers in a terminal umbel borne in the axils of the lower side of a whorl of leaves. The style is trifid and the seeds are winged. In our combined analysis, subgenus *Petillum* is moderately supported (83 BP).

Subgenus *Petilium* is sister to *F. sewerzowi* (100 BP) in a clade also containing *F. pallidiflora* and *F. persica* (B1, Fig. 4, 90 BP). The Central Asian *F. sewerzowi* is often given generic rank as the sole member of *Korolkowia* (Komarov, 1935; Turrill and Sealy, 1980), but this is clearly not appropriate. Bentham and Hooker (1883) included *F. sewerzowi* in the primarily North American section *Liliorhiza*, whereas Rix (2001) treated it as a monotypic subgenus of *Fritillaria* following Baker (1874). Like the species of section *Petilium*, it is a sturdy plant (20–50 cm tall) with many, more or less racemose flowers (8–10 or more) and a large bulb composed mainly of one massive scale (3–5 cm). The style is entire. *Fritillaria persica* from western Asia, which constitutes the monotypic subgenus *Theresia*, likewise has a bulb consisting of only one massive fleshy scale, second in size only to *F. imperialis*, and numerous racemose flowers (over 30) on a tall stem (up to 100 cm). The style is also entire.

Fritillaria pallidiflora has always been considered a member of the large Eurasian subgenus *Fritillaria* (Boissier, 1882; Bentham and Hooker, 1883; Turrill and Sealy, 1980; Rix, 2001). However, Bentham and Hooker, 1883 noted in their description of section *Petilium* that the then single species, *F. imperialis*, has characteristics not too far from *F. pallidiflora* in their section *Fritillaria*, and *F. pallidiflora* does have some similarities with *F. sewerzowi*, *F. persica*, and especially with the species of subgenus *Petilium*. *Fritillaria pallidiflora* has bulbs up to 4 cm formed by two to three, thick, fleshy scales and a strong, stout stem (up to 80 cm) bearing 1–5 racemose flowers with trifid styles. The characters of *F. pallidiflora* that link it to these other subgenera have not been considered before, and additional morphological studies are needed to investigate this finding and consider the placement of this species in relation to its closest relatives. At present, *F. pallidiflora* could be treated as an additional subgenus, but alternatively a simpler taxonomy would be to include *F. sewerzowi*, *F. persica*, and *F. pallidiflora* in an expanded subgenus *Petilium*, supported by the presence of massive bulbs consisting of one to a few large, fleshy scales and a sturdy overall habit. A detailed morphological comparison may reveal more characters to support this treatment.

4.3.3. *Fritillaria* subgenus *Fritillaria* and subgenus *Japonica*

Fritillaria subgenus *Fritillaria* is the largest subgenus comprising more than half of the species and including the type species, *F. meleagris*. The species in this subgenus are widely distributed from western Europe and the Mediterranean region to eastern Asia and are characterized by having the “typical” *Fritillaria* bulb, which consists of two fleshy subglobose scales more or less tunicated by the remains of the scales of the previous year or years. There may be three or four scales if one or

both of the scales from the previous year have persisted into a second season. The subgenus is normally subdivided into two sections based on the style (e.g., Turrill and Sealy, 1980). Species with a clearly trifid style are included in section *Fritillaria*, whereas species with an undivided style or a style that is only shortly trilobulate at the apex are placed in section *Olostyleae*. Rix (2001) subdivided section *Olostyleae* into 6 series and section *Fritillaria* into 10.

Except for *F. pallidiflora*, which is closely related to the species in subgenera *Petilium*, *Theresia*, and *Korolkowia* in our combined analysis (Fig. 4, clade B1, see Section 4.3.2), all the included species of subgenus *Fritillaria* as usually circumscribed (e.g., Rix, 2001) form a clade (B2). *Fritillaria japonica* is sister to the remainder of subgenus *Fritillaria* in the combined analysis and in the plastid analysis, but in the ITS analysis this species is more closely related to the *Petilium*, *Theresia*, *Korolkowia* clade (B1, Fig. 3), although this was only weakly supported (60 BP). *F. japonica* and the similar *F. amabilis* Koidzumi (not included in this study) were considered distinctive within the genus by Turrill and Sealy (1980), but they nonetheless placed them in section *Fritillaria* because of the bulb type. Both species are dwarf, single-flowered plants from Japan with a slender stem bearing, three, linear verticillate leaves at the top of the stem. Two broader oblong to elliptic and opposite leaves are borne ca. 1 cm below the upper three, and the small campanulate flower is borne on a short pedicel among them (Turrill and Sealy, 1980). In a recent classification, Rix (2001) placed *F. japonica* and *F. amabilis* in a new subgenus, *Japonica* Rix, together with *F. ayakoana* I. Maruyama & N. Naruhashi, *F. koidzumiana* Ohwi, and *F. shikokiana* N. Narushi. The placement of *F. japonica* is ambiguous in our study, and it would be worthwhile to include more of the four other species of subgenus *Japonica* in the analysis to get a better indication of the placement of these peculiar species. At present, they are best treated as a subgenus in their own right, as proposed by Rix (2001).

Most of the species included in this study belong to subgenus *Fritillaria* section *Fritillaria*, but *F. alburyana*, *F. caucasica*, and *F. minuta* belong to three series in section *Olostyleae* in the classification of Rix (2001). However these species do not form a clade, and, as previously questioned by Turrill and Sealy (1980), the style character is invalid for subdividing subgenus *Fritillaria*. Relationships within subgenus *Fritillaria* are not well resolved in our study. However, the series suggested by Rix (2001) are not supported as natural entities, the species from several series being intermingled.

4.3.4. *Fritillaria* subgenus *Rhinopetalum*

Fritillaria gibbosa and *F. karelinii* are sometimes grouped with *F. ariana* (Loz.-Lozinsk. & Vved.) E.M. Rix, *F. bucharica* Regel, and *F. stenantha* Regel in

subgenus *Rhinopetalum*, which was considered a genus in its own right by Komarov (1935). Bakshi-Khaniki and Persson (1997) recently supported the generic rank of *Rhinopetalum* based on the unique nectaries, which are deeply impressed and have a slit-like orifice on the tepals of these species. However, according to our analyses, this group of species is nested in genus *Fritillaria*. The inclusion of *F. gibbosa* and *F. karelinii* in the subgenus *Fritillaria* clade is only weakly supported (59 BP) in the combined analysis, and based on our results it is appropriate to follow Baker (1874) and Rix (2001) and rank *Rhinopetalum* as a subgenus. However, if *F. japonica* is not given subgeneric rank (Rix, 2001; Section 4.3.1) but remains in subgenus *Fritillaria*, then the *Rhinopetalum* species will have to be included in this subgenus as well to maintain monophyly (clade B2, Fig. 4).

4.4. Biogeographic patterns

Liliaceae *sensu* Chase et al. (2000) probably dates back to the beginning of the Paleocene or the end of the Cretaceous period (~65 million years ago, mya). Using a molecular clock approach in a broader angiosperm analysis with a limited sampling of specific groups (i.e., Liliales), Wikström et al. (2001) dated the split of Liliaceae from Smilacaceae to about 65 mya, and the diversification of the crown group dates to about 50 mya. In a study of Liliales with extended sampling, Vinnersten and Bremer (2001) dated the split of Liliaceae and Smilacaceae to about 55 mya, and the diversification of the crown clade of Liliaceae to 40–45 mya.

Vinnersten and Bremer (2001) also discussed the historical biogeography of Liliales and proposed that Liliaceae originated in North America and expanded to Eurasia 30–40 mya, where subfamily Lilioideae originated. If this were correct, then *Erythronium* must have at a later stage re-colonized western America, where most of the diversity of the genus is now present. *Lloydia* is also represented in North America, although abundant in Eurasia (Tamura, 1998). Based on our results, it is also clear that two invasions of North America across the Bering Straits have taken place within the *Lilium*/*Fritillaria* clade, once within in each genus. In the predominantly North American *Fritillaria* subgenus *Liliorhiza* (A, Fig. 4), *F. maximowiczii* (southeastern Russia) and *F. camtschaticensis* (ranging into Japan and Siberia) form a grade to the strictly American species reflecting their probable origin in Asia, followed by a later dispersal to North America. The isolated Chinese *F. davidii*, which was previously included in subgenus *Liliorhiza* due to the presence of rice-grain bulbils, is more closely related to the Eurasian clade (B, Fig. 4) according to our results. In *Lilium*, the strongly supported North American clade (C1, Fig. 4) forms a polytomy relative to the two weakly supported Asian–European clades (C2 and C3, Fig. 4).

4.5. Evolution of bulb structure and function of underground bulbils

A variety of storage organs is found in Liliaceae *sensu* Chase et al. (2000). Some species of Calochortaceae *sensu* Tamura (1998) and Liliaceae subfamily Medeoloideae are rhizomatous, whereas subfamily Lilioideae is composed of species with bulbs of various types, which are presumed to be derived from rhizomes (Tamura, 1998). The bulbs of some *Lilium* species (i.e., *L. canadense* L. and *L. pardalinum* Kellogg) may also form rhizomes (Comber, 1949; Tamura, 1998). Rhizomatous storage organs are normally found in species adapted to remain photosynthetically active over long growing seasons in shaded habitats (i.e., the forest floor), whereas large storage organs like bulbs and corms can rapidly develop shoots and are considered adaptations to short growing seasons in open habitats (i.e., the so-called spring geophytes; Patterson and Givnish, 2002 and references therein). Most species of *Fritillaria* are hardy and occur over a wide range of climate and habitats, although about half of them prefer full sun in open habitats (Beetle, 1944).

According to the relationships between genera within Liliaceae *sensu* Chase et al. (2000) that we obtained in the initial large *matK* analysis (Fig. 1), bulbs seem to have arisen independently from rhizomatous storage organs in *Calochortus* and Lilioideae (tribes Liliae and Tulipeae) as was also pointed out by Patterson and Givnish (2002) in their discussion of the evolution of storage organs in Liliales. *Clintonia*, *Medeola*, *Tricyrtis*, *Prosartes*, *Scoliopus*, and *Streptopus* all have rhizomatous storage organs (Patterson and Givnish, 2002; Tamura, 1998). Tunicated bulbs are found both in *Calochortus*, Tulipeae, and parts of tribe Liliae and could be the ancestral type. Within tribe Liliae, *Lilium* (including *Nomocharis*) has naked bulbs consisting of several fleshy scales.

Fritillaria presents a variety of bulb types. In our combined analysis (Fig. 4), we find a clade of Eurasian species (B), which consists of two sister clades (B1 and B2). In clade B2, comprising subgenera *Fritillaria*, *Rhinopetalum*, and *Japonica*, we find the typical *Fritillaria* bulb, which consists of two or three fleshy scales more or less tunicated depending on the remains of scales of the previous year or years. Clade B1 includes species belonging to subgenus *Petilium*, the monotypic subgenera *Theresia* and *Korolkowia*, and *F. pallidiflora*. The bulb of *F. pallidiflora* consist of two to three scales similar to the bulbs of species in subgenus *Fritillaria*, although it is much larger. The bulbs of *F. sewerzowii* (subgenus *Korolkowia*) and *F. persica* (subgenus *Theresia*) are both large and composed of only one massive, fleshy scale, whereas the large bulbs of subgenus *Petilium* species consist of a few large, erect, imbricate scales.

In the predominantly North American *F.* subgenus *Liliorhiza* (clade A, Fig. 4), we find naked bulbs with numerous scales resembling the *Lilium* type, but with numerous rice-grain bulbils attached. Bulbils normally arise from the axil of a leaf or the inflorescence (e.g., the aerial bulbils in several species of *Lilium* and *Allium*; Hickey and King, 2000). In the monotypic subgenus *Davidii*, which is sister to the clade of Eurasian species (clade B, Fig. 4), we find a similar type of bulb with bulbils, which appears to have developed independently from the bulb type in subgenus *Liliorhiza* or alternatively could have evolved in an ancestor of genus *Fritillaria* followed by loss in the clade sister to *F. davidii* (Fig. 4).

Bulbils are not unique to *Fritillaria* within Liliaceae and also found in some species of *Lilium* and *Gagea*. In species of *Fritillaria*, the bulbils are formed in the axils of the scale leaves, whereas the bulbils occasionally present in species of *Nomocharis* and *Lilium* (e.g., *L. bulbiferum* L.) are aerial and formed in cauline leaf axils (Synge, 1980; Tamura, 1998). In species of *Gagea*, bulbils are normally developed in the axils of the scale leaves as in species of *Fritillaria*, but aerial bulbils are sometimes formed in the axils of the cauline foliage leaves as in species of *Lilium* (Levichev, 1999; Tamura, 1998).

Regardless of their origin, bulbils function as a means of vegetative propagation, and species that produce bulbils usually multiply more vigorously than species that reproduce only by seeds. As an example, populations of *Allium vineale* L. (Alliaceae) include individuals with three modes of reproduction: sexual flowers, aerial bulbils, and below-ground asexual offsets. Ronsheim (1994; Ronsheim and Bever, 2000) studied the advantages of the different reproductive modes in *A. vineale* and found that seeds generally suffer higher predation rates and lower germination and survival rates; they show no dormancy in the field and are dispersed no further than the aerial bulbils. Likewise, diploids of *Butomus umbellatus* L. (Butomaceae) produce both abundant viable seeds and hundreds of small aerial bulbils. The seeds appear to be out-competed by the bulbils, which disperse on the water surface, sometimes over long distances (Eckert et al., 2003).

In the Cape flora of South Africa, geophytes constitute more than 16% of the flora, and the nutritious bulbs and corms are consumed by mole rats, which also participate in the dispersal of these bulbs (Johnson, 1992). The highly palatable segmented corms of *Micranthus junceus* N.E. Brown (Iridaceae) are broken apart by the mole rats, and uneaten segments are carried away and stored in the burrows (Lovegrove and Jarvis, 1986). Many Cape geophytes (i.e., species of *Moraea*, Iridaceae) also have clusters of cormlets or bulbils. In an attempt to get to the main bulb, the bulbils are dislodged by the mole rat and dispersed throughout the burrow system (Johnson, 1992). Likewise, it was proposed that mole-voles are a

primary dispersal agent of geophytes in the Spanish Pyrenees, leaving most of the small storage organs uneaten (Borghi and Giannoni, 1997). The bulbils in species of *Fritillaria* are produced in the axils of the underground scale leaves and are less easily dispersed above ground. Bulbils may instead be dispersed underground by burrowing animals. However, other ecological conditions may also determine the success of bulbils in comparison with reproduction and dispersal by seeds.

In summary, phylogenetic analysis of DNA sequences from three regions using maximum parsimony demonstrated *Fritillaria* and *Lilium* (the latter including *Nomocharis*) to be sister taxa. *Fritillaria* sections *Fritillaria* and *Liliorhiza* are supported in part, and some of the most enigmatic species usually included in *Fritillaria* (sections *Petilium* and *Theresia* and the monotypic genus *Korolkowia*) are closely related. The results generally support a new classification of *Fritillaria* proposed by Rix, particularly at higher levels. We postulate independent origins of the underground bulbils found in *F. davidii* and the remainder of subgenus *Liliorhiza* and suggest that bulbils are an adaptation to underground dispersal.

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