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Molecular systematics of *Lilium* and allied genera (Liliaceae): phylogenetic relationships among *Lilium* and related genera based on the *rbcL* and *matK* gene sequence data

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Abstract

Coding regions of the *rbcL* and *matK* genes of cpDNA were sequenced to analyze phylogenetic relationships of the family Liliaceae *sensu stricto*, including the major 16 genera of Medeoloideae and Lilioideae of the Liliaceae, in reference to several genera such as *Scoliopus*, *Uvularia*, *Disporum*, and *Trillium* used as outgoups. The results were congruent with the taxonomic concept of Liliaceae *sensu stricto* recently proposed by Tamura (1998). The inter- and infrageneric relationships in the genus *Lilium* and allied taxa were then analyzed based upon the *rbcL* and *matK* gene sequencing data, using *Medeola* and *Erythronium* as outgroups. The *rbcL* gene has evolved more slowly than *matK* and its phylogenetic resolution has been poor as a result of the low base substitution rates; whereas the *matK* gene has shown a much higher base substitution: 104 variable sites (including 80 informative sites) out of 1641 base pairs were detected. In addition, a remarkably high number of indels, i.e. 19 insertion/deletion events, were detected in the *matK* gene, which provided us with new evidence for structural changes of this gene within the genus *Lilium* and allied taxa. Phylogenetic analyses based on the majority rule of the sequence data of *matK* gene revealed that the genus *Lilium* consists of three different major clades, including taxa that were placed into different sections by earlier taxonomic treatments, and thus the results of molecular systematic analysis was not congruent with sectional delimitations of the genus *Lilium* based on the morphological characters. *Nomocharis pardanthina* and *Nomocharis saluenensis* were ingroup taxa of *Lilium*. *Notholirion*, *Cardiocrinum*, and *Fritillaria* turned out to be sister groups to *Lilium*. An evaluation of the morphological and life-history characteristics was also attempted in light of the molecular phylogeny.

Keywords: Cardiocrinum, Fritillaria, Liliales, *Lilium, matK,* molecular phylogeny, *Nomocharis, Notholirion, rbcL.*

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Introduction

The concept of the family Liliaceae has been a subject of considerable dispute in the history of the Monocots taxonomy (Krause 1930; Hutchinson 1959; Dahlgren *et al*. 1985; Takhtajan 1987). In recent years, a number of new concepts have been proposed on the delimitation of the Liliaceae *sensu stricto* based on the re-evaluation of morphological traits and various other criteria (Takhtajan 1997; Tamura 1998).

Takhtajan (1987) included the following eight genera in the Liliaceae *sensu stricto*: *Erythronium* and *Tulipa* (subfamily Tulipeae); *Cardiocrinum, Lilium, Notholirion, Nomocharis, Fritillaria* and *Rhinopetalum* (subfamily Lilieae). However, he regarded *Medeola* as a monotypic genus of the Medeolaceae, and he included *Clintonia* tentatively in the separate family Convallariaceae. His newly

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proposed system for the family Liliaceae *sensu stricto* (Takhtajan 1997) follows the same concept he proposed in 1987. Tamura (1998), however, recognized two subfamilies in the Liliaceae *sensu stricto*, Medeoloideae (*Clintonia* and *Medeola*) and Lilioideae (*Erythronium, Tulipa, Gagea* and *Lloydia* (tribe Tulipeae); *Cardiocrinum, Lilium, Fritillaria, Nomocharis* and *Notholirion* (tribe Lilieae)). Thus there are still differing taxonomic viewpoints concerning the members of the family Liliaceae, and of the families within the Liliales.

The genus *Lilium*, the type genus of the Liliaceae *sensu stricto*, consists of approximately 100 species that are widespread primarily in the northern hemisphere with a pronounced centering of distribution around south-western and Himalayan Asia–China (Krause 1930; Comber 1949; Woodcock & Stearn 1950; Dahlgren *et al*. 1985). The long-standing popularity of the *Lilium* as ornamental plants is because of their large, showy flowers that often have a strong fragrance. Because of this horticultural interest, breeding studies with an incalculable number of resulting hybrids have been conducted. Among the so-called ‘true lilies’, four genera – *Cardiocrinum, Nomocharis, Notholirion* and *Lilium* – have been included (Buxbaum 1937; Woodcock & Stearn 1950). A phylogenetic tree of the genus *Lilium* has been proposed based on the results of cytogenetic and interspecific hybridization studies (Lighty 1960, 1968; Asano 1986; Noda 1987).

However, inter- and/or infrageneric classifications of the genus *Lilium* and allied groups have been an issue of considerable dispute and taxonomic systems have repeatedly changed (see Table 1). Wilson (1925) recognized four subgenera, *Notholirion, Cardiocrinum, Eulirion* and *Lophophorum*, and within *Eulirion* four sections were distinguished. However, in Comber’s revision (1949) only two subgenera (*Cardiocrinum* and *Eulirion*), and seven sections within *Eulirion*, were recognized. The most recent classification scheme, that of Liang (1980) distinguishes *Lilium, Cardiocrinum, Nomocharis* and *Notholirion* as independent genera and recognizes eight sections in *Lilium* (Table 1). Sealy (1983) and Liang (1984) monographed the genus *Nomocharis*, in which Sealy (*l. c.*) transferred five *Nomocharis* species into the genus *Lilium*. This suggests that *Nomocharis* and some members of *Lilium* are similar enough to cause taxonomic confusion between these two genera.

The purpose of the present study is two-fold. First, the study aims to re-evaluate the systematic status of the family Liliaceae *sensu stricto* in light of the molecular sequencing data of *rbcL* (a large subunit of ribulose-1,5-bisphosphate-carboxylase) and *matK* (matulase) genes of *cp*DNA for 16 genera, including the major members of Medeoloideae and Lilioideae of the Liliaceae, in reference to several other outgroup genera referred to Liliaceae

Table 1 The genus *Lilium* and allied genera, and changes in classification systems

Reichenbach, 1830	Endlicher, 1836	Baker, 1871	Ascherson & Graebner, 1905	Wilson, 1925	Comber, 1949	Liang, 1980
*Lilium	*Lilium	*Lilium	*Lilium	*Lilium	*Lilium	*Lilium
sect. *Lilium*	sect. *Cardiocrinum*	subgenus *Notholirion*	subgenus *Cardiocrinum*	subgenus *Notholirion*	subgenus *Cardiocrinum*	sect. *Martagon*
sect. *Martagon*	sect. *Amblirion* (=*Fritillaria*)	subgenus *Lilium*	subgenus *Eulirion*	subgenus *Cardiocrinum*	subgenus *Eulirion*	sect. *Pseudolillium*
	sect. *Eulirion*	group *Eulirion*	sect. *Martagon*	subgenus *Lophophorum*	sect. *Martagon*	sect. *Archelirion*
	sect. *Martagon*	group *Martagon*	sect. *Isolirion*	subgenus *Eulirion*	sect. *Pseudolirium*	sect. *Asteridium*
	sect. *Pseudolirium*	group *Isolirion*	sect. *Archelirion*	sect. *Martagon*	sect. *Liriotypus*	sect. *Sinomartagon*
		group *Archelirion*	sect. *Liriotypus*	sect. *pseudolirium*	sect. *Archelirion*	sect. *Dimorphophyllum*
				sect. *Archelirion*	sect. *Sinomartagon*	sect. *Regalia*
				sect. *Leucolirion*	sect. *Leucolirion*	sect. *Lophophorum*
					sect. *Daurolirion*	sect. *Concolor*
						sect. *Henryi*
						**Nomocharis*
						**Cardiocrinum*
						**Notholirion*

sensu lato (Krause 1930). Second, the study aims to analyze the affinities and phylogenetic relationships of taxa that have been referred to *Lilium* and several allied genera by analyzing their *rbcL* and *matK* gene sequences, because inter- or infrageneric delimitation of *Lilium sensu lato* has been a controversial taxonomic issue.

In the course of summarizing all evidence available, an attempt was also made to re-evaluate the diagnostic value of morphological characters and life-history traits of *Lilium* for establishing their systematic positions (Comber 1949) and trends of evolutionary divergence of infrageneric taxa by overlaying these characters on the molecular tree constructed in this study.

Methods

Plant samples

Thirty-five *Lilium* species belonging to seven sections, one *Cardiocrinum* species, two *Nomocharis* species belonging to two sections, one *Notholirion* species and one *Fritillaria* species were sampled and analyzed for *rbcL* and *matK* genes. Several other genera analyzed for the *rbcL* and *matK* gene were also included in order to obtain a general picture of the topology of Liliiflorae (sensu Dahlgren *et al*. 1985). *Erythronium japonicum* and *Medeola virginiana* were used as outgroup taxa, as they have been placed in neighboring positions in the Liliales (Takhtajan 1997; Tamura 1998). Several additional genera analyzed for the *rbcL* and *matK* gene were also included in order to obtain a general picture of the topology of Liliiflorae (*sensu* Dahlgren *et al*. 1985). Voucher specimens of the plants analyzed are deposited in the Herbarium of Kyoto University (KYO). The *rbcL* and *matK* sequencing data of all the species and outgroup used in this study are registered in the DNA Data Bank of Japan (DDBJ) (Table 2).

DNA extraction

Total genomic DNA was extracted from silica-gel-dried leaves using the CTAB method of Doyle & Doyle (1987), except that liquid nitrogen was used to assist in the grinding of plant tissue. In many cases, the same DNA as those used in recent *cp*DNA restriction site analyses (Shinwari *et al*. 1994; Kato *et al*. 1995) were used to generate *matK* sequences.

Polymerase chain reaction for the rbcL *gene*

The PCR employed to amplify the 1411 bp of the *rbcL* gene used two primers that anneal to the 5′ end, *rbcL*N′: 5′-ATGTCACCACAAACAGAAACT-3′, and just downstream of the 3′ end of the *rbcL* coding region, DBRBAS2: 5′-GCTTGAATTCGAATTTGATC-3′. To obtain the sequence of the 5′ end of *rbcL* gene, PCR was conducted using an additional primer that anneals to the *atp*β (*atp*β 2325′-CCGTCCGTAGCATCATAGC-3′), upstream from the *rbcL* gene (Table 3). The amplification reaction mixture (100 mL) contained 50–100 ng of total DNA, 40 pmol of each primer, 0.2 mmol/L of dNTP, 50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.8, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100 (McPherson *et al*. 1991, 1995) and 2.0 units of Taq DNA polymerase (Wako Chemicals, Tokyo, Japan). Amplification was conducted in a DNA Thermal Cycler (Cetus Model; Perkin Elmer, Cetus, CA, USA) for 35 cycles. Each cycle consisted of a denaturing step of 1 min at 94°C, an annealing step of 2 min at 54°C and an extension step of 3 min at 72°C. After the last cycle, a final extension step (10 min, 72°C) was added. The amplified DNA was subjected to electrophoresis through 1% agarose gel and excised from the gel. The DNA was purified by glass-milk extraction (Gene Clean II, Bio101; Vista, CA, USA) and re-suspended in 20 mL of TE (10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA). The final yield averaged about 4 mg of DNA, enough for eight sequencing reactions.

Polymerase chain reaction of matK *gene*

The *matK* gene was amplified using the Taq polymerase (Toyobo, Tokyo, Japan) and universal primers, *trnK*-3914FM and *trnK*-2R of Johnson & Soltis (1995). PCR sequence primers used in the present study are shown in Table 3 (Johnson & Soltis 1995; Ooi *et al*. 1995; Yoshida, unpublished data).

For the PCR amplification, each reaction mixture (100 µL) contained 54 µL of sterile water, 10 µL of 10 × Taq polymerase reaction buffer (Toyobo), 10 µL of 25 mmol/L $MgCl_2$, 16 µL of 1.25 mmol/L dNTP (Toyobo), 4 µL of each of the two primers (40 pmol), 0.4 µL (2 units) of Taq polymerase (Toyobo), and 2 µL of genomic DNA template (50–100 ng). Amplification was done in a DNA Thermal Cycler (Perkin Elmer) for 35 cycles. Each PCR cycle proceeded in the following manner: (i) 1 min at 94°C to denature the double-stranded template DNA; (ii) 2 min at 50°C to anneal primers to single-stranded DNA; and (iii) 3 min at 72°C to extend primers. The first cycle was preceded by an initial denaturation step of 2 min at 94°C; a final extension at 72°C for 7 min followed completion of the 35 cycles. Each set of reactions was monitored by the inclusion of a negative (no template) control.

To remove unused amplifying primers and dNTP, the PCR product was electrophoresed in a 1% agarose gel (using 1× TAE as the gel buffer) stained with ethidium bromide and then excised under low-wave-length ultraviolet light with a scalpel.

Following cycle sequencing, the reactions were purified using the ‘Ethanol Precipitation Protocol 1’ (manufac-

Table 2 Plant material analysed in the present study

				DDBJ accession number	
Genus	Species	Locality	Collector(s)	*rbcL*	*matK*
Trillium	*T. underwoodii* Small	USA: Florida, Gaden Co., Flat Riner	M. Ohra *et al.*	–	AB017412
	T. grandiflorum (Michaux) Salisb.	USA: Pennsylvania, Westmoreland Co.	S. Kawano *et al.*	D28164	–
Scoliopus	*S. bigelovii* Torr.	USA: California, Humboldt Co.	S. Kawano *et al.*	D28162	AB024394
Clintonia	*C. borealis* (Alt.) Rafin	USA: Wisconsin, Marathon Co.	S. Kawano *et al.*	D17372	AB024542
Amana	*A. edulis* (Miq.) Honda	Japan: Tokyo	M. Iiizumi	AB024385	AB024388
Gagea	*G. lutea* (L.) Ker-Gawl.	Japan: Akita Pref., Tubaki	Y. Horii	AB024389	–
Uvularia	*U. floridana* Chapman	USA: Florida, Gadsen Co., Flat Creak	S. Kawano	AB009949	AB024396
Disporum	*D. sessile* Don	Japan: Toyama Pref., Mt. Tateyama	S. Kawano	D17376	AB024543
Tulipa	*T. turkestanica*	Turkey	unknown	AB037378	AB024386
Medeola	*M. virginiana* L.	USA: Pennsylvania, Somerset Co.	S. Kawano	D28158	AB024543
Erythronium	*E. japonicum* Decne.	Japan: Toyama Pref., Yatsuo-machi	S. Kawano	D28156	AB024387
Fritillaria	*F. koidzumiana* Ohwi	Japan: Toyama Pref., Yatsuo-machi	K. Hayashi	AB034939	AB024390
Notholirion	*N. thomsonianum* (Royle) Stapf**	Western Himalaya	unknown	AB034919	AB024390
Cardiocrinum	*C. cordatum* (Thunb.) Makino	Japan: Osaka Pref., Nikawabe	K. Hayashi	AB034918	AB024390
Nomocharis	*N. pardanthina* Franch.**	China: Yunnan	unknown	–	AB030842
	N. saluenensis Balf. f.*	China: Yunnan	Yurigahara	AB034938	AB024391
Lilium	Section *Martagon*				
	L. hansonii Leichtlin	Korea: Isl. Ullung	S. Kawano *et al.*	AB034930	AB030871
	L. martagon L.*	South Germany	Yurigahara	–	AB030872
	L. medeoloides A. Gray	Japan: Akita Pref., Maki	K. Hayashi	AB034931	AB030873
	L. tsingtauense Gilg.	Korea: Mt. Haein	K. Hayashi *et al.*	–	AB030874
	Section *Pseudolilium*				
	L. columbianii Hanson	USA: Oregon, Manrion Forks	K. Hayashi *et al.*	AB034927	AB030847
	L. washingtonianum Kellog	USA: Oregon, Trillium Creek	K. Hayashi *et al.*	–	AB030848
	L. pardalinum Kellog.*	USA: Oregon	E. Mirro	–	AB030845
	L. superbum L.	USA: Pennsylvania, Westmorland Co. Donegal Township	K. Hayashi *et al.*	AB034926	AB024546
	L. michiganense Farwell*	USA: Oregon	E. Mirro	–	AB030844
	L. canadense L.	USA: New Hampshere, Swiftwater	K. Hayashi *et al.*	–	AB030843
	L. philadelphicum L.	USA: Pennsylvania, Penn Roosevelt State Park	K. Hayashi *et al.*	AB034925	AB030846
	L. philadelphicum var. *andinum* (Nutt) Ker-Gawle	Canada: Ontario, Algonquin Provincial Park	K. Hayashi *et al.*	–	AB037377

Section *Liliotypus*				
L. bulbiferum L.*	South Germany	Yurigahara	AB034929	AB030864
L. candidum L.*	Palestine	Yurigahara	AB034928	AB024545
L. pomponium L.*	South France	F. D. Hanson	–	AB030865
L. pyrenicum Gouan	South-west France	Yurigahara	–	AB030866
Section *Archelirion*				
L. alexsandrae hort. Wallace	Japan: Kagoshima Pref., Amami-Oshima	K. Hayashi	AB034920	AB030849
L. japonicum Thunb.	Japan: Osaka Pref., Kobuka	K. Hayashi	AB034921	AB030850
L. nobilissimum Makino**	Japan: Kagoshima Pref., Kuchino-Shima	unknown	–	AB030851
L. rubellum Baker	Japan: Niigata Pref., Kirinzan	H. Kato	–	AB030852
L. speciosum Thunb.	Japan: Fukuoka Pref., Munakata	K. Hayashi	AB034922	AB030853
Section *Sinomartagon*				
L. cailosum Sieb.	Japan: Fukuoka Pref., Hiraodai	K. Hayashi	–	AB030854
L. cernuum Komar.*	Republic of Korea	Yurigahara	–	AB030855
L. pumilum Delile**	China: Jilin	unknown	–	AB030857
L. concolor Salisb.*	Japan: Kochi Pref.	unknown	–	AB030856
L. henryi Baker.**	Central China	unknown	AB034924	AB030858
L. duchartrei Franch.**	South-west China	unknown	–	AB030862
L. lancifolium Thunb. (2X)	Japan: Nagasaki Pref., Komota	K. Hayashi	AB034937	AB030859
L. leichtlinii var. *maximowiczii* (Regel) Baker	Japan: Nara Pref., Musashi	K. Hayashi	AB034932	AB030860
L. rosthomii Diels.**	China: Sichuan	unknown	–	AB030861
L. bakerianum Coll. et Hemst.**	China: Yunnan	unknown	AB034923	AB024544
L. nanum Klotzsch*	China: Yunnan	unknown	–	AB030863
L. mackliniae Sealy*	India: Manipur	unknown	–	AB030877
L. fargeslii Franch.**	China: Yunnan	unknown	–	AB030878
Section *Leucolirion*				
L. sargentiae Wils.	China: Sichuan	S. Sakamoto	–	AB030870
L. regale Wilson.**	China: Sichuan	unknown	–	AB030869
L. formosanum Wallace	Thailand: Mt. Keitou	M. Shimizu	AB034933	AB030867
L. longiflorum Thunb.	Japan: Okinawa Pref., Ryukyu Islands, Gaja	S. Noda	AB034934	–
L. leucanthum Baker*	China: Gansu	unknown	–	AB030868
Section *Daurolirion*				
L. maculatum Thunb.	Japan: Shizuoka Pref., Shimoda	K. Hayashi	AB034932	AB030875
L. maculatum ssp. *dauricum* (Baker) Hara	Japan: Hokkaido, Esan Nanatsuiwa	K. Hayashi	AB034935	AB030876

*Material collected from cultivated plants in Yurigahara Park. **Material collected from cultivated plants which I bought from a commercial farm.

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Table 3 PCR sequence primers used in the present study. Location of the 5′ end base of the primer is indicated with regard to the site number of the *Nicotiana tabacum trn K* and *matL* gene (Sugita *et al.* 1985)

	Primer	Sequence	Location	Strand	Designed by
rbcL					
	rbcL N′	5′-ATGTCACCACCACAAACAGAAACT-3′	1–18	sense	Terachi *et al.* 1987
	S1	5′-AGGACGATGCTACCACATCG-3′	243–263	sense	Terachi *et al.* 1987
	S2	5′-AAAACTTTCCAAGGCCC-3	435–451	sense	Terachi *et al.* 1987
	S3	5′-TTTATGCGTTGGAGAGACCG-3′	631–650	sense	Terachi *et al.* 1987
	S4	5′-AATGCATGCAGTTATTG-3′	887–903	sense	Terachi *et al.* 1987
	S5	5′-GGTATTCATGTTTGGCA-3′	1141–1158	sense	Terachi *et al.* 1987
	DBRBAS1	5′-TTACAGCTTGTACACACGC-3′	1295–1276	sense	Terachi *et al.* 1987
	DBRBAS2	5′-GCTTGAATTCGAATTTGATC-3′	1411–1392	antisense	Terachi *et al.* 1987
	TRRV1	5′-TAGAGACCCAATCTTGAGTG-3′	1111–1092	antisense	Terachi *et al.* 1987
	RV5	5′-CCGTAGTTCTTTGCGGATAA-3′	557–538	antisense	Terauchi *et al.* unpubl.
	RV4	5′-TCAGTCCACACACAGTTGTCCA-3′	215–196	antisense	Terauchi *et al.* unpubl.
	atp β 232	5′-CCGTCCGTAGCATCATAGC-3′	*atp* β 232	antisense	Howe *et al.* 1985; Moon *et al.* 1987
matK					
F1	*trn K*-3914FM	5′-ATCTGGGTTGCTAACTCAATGG-3′	4–19	sense	Johnson & Soltis, 1994
F2	*mat* K-FF74	5′-ATACCCTGTTCGGACCATATTG-3′	669–689	sense	Yoshida & Hayashi*
F3	*mat* K-FL32	5′-CTGTCCTCCGTAAGAAC-3′	713–732	sense	Yoshida & Hayashi*
F4	*mat* K-AF	5′-CTATATCCACTTATCTTTCAGGAGT-3′	804–828	sense	Ooi *et al.* 1995
F5	*mat* K-BFM	5′-TCAAAGGGATTTGCGTTTATTGTGG-3′	1038–1062	sense	Hayashi, 1998
F6	*mat* K-EF1	5′-TCAAAGGGATTTGCGTTTATTGTGG-3′	1250–1270	sense	Youshida upubl.
F7	*mat* K-EF2	5′-CTCATGAAGAAATGGAGATATTACC-3′	1638–1662	sense	Yoshida unpubl.
F8	*mat* K-CF	5′-TTGATCGATTTGGTCGGATATGTAG-3′	2057–2080	sense	Yoshida & Hayashi*
R1	*trnK*-2R	5′-AACTAGTCGGATGGAGTAG-3′	2573–2554	antisense	Steele & Vilgalys, 1994
R2	*mat* K-8R	5′-AAAGTTCTAGCACAAGAAAGTCGA-3′	2080–2057	antisense	Ooi *et al.*, 1995
R3	*mat* K-RM	5′-CTACATATCCGACCAAATCGATCAA-3′	1990–1966	antisense	Hayashi, 1998
R4	*mat* K-ER1	5′-CATCTTGAATCCAGTATTGAAGG-3′	1662–1638	antisense	Yoshida unpubl.
R5	*mat* K-ER2	5′-GGTAATATCTCCATTTCTTCATGAG-3′	1270–1250	antisense	Yoshida unpubl.
R6	*mat* K-AR	5′-CTGTTGATACATTCGA-3′	956–941	antisense	Yoshida & Hayashi*

*Designed in this study. The location was based on the starting position of *trn K* (5′).

turer's instructions: Perkin Elmer, Revision A, August 1995) or the 'Ethanol/Sodium Acetate Precipitation Protocol' (Perkin-Elmer, 1997) to remove unincorporated dye terminators and then completely dried in a vacuum. The reaction pellets were resuspended in 4 µL of loading buffer (five parts deionized foramide to one part 50 mmol/L EDTA (pH = 8.0)) and analyzed in an Applied Biosystems 373 A DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using 6% acrylamide gel run in 1 × TBE buffer.

DNA sequencing of the rbcL *and* matK *genes*

For sequencing the *rbcL* and *matK* genes, purified double-stranded DNA were then used in cycle sequencing reactions that were conducted using the Prism™ Dye Deoxy Terminator Cycle Sequencing Ready Reaction Kit or ABI Prism™ BIG Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The cycle sequencing reaction mixture contained 80 or 40 ng of template DNA, 8 µL of terminator premix, 3 µL of primers (3.2 pmol) and the appropriate amount of sterile water for a total volume of 20 µL. The cycle sequencing involved 25 cycles of denaturation for 30 s at 96°C, annealing for 15 s at 50°C and extension for 4 min at 60°C. Reaction mixtures were subsequently stored at 4°C.

The location and base composition of each of the primers used in this study are given in Table 3. Following cycle sequencing, the reactions were purified using the 'Ethanol Precipitation Protocol 1' (Perkin Elmer) to remove unincorporated dye terminators and then completely dried in a vacuum. The reaction pellets were resuspended in 6 µL of loading buffer (five parts deionized foramide to one part 25 mmol/L EDTA–blue dextrine mixture) and analyzed in an ABI Prism™ 377 DNA Sequencer using 50% Long Ranger gel solution (Applied Biosystems) run in 1 × TBE buffer. For sequencing the *rbcL* gene, the purified double-stranded PCR product was used as a template for direct sequencing with an autosequencer (ABI 373 A) and Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Data analysis of rbcL *and* matK *genes*

The *matK* sequences were visually aligned with Seq Ed version 1.0.3 (Applied Biosystems); the few insertion/deletion events (indels) did not hinder alignment. Each indel was treated as a missing character or scored conservatively as a single evolutionary event in separate analyses. Phylogenetic analyses using the maximum parsimony method were performed with PAUP version 3.1.1 (Swofford 1993). The most parsimonious trees were obtained using the heuristic search option involving 100 replications of random addition sequence and tree-bisection–reconnection (TBR) branch-swapping. All characters were specified as unweighted. To obtain confidence limits for various clades, bootstrap analysis (Felsenstein 1995) was conducted. Bootstrap values with 1000 replications were calculated using the heuristic search option (with TBR branch-swapping and simple addition sequence algorithms).

Results

Phylogeny of Liliaceae sensu stricto *as revealed by* rbcL *gene sequencing data*

Partial sequences of *rbcL* gene (1390 bp) were determined for 14 selected taxa, including *Erythronium*, *Gagea*, *Amana*, *Tulipa*, *Cardiocrinum*, *Notholirion*, *Fritillaria*, *Lilium*, *Nomocharis*, *Medeola* and *Clintonia*, using *Scoliopus*, *Trillium*, *Disporum* and *Uvularia* as outgroups. A total of 180 variable nucleotide positions was detected among the ingroup taxa; 114 of these were potentially informative. A strict consensus tree (50% majority rule consensus tree) of the *rbcL* gene with its bootstrap values is shown in Fig. 1. The tree showed two major clades, one consisting of five genera – *Notholirion, Cardiocrinum, Fritillaria, Lilium,* and *Nomocharis* – and a second consisting of four genera – *Erythronium, Tulipa, Amana* and *Gagea*. *Medeola* and *Clintonia* are obviously somewhat a distantly related sister group.

Phylogeny of Liliaceae sensu stricto *as revealed by* matK *gene sequencing data*

The results of phylogenetic analysis using the *matK* gene for 14 selected genera, including *Erythronium*, *Gagea*, *Amana*, *Tulipa*, *Cardiocrinum*, *Notholirion*, *Fritillaria*, *Lilium*, *Nomocharis*, *Medeola*, *Clintonia* and *Scoliopus*, using *Disporum*, *Uvularia* and *Trillium* as outgroups, clearly revealed the phylogenetic positions of genera referred to Liliaceae *sensu stricto* and their infra-familial positions. The *matK* tree (50% majority rule consensus tree) obtained is shown in Fig. 2. The tree obtained for *matK* was very similar to the *rbcL* tree (Fig. 2) which showed two major clades, one consisting of five genera – *Notholirion, Cardiocrinum, Fritillaria, Lilium* and *Nomocharis* – and a second consisting of four genera – *Erythronium, Tulipa, Amana* and *Gagea. Medeola* and *Clintonia* are a distantly related sister group.

Sequence variation and divergence rates in rbcL *gene*

The 18 *Lilium* species and one species each of the following four genera, *Nomocharis, Notholirion, Fritillaria* and *Cardiocrinum*, and also including *Erythronium* and *Medeola* were sequenced for *rbcL* gene (Table 2). Topology obtained by the MP method for the *rbcL* gene tree (50% majority rule consensus tree) is illustrated in Fig. 3. The results of phylogenetic analysis showed that *Lilium, Cardiocrinum, Nomocharis, Notholirion* and *Fritillaria* constituted a single large clade with a high bootstrap value of 92%, which at least indicates a close relationship of these genera that have been referred to the Liliaceae (*sensu* Krause 1930; Takhtajan 1997) (Fig. 3). However, the phylogenetic resolution of these genera and species analyzed was exceedingly poor as a result of low base-substitution rates in *rbcL*. The *rbcL* gene should therefore be considered to be highly conserved, at least within this group of Liliales.

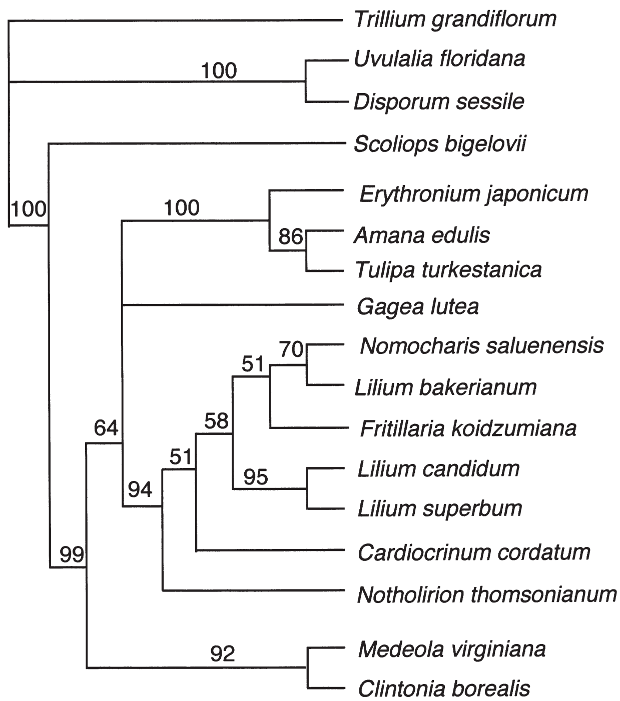

Fig. 1 The 50% majority-rule consensus tree obtained from the phylogenetic analysis of *rbcL* gene sequences for 17 taxa of Liliaceae and *Trillium* as an outgroup (× 1000 replications). The length of the shortest tree (L) was 282 steps; a consistency index (CI), 0.794; a homoplasy index (HI), 0.206; and a retention index (RI), 0.791. Percentages above branches are bootstrap values.

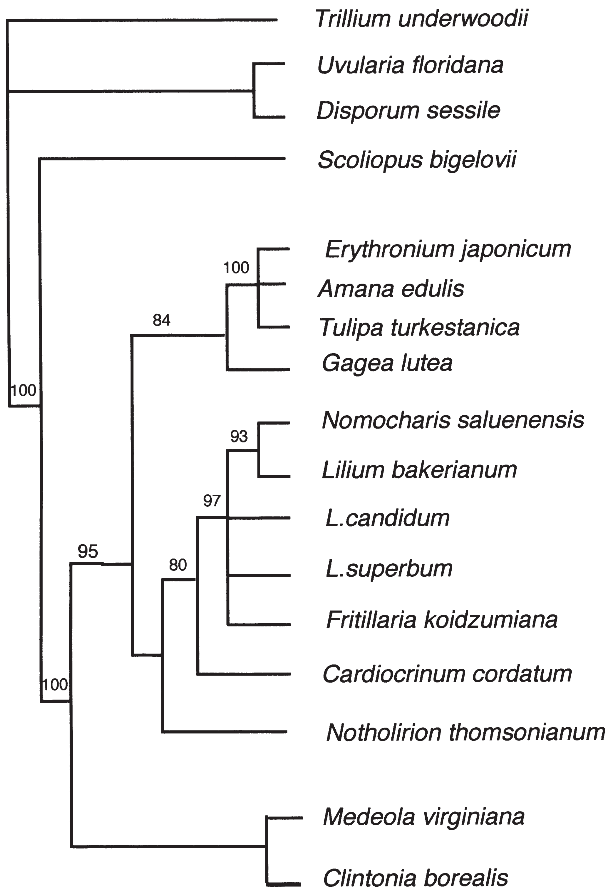

Fig. 2 The 50% majority-rule consensus tree obtained from the phylogenetic analysis of *matK* gene sequences 17 taxa of Liliaceae and Trillium as an outgroup. Percentages above branches are bootstrap values (× 1000 replications). The length of the shortest tree (L) was 642 steps; a consistency index (CI), of 0.824, a homoplasy index (HI) of 0.176, and a retention index (RI) of 0.827.

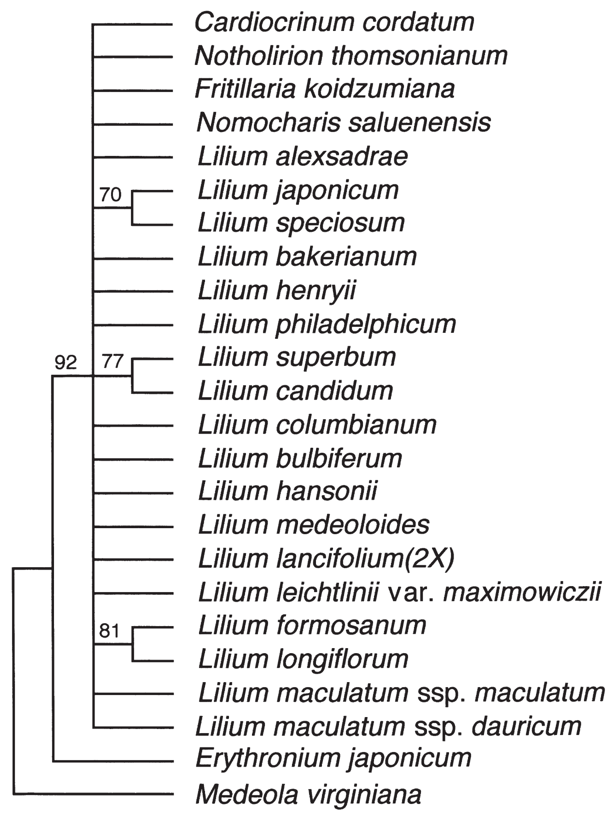

Fig. 3 The 50% majority-rule consensus tree obtained from the phylogenetic analysis of *rbcL* gene sequences for 22 taxa of Liliaceae *sensu stricto* and *Medeola* and *Erythronium* as outgroups. Percentages above branches are bootstrap values. The length of the shortest tree (L) was 144 steps, a consistency index (CI) of 0.583, a homoplasy index (HI) of 0.417, and a retention index (RI) of 0.286.

Among the 18 *Lilium* taxa sequenced, relatedness was shown for only three pairs of species as follows. (i) *L. japonicum and L. speciosum*, with a bootstrap value of 70%; (ii) *L. superbum and L. candidum*, with a bootstrap value of 77%; and (iii) *L. formosanum and L. longiflorum*, with a bootstrap value of 81%. The first and third clades matched the topology obtained by *matK* gene sequencing data but *L. superbum* (section *Pseudolilium*) and *L. candidum* (section *Liriotypus*) demonstrated affinities with different species belonging to different clades in the *matK* tree (cf. Fig. 4).

Sequence variation and divergence rates in matK *gene*

In the present study, sequencing of the *matK* (1641 bp) gene was conducted for the 39 *Lilium* species and two subspecies, two *Nomocharis*, two *Fritillaria*, one *Notholirion* and one *Cardiocrinum* species with *Medeola virginiana* and *Erythronium japonicum* used as outgroups.

The *matK* gene tree (50% majority rule consensus) obtained is shown in Fig. 4. The genus *Lilium* is now divided into seven (Comber 1949) or 10 sections (Liang 1980) (only for Chinese taxa) (cf. Table 1). Thus in this study at least one or more taxa of each section, taxa of closely related genera – *Nomocharis*, *Cardiocrinum*, *Notholirion* and *Fritillaria* – were selected and their *matK* gene sequences analyzed. The number of base substitutions ranged from one to 35 among the *Lilium* and *Nomocharis* taxa examined (Table 4). Of 42 taxa, including 40 *Lilium* and two *Nomocharis*, three distinct clades were distinguished (Fig. 4): (i) including 16 *Lilium* and two *Nomocharis* species, with a bootstrap value of 100%; (ii) including 18 *Lilium* taxa (including two subspecies), with a bootstrap value of 100%; and (iii) including six species, with a bootstrap value of 100%. *Fritillaria* (100% bootstrap values) was a sister to all *Lilium* and *Nomocharis* taxa examined. Furthermore, *Cardiocrinum* turned out to be a sister group to *Lilium*, *Nomocharis* and *Fritillaria* with a very high bootstrap value of 100%. *Notholirion* is a sister

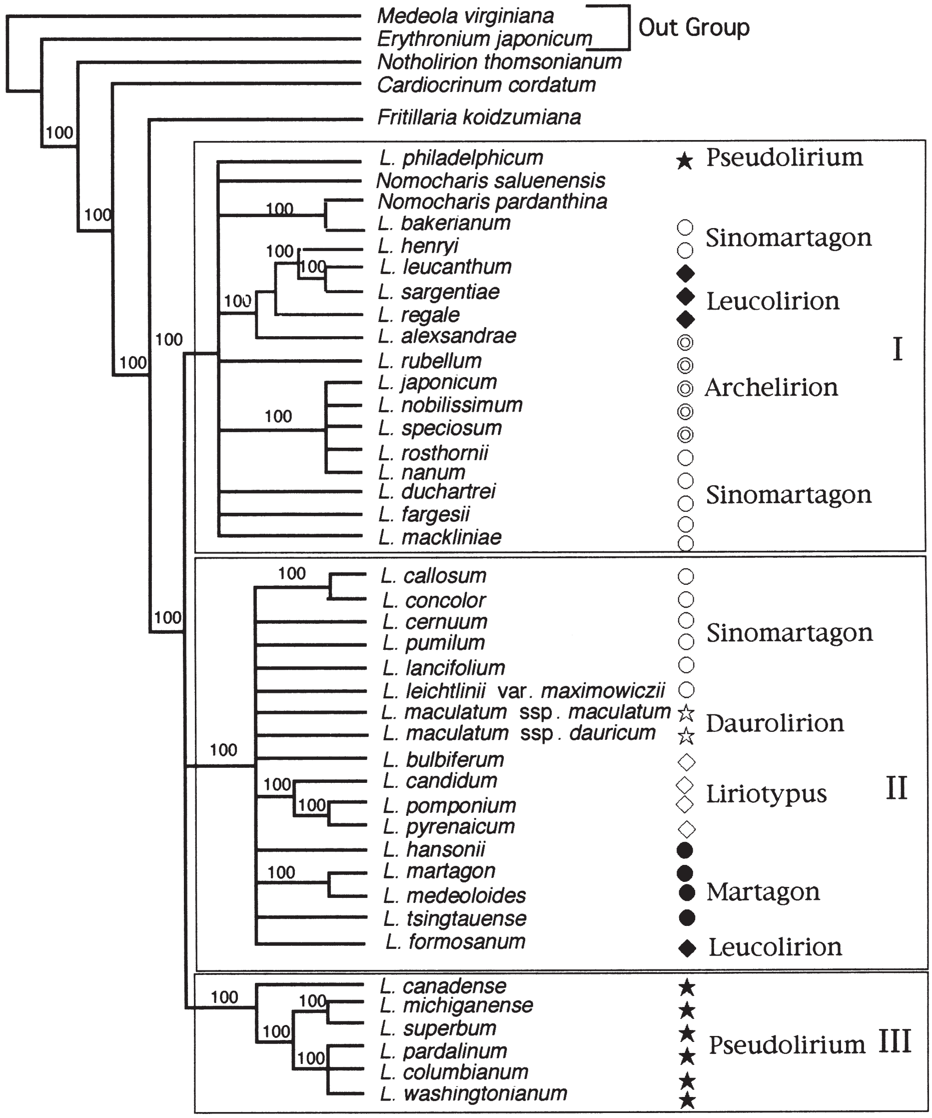

Fig. 4 The 50% majority-rule consensus tree obtained from the phylogenetic analysis of *matK* gene sequences for 49 taxa of Liliaceae *sensu stricto*, using *Medeola* and *Erythronium* as outgroups (× 1000 replications). Percentages above branches are bootstrap values. The length of the shortest tree (L) was 338, a consistency index (CI) of 0.805, a retention index (RI) of 0.813, and a homoplasy index of 0.195. cf. Although *L. longiflorum* was omitted from the dendrogram due to some undetermined parts included in the base sequence data, this species no doubt forms a pair with *L. formosanum*, as shown in Fig. 3.

group to the former four genera, with a bootstrap value of 100%.

The first clade can be divided further into three subclades and six isolated species (Fig. 4) as follows. (i) *Nomocharis pardanthina* and *L. bakerianum* formed a pair, with a bootstrap value of 100%; (ii) five species (*L. alexsandrae, L. henryi, L. leucanthum, L. regale* and *L. sargenitae*) constituted a clade, with a bootstrap value of 100%; (iii) five species (*L. japonicum, L. nobilissimum, L. rosthorni, L. speciosum* and *L. nanum*) constituted a clade, with a bootstrap value of 100%; (iv) six species (*Nomocharis salunensis, L. philadelphicum, L. rubellum, L. duchartrei, L. fargesii* and *L. mackliniae*) were independent lineages. All these species are distributed from the Japanese Islands to Burma, southwestern China and the Himalayan regions (Sino–Japanese element, Kitamura *et al*. 1957), except for *L. philadelphicum* (including var. *andenum*) which occurs in western to eastern North America (Fernald 1950; Feldmaier & McRae 1982).

The second clade constitutes a large single clade with a bootstrap value of 100%, with more or less four distinct subclades as follows. (i) The first subclade consists of a pair of species (*L. callosum* and *L. concolor*) with a bootstrap value of 100% (a typical Manchuria–Korean element; Kitamura *et al*. 1957); (ii) the second subclade consists of three Mediterranean species (*L. candidum, L. pyrenaicum* and *L. pomponium*) with a bootstrap value of 100%; (iii) the third subclade consists of a pair of species (*L. medeoloides* and *L. martagon*) with a bootstrap value of 100%. All the remaining eight taxa (*L. maculatum* ssp. *dauricum and* ssp. *maculatum, L. hansonii, L. lancifolium, L. leichtlinii* var. *maximowiczii, L. pumilum, L. tsingtauense, L. bulbiferum* and *L. cernuum*) were parallel, forming no branches. These species are a typical cool-temperate

Table 4 Base pair differences (above diagonal) and number of base substitutions per site (below diagonal, calculated according to Kimura, 1981) of *matK* sequences from 46 taxa of *Lilium* and allied taxa

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1 *Medeola virginiana*	–	87	91	83	98	87	90	92	90	91	93	97	92	91	91	90	90	93	90	93	92	94	94
2 *Erythronium japonicum*	0.057	–	86	78	94	81	86	85	86	87	89	91	88	87	87	86	86	89	86	86	85	87	87
3 *Notholirion thomsonianum*	0.060	0.057	–	28	48	37	36	41	39	40	42	44	41	38	40	41	41	39	41	41	40	42	42
4 *Cardiocrinum cordatum*	0.055	0.052	0.018	–	39	26	28	30	28	29	31	33	30	29	31	30	30	32	30	30	29	30	31
5 *Fritillaria koidzumiana*	0.064	0.062	0.031	0.026	–	28	28	32	32	33	33	35	32	29	33	32	32	32	32	32	31	33	33
6 *Nomocharis saluenensis*	0.057	0.053	0.024	0.017	0.018	–	6	14	14	15	15	13	14	13	9	8	8	10	8	14	13	15	15
7 *Nomocharis pardanthina*	0.059	0.057	0.024	0.018	0.018	0.004	–	16	16	17	17	15	16	13	11	10	10	10	10	16	15	17	17
8 *Lilium canadense*	0.060	0.056	0.027	0.020	0.021	0.009	0.010	–	6	7	11	23	12	9	19	18	18	20	18	12	7	13	13
9 *Lilium michiganense*	0.059	0.057	0.025	0.018	0.021	0.009	0.010	0.004	–	1	7	23	8	5	19	18	18	20	18	18	13	19	19
10 *Lilium superbum*	0.060	0.057	0.026	0.019	0.021	0.010	0.011	0.005	0.001	–	8	24	9	6	20	19	19	21	19	19	14	20	20
11 *Lilium pardalinum*	0.061	0.059	0.027	0.020	0.021	0.010	0.011	0.007	0.005	0.005	–	24	7	4	20	19	19	21	19	19	16	20	20
12 *Lilium philadelphicum*	0.064	0.060	0.029	0.022	0.023	0.008	0.010	0.015	0.015	0.016	0.016	–	23	22	18	17	17	19	17	23	22	24	24
13 *Lilium columbianum*	0.060	0.058	0.027	0.020	0.021	0.009	0.010	0.008	0.005	0.006	0.005	0.015	–	5	19	18	18	20	18	14	17	15	15
14 *Lilium washingtonianum*	0.060	0.057	0.025	0.019	0.019	0.008	0.008	0.006	0.003	0.004	0.003	0.014	0.003	–	18	17	17	17	17	17	14	18	18
15 *Lilium alexsandrae*	0.060	0.057	0.026	0.020	0.021	0.006	0.007	0.012	0.012	0.013	0.013	0.012	0.012	0.012	–	9	11	15	9	19	18	20	20
16 *Lilium japonicum*	0.059	0.057	0.027	0.020	0.021	0.005	0.006	0.012	0.012	0.012	0.012	0.011	0.012	0.011	0.006	–	2	14	0	18	17	19	19
17 *Lilium nobilissimum*	0.059	0.057	0.027	0.020	0.021	0.005	0.006	0.012	0.012	0.012	0.012	0.011	0.012	0.011	0.007	0.001	–	14	2	18	17	19	19
18 *Lilium rubellum*	0.062	0.059	0.026	0.021	0.021	0.007	0.007	0.013	0.013	0.014	0.014	0.012	0.013	0.011	0.010	0.009	0.009	–	14	20	19	21	21
19 *Lilium speciosum*	0.059	0.057	0.027	0.020	0.021	0.005	0.006	0.012	0.012	0.012	0.012	0.011	0.012	0.011	0.006	0.000	0.001	0.009	–	18	17	19	19
20 *Lilium callosum*	0.061	0.057	0.027	0.020	0.021	0.009	0.010	0.008	0.012	0.012	0.012	0.015	0.009	0.011	0.012	0.012	0.012	0.013	0.012	–	5	1	3
21 *Lilium cernuum*	0.060	0.056	0.026	0.019	0.020	0.008	0.010	0.005	0.008	0.009	0.010	0.014	0.011	0.009	0.012	0.011	0.011	0.012	0.011	0.003	–	6	6
22 *Lilium concolor*	0.062	0.057	0.027	0.020	0.021	0.010	0.011	0.008	0.012	0.013	0.013	0.016	0.010	0.012	0.013	0.012	0.012	0.014	0.012	0.001	0.004	–	4
23 *Lilium pumilum*	0.062	0.057	0.027	0.020	0.021	0.010	0.011	0.008	0.012	0.013	0.013	0.016	0.010	0.012	0.013	0.012	0.012	0.014	0.012	0.002	0.004	0.003	–
24 *Lilium henryi*	0.059	0.057	0.025	0.020	0.022	0.006	0.008	0.013	0.013	0.014	0.014	0.012	0.013	0.012	0.008	0.009	0.009	0.011	0.009	0.013	0.012	0.014	0.014
25 *Lilium lancifolium*	0.061	0.057	0.027	0.020	0.021	0.009	0.010	0.008	0.012	0.012	0.012	0.015	0.009	0.011	0.012	0.012	0.012	0.013	0.012	0.001	0.003	0.002	0.002
26 *Lilium leichtlinii* var. *maximowiczii*	0.061	0.057	0.027	0.020	0.021	0.009	0.010	0.008	0.012	0.012	0.012	0.015	0.009	0.011	0.012	0.012	0.012	0.013	0.012	0.001	0.003	0.002	0.002
27 *Lilium rosthorni*	0.057	0.055	0.025	0.018	0.019	0.003	0.005	0.010	0.010	0.010	0.010	0.009	0.010	0.009	0.006	0.002	0.002	0.007	0.002	0.010	0.009	0.010	0.010
28 *Lilium duchartrei*	0.059	0.057	0.023	0.017	0.019	0.004	0.005	0.010	0.010	0.011	0.011	0.008	0.010	0.010	0.007	0.006	0.006	0.008	0.006	0.010	0.010	0.010	0.010
29 *Lilium bakerianum*	0.059	0.055	0.025	0.018	0.019	0.003	0.005	0.010	0.010	0.011	0.011	0.010	0.010	0.010	0.007	0.006	0.006	0.008	0.006	0.010	0.010	0.011	0.011
30 *Lilium nanum*	0.058	0.056	0.026	0.019	0.020	0.005	0.006	0.011	0.011	0.012	0.012	0.010	0.011	0.010	0.006	0.002	0.002	0.009	0.002	0.011	0.010	0.012	0.012
31 *Lilium bulbiferum*	0.061	0.057	0.025	0.020	0.019	0.009	0.009	0.008	0.012	0.012	0.012	0.015	0.009	0.010	0.012	0.012	0.012	0.012	0.012	0.003	0.005	0.003	0.003
32 *Lilium candidum*	0.062	0.057	0.029	0.022	0.022	0.011	0.012	0.010	0.014	0.014	0.014	0.017	0.012	0.013	0.014	0.014	0.014	0.015	0.014	0.007	0.008	0.008	0.008
33 *Lilium pomponium*	0.062	0.058	0.027	0.020	0.021	0.010	0.012	0.009	0.013	0.014	0.014	0.015	0.012	0.012	0.014	0.013	0.013	0.014	0.013	0.006	0.007	0.007	0.007
34 *Lilium pyrenaicum*	0.062	0.057	0.025	0.018	0.020	0.010	0.011	0.008	0.012	0.013	0.013	0.014	0.011	0.012	0.013	0.012	0.012	0.014	0.012	0.006	0.006	0.006	0.006
35 *Lilium formosanum*	0.060	0.058	0.027	0.021	0.021	0.010	0.010	0.009	0.013	0.014	0.014	0.016	0.010	0.011	0.014	0.013	0.013	0.013	0.013	0.005	0.007	0.006	0.006
36 *Lilium leucanthum*	0.059	0.058	0.026	0.021	0.022	0.006	0.008	0.013	0.013	0.014	0.014	0.012	0.013	0.012	0.008	0.009	0.009	0.011	0.009	0.013	0.012	0.014	0.014
37 *Lilium regale*	0.060	0.057	0.026	0.020	0.021	0.006	0.007	0.012	0.012	0.013	0.013	0.012	0.010	0.012	0.008	0.008	0.008	0.010	0.008	0.010	0.012	0.010	0.010
38 *Lilium sargentiae*	0.060	0.059	0.027	0.022	0.023	0.007	0.008	0.014	0.014	0.014	0.014	0.013	0.014	0.013	0.009	0.010	0.010	0.011	0.010	0.014	0.013	0.014	0.014
39 *Lilium hansonii*	0.061	0.057	0.027	0.020	0.021	0.009	0.010	0.008	0.012	0.012	0.012	0.015	0.009	0.011	0.012	0.012	0.012	0.013	0.012	0.001	0.003	0.002	0.002
40 *Lilium martagon*	0.059	0.056	0.026	0.019	0.020	0.008	0.010	0.007	0.011	0.012	0.012	0.014	0.011	0.010	0.012	0.011	0.011	0.012	0.011	0.005	0.004	0.005	0.005
41 *Lilium medeoloides*	0.060	0.057	0.027	0.020	0.021	0.010	0.011	0.008	0.012	0.013	0.013	0.016	0.010	0.012	0.013	0.012	0.012	0.014	0.012	0.003	0.005	0.004	0.004
42 *Lilium tsingtauense*	0.062	0.058	0.027	0.021	0.022	0.010	0.012	0.009	0.013	0.014	0.014	0.016	0.010	0.012	0.014	0.013	0.013	0.014	0.013	0.003	0.005	0.003	0.003
43 *Lilium maculatum* ssp. *maculatum*	0.061	0.057	0.027	0.020	0.021	0.008	0.009	0.008	0.012	0.012	0.012	0.014	0.009	0.011	0.011	0.010	0.010	0.010	0.010	0.001	0.003	0.002	0.002
44 *Lilium maculatum* ssp. *dauricum*	0.060	0.056	0.026	0.019	0.020	0.008	0.010	0.007	0.011	0.012	0.012	0.014	0.008	0.010	0.012	0.011	0.011	0.012	0.011	0.001	0.003	0.001	0.001
45 *Lilium mackliniae*	0.058	0.055	0.024	0.017	0.018	0.003	0.003	0.009	0.009	0.010	0.010	0.008	0.009	0.008	0.006	0.005	0.005	0.007	0.005	0.009	0.008	0.010	0.010
46 *Lilium fargesii*	0.060	0.059	0.026	0.019	0.022	0.006	0.008	0.013	0.013	0.014	0.014	0.012	0.013	0.012	0.010	0.009	0.009	0.011	0.009	0.013	0.012	0.014	0.014

Table 4 *Continued*

	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
1 *Medeola virginiana*	90	93	93	87	90	90	89	93	94	95	94	92	90	91	91	93	90	92	95	93	92	88	92
2 *Erythronium japonicum*	87	86	86	83	86	84	85	86	87	88	86	88	88	87	89	86	85	87	88	86	85	84	89
3 *Northolirion thomsonianum*	39	41	41	38	35	39	40	39	44	41	39	41	40	40	41	41	40	42	42	41	40	37	40
4 *Cardiocrinum cordatum*	31	30	30	27	26	28	29	30	33	30	28	32	32	31	33	30	29	31	32	30	29	26	29
5 *Fritillaria koidzumiana*	34	32	32	29	30	30	31	30	33	32	31	32	34	33	35	32	31	33	34	32	31	28	34
6 *Nomocharis saluenensis*	10	14	14	5	6	4	7	14	17	16	15	16	10	9	11	14	13	15	16	12	13	4	10
7 *Nomocharis pardanthina*	12	16	16	7	8	8	9	14	19	18	17	16	12	11	13	16	15	17	18	14	15	4	12
8 *Lilium canadense*	20	12	12	15	16	16	17	12	15	14	13	14	20	19	21	12	11	13	14	12	11	14	20
9 *Lilium michiganense*	20	18	18	15	16	16	17	18	21	20	19	20	20	19	21	18	17	19	20	18	17	14	20
10 *Lilium superbum*	21	19	19	16	17	17	18	19	22	21	20	21	21	20	22	19	18	20	21	19	18	15	21
11 *Lilium pardalinum*	21	19	19	16	17	17	18	19	22	21	20	21	21	20	22	19	18	20	21	19	18	15	21
12 *Lilium philadelphicum*	19	23	23	14	13	15	16	23	26	23	22	25	19	18	20	23	22	24	25	21	22	13	19
13 *Lilium columbianum*	20	14	14	15	15	16	17	14	19	18	17	16	20	15	21	14	17	15	16	14	13	14	20
14 *Lilium washingto-nianum*	19	17	17	14	15	15	16	15	20	19	18	17	19	18	20	17	16	18	19	17	16	13	19
15 *Lilium alexsandrae*	13	19	19	10	11	11	10	19	22	21	20	21	13	12	14	19	18	20	21	17	18	9	15
16 *Lilium japonicum*	14	18	18	3	10	10	3	18	21	20	19	20	14	13	15	18	17	19	20	16	17	8	14
17 *Lilium nobilissimum*	14	18	18	3	10	10	3	18	21	20	19	20	14	13	15	18	17	19	20	16	17	8	14
18 *Lilium rubellum*	16	20	20	11	12	12	13	18	23	22	21	20	16	15	17	20	19	21	22	18	19	10	16
19 *Lilium speciosum*	14	18	18	3	10	10	3	18	21	20	19	20	14	13	15	18	17	19	20	16	17	8	14
20 *Lilium callosum*	20	2	2	15	15	16	17	4	11	10	9	8	20	15	21	2	7	5	4	2	1	14	20
21 *Lilium cernuum*	19	5	5	14	15	15	16	7	12	11	10	11	19	18	20	5	6	8	7	5	4	13	19
22 *Lilium concolor*	21	3	3	16	16	17	18	5	12	11	10	9	21	16	22	3	8	6	5	3	2	15	21
23 *Lilium pumilum*	21	3	3	16	16	17	18	5	12	11	10	9	21	16	22	3	8	6	5	3	2	15	21
24 *Lilium henryi*	–	20	20	11	12	12	13	20	23	22	19	22	4	9	5	20	19	21	22	18	19	10	14
25 *Lilium lancifolium*	0.013	–	2	15	15	16	17	4	11	10	9	8	20	15	21	2	7	5	4	2	1	14	20
26 *Lilium leichtlinii* var. *maximowiczii*	0.013	0.001	–	15	15	16	17	4	11	10	9	8	20	15	21	2	7	5	4	2	1	14	20
27 *Lilium rosthorni*	0.007	0.010	0.010	–	7	7	2	15	18	17	16	17	11	10	12	15	14	16	17	13	14	5	11
28 *Lilium duchartrei*	0.008	0.010	0.010	0.005	–	8	9	15	18	15	14	17	12	10	13	15	15	16	17	13	14	6	10
29 *Lilium bakerianum*	0.008	0.010	0.010	0.005	0.005	–	9	16	17	16	15	18	12	11	13	16	15	17	18	14	15	6	12
30 *Lilium nanum*	0.008	0.011	0.011	0.001	0.006	0.006	–	17	20	19	18	19	13	12	14	17	16	18	19	15	16	7	13
31 *Lilium bulbiferum*	0.013	0.003	0.003	0.010	0.010	0.010	0.011	–	11	10	9	6	20	15	21	4	7	5	6	4	3	14	20
32 *Lilium candidum*	0.015	0.007	0.007	0.012	0.012	0.011	0.013	0.007	–	7	6	13	23	20	24	11	11	11	13	11	10	17	23
33 *Lilium pomponium*	0.014	0.006	0.006	0.011	0.010	0.010	0.012	0.006	0.005	–	3	12	22	19	23	10	11	11	12	10	9	16	22
34 *Lilium pyrenaicum*	0.012	0.006	0.006	0.010	0.009	0.010	0.012	0.006	0.004	0.002	–	11	21	18	22	9	10	10	11	9	8	15	19
35 *Lilium formosanum*	0.014	0.005	0.005	0.011	0.011	0.012	0.012	0.004	0.008	0.008	0.007	–	22	17	23	8	11	9	10	8	7	16	22
36 *Lilium leucanthum*	0.003	0.013	0.013	0.007	0.008	0.008	0.008	0.013	0.015	0.014	0.014	0.014	–	5	1	20	19	21	22	18	19	10	16
37 *Lilium regale*	0.006	0.010	0.010	0.006	0.006	0.007	0.008	0.010	0.013	0.012	0.012	0.011	0.003	–	6	15	18	16	17	13	14	9	15
38 *Lilium sargentiae*	0.003	0.014	0.014	0.008	0.008	0.008	0.009	0.014	0.016	0.015	0.014	0.015	0.001	0.004	–	21	20	22	23	19	20	11	17
39 *Lilium hansonii*	0.013	0.001	0.001	0.010	0.010	0.010	0.011	0.003	0.007	0.006	0.006	0.005	0.013	0.010	0.014	–	7	5	4	2	1	14	20
40 *Lilium martagon*	0.012	0.005	0.005	0.009	0.010	0.010	0.010	0.005	0.007	0.007	0.006	0.007	0.012	0.012	0.013	0.005	–	4	9	7	6	13	19
41 *Lilium medeoloides*	0.014	0.003	0.003	0.010	0.010	0.011	0.012	0.003	0.007	0.007	0.006	0.006	0.014	0.010	0.014	0.003	0.003	–	7	5	4	15	21
42 *Lilium tsingtauense*	0.014	0.003	0.003	0.011	0.011	0.012	0.012	0.004	0.008	0.008	0.007	0.006	0.014	0.011	0.015	0.003	0.006	0.005	–	4	3	16	22
43 *Lilium maculatum* ssp. *maculatum*	0.012	0.001	0.001	0.008	0.008	0.009	0.010	0.003	0.007	0.006	0.006	0.005	0.012	0.008	0.012	0.001	0.005	0.003	0.003	–	1	12	18
44 *Lilium maculatum* ssp. *dauricum*	0.012	0.001	0.001	0.009	0.009	0.010	0.010	0.002	0.007	0.006	0.005	0.005	0.012	0.009	0.013	0.001	0.004	0.003	0.002	0.001	–	13	19
45 *Lilium mackliniae*	0.006	0.009	0.009	0.003	0.004	0.004	0.005	0.009	0.011	0.010	0.010	0.010	0.006	0.006	0.007	0.009	0.008	0.010	0.010	0.008	0.008	–	10
46 *Lilium fargesii*	0.009	0.013	0.013	0.007	0.006	0.008	0.008	0.013	0.015	0.014	0.012	0.014	0.010	0.010	0.011	0.013	0.012	0.014	0.014	0.012	0.012	0.006	–

Asiatic element, except for *L. bulbiferum*, which is a central European species.

The third clade with a bootstrap value of 100% includes six species which are all North American (*L. canadense*, *L. michganense*, *L. superbum*, *L. pardalinum*, *L. washigtonianum* and *L. columbianum*) (Fig. 4). All the species in this clade occur in North America; three species, *L. canadense*, *L. michiganense* and *L. superbum*, are eastern species, while *L. pardalinum*, *L. washingtonianum* and *L. columbianum* are typical west coast species in their distribution (Feldmaier & McRae 1982).

Insertion-deletion events in the matK *gene of* Lilium *and allied genera*

No indels have been found in the *rbcL* gene of all higher plants so far examined, but in the case of the *matK* gene indels have been recorded from various higher plant taxa (Johnson & Soltis 1995). In reference to the sequences of the *matK* gene of tobacco (*Nicotiana tabacum*) (Sugita *et al*. 1985), indels were examined in *Lilium* and allied genera and also in taxa used as outgroups.

In this study 19 indels (insertion/deletion events) (Table 5) were discovered in the *matK* gene of *Lilium* and allied genera, as follows.

(a) Twelve deletions of 6 bp: 115–120 bp [I]; 156–161 bp [III]; 265–270 bp [IV], except for *Notholirion*; 286–291 bp [V]; 394–399 bp [VI]; 338–444 bp [VII]; 643–648 bp [X], except for *Cardiocrinum* and *L. rubellum*, in both of which 18 bp are lacking; 856–861 bp [XIII], found only in *L. candidum*; 889–894 bp [XIV]; 1176–1182 bp [XV]; 1519–1525 bp [XVI], excepting four taxa, *L. japonicum*, *L. nobilissimum*, *L. speciosum*, and *L. alexandrae*, in

Table 5 Indels to *matK* gene of the genus *Lilium* and allied genera

Indel bp / Taxa	115–120 (I) 109–123 (II)	156–161 (III)	265–270 (IV)	286–291 (V)	394–399 (VI)	338–444 (VII)	619–630 (VIII) / 631–642 (IX) / 643–648 (X) / 649–666 (XII)
Medeola virginiana	TTAAAT------AGT	T------G	A------T	C------A	A------G	T------C	GAATAGTTTTATT------------------CAGAATAATAAAACTATTT
Erythronium japonicum	A------G	T------G	A------C	C------A	A------G	T------C	GAATAGTTTTATT------------------CAGAATAATAAAACTATTT
Fritillaria koidzumiana	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Notholirion thomsoniana	---------------	T------G	ATATAGAT	C------A	A------G	T------C	G------------AATAAAACTATT------CAGAGTAATAACACTTTTT
Cardiocrinum cordatum	T------G	T------G	A------T	C------A	A------G	A------G	G------------AATAAAACTATT------------------------T
Nomocharis pardantina	CAAT------G	T------G	A------T	C------A	A------G	A------G	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium rubellum	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------------------------T
Lilium candidum	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium martagon	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium medeoloides	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium henryi	T------G	T------G	A------T	A------T	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium leucanthum	T------G	T------G	A------T	A------T	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium sargentiae	T------G	T------G	A------T	A------T	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium regale	T------G	T------G	A------T	A------T	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium japonicum	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium nobilissimum	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium speciosum	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
Lilium alexsandrae	T------G	T------G	A------T	C------A	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT
All other taxa	T------G	T------G	A------T	A------T	A------G	T------C	G------------AATAAAACTATT------CAGGATAATAAAACTATTT

Taxa	838–853 (XI) / 856–861 (XIII)	889–894 (XIV)	1176–1182 (XV)	1519–1525 (XVI)	1546–1548 (XVII)	1632–1637 (XVIII) 1626–1637 (XIX)
Medeola virginiana	TATT---------------ATAGTAGTAT	G------T	T------A	T------T	T---C	T------T
Erythronium japonicum	T--------------------ATAGTGT	G------T	T------A	T------T	T---C	T------T
Fritillaria koidzumiana	TATT---------------ATAATAGTGT	G------T	G------A	T------T	T---C	C------------T
Notholirion thomsoniana	TATT---------------ATAATAGTGT	G------T	T------A	T------T	T---C	T------T
Cardiocrinum cordatum	TATT---------------ATAATAGTGT	G------T	G------A	T------T	T---T	T------T
Nomocharis pardantina	TATT---------------ATAATAGTGT	G------T	G------A	T------T	T---C	T------------T
Lilium rubellum	TATT---------------ATAATAGTGT	G------T	G------A	T------T	T---C	T------------T
Lilium candidum	TATT---------------ATA------T	G------T	G------A	T------T	T---C	T------------T
Lilium martagon	TATT---------------ATAATAGTAT	T------A	G------A	T------T	T---C	T------------T
Lilium medeoloides	TATT---------------ATAATAGTAT	T------A	T------A	T------T	T---C	T------------T
Lilium henryi	TATT---------------ATAATAGTGT	T------A	T------A	T------T	T---C	T------------T
Lilium leucanthum	TATT---------------ATAATAGTGT	T------A	T------A	T------T	T---C	T------------T
Lilium sargentiae	TATT---------------ATAATAGTGT	T------A	T------A	T------T	T---C	T------------T
Lilium regale	TATT---------------ATAATAGTGT	T------A	T------A	T------T	T---C	T------------T
Lilium japonicum	TATT---------------ATAATAGTGT	T------A	T------A	TCTTTCTT	T---C	T------------T
Lilium nobilissimum	TATT---------------ATAATAGTGT	T------A	T------A	TCTTTCTT	T---C	T------------T
Lilium speciosum	TATT---------------ATAATAGTGT	T------A	T------A	TCTTTCTT	T---C	T------------T
Lilium alexsandrae	TATT---------------ATAATAGTGT	T------A	T------A	TCTTTCTT	T---C	T------------T
All other taxa	TATT---------------ATAATAGTGT	T------A	T------A	T------T	T---C	T------------T

which 6 bp insertion (CTTTCT) occurs; 1632–1637 bp [XVIII], found only in four taxa, *Medeola, Erythronium, Notholirion,* and *Cardiocrinum*.

(b) Two deletions of 15 bp: 109–123 bp [II], only found in *Notholirion thomsoniana*, and 838–853 bp [XI] in all taxa except for *Erythronium* (835–855 bp).

(c) A reciprocal inversion and deletion of 12 bp: 619–630 bp [VIII] and 631–642 bp [IX]; in *Medeola* and *Erythronium* 12 bp are lacking in 631–642 bp, whereas in the remaining 17 taxa examined (cf. Table 5), 12 bp of 619–630 bp are a deletion and 12 bp of 631–642 bp are an inverted insertion.

(d) One deletion of 3 bp: 1546–1548 bp [XVII].

(e) One deletion of 18 bp: 649–666 bp [XII] in two taxa, *C. cordatum* and *L. rubellum*.

(f) One deletion of 12 bp: 1626–1637 bp [XIX] in the remaining 15 taxa, except for the above four genera.

Amino acid topology obtained by the MP method of matK *gene, and its evaluation*

In Fig. 5, the amino acid topology (50% majority rule consensus tree) based on translation of the *matK* gene base sequence data is presented. Basically, three major *Lilium* clades in the tree based on amino acid data were corresponding to those obtained by the base sequence data (Fig. 4). However, *Fritillaria* was an ingroup taxon of *Lilium*. Closely related genera, such as *Cardiocrinum, Notholirion* and *Nomocharis*, occupy almost the same phylogenetic positions in the amino acid tree as in the base sequence tree (Fig. 4).

The numbers of synonymous and non-synonymous base substitutions and codon usage in the taxa examined in the present analyses are important, but as the numbers of base substitutions at the first and second codons were not so high, striking differences were not visible in the amino acid tree for *Lilium* and related taxa, in sharp contrast to what we have recently obtained in the Trilliaceae (Kazempour Osaloo & Kawano 1999; Kazempour Osaloo *et al.* 1999).

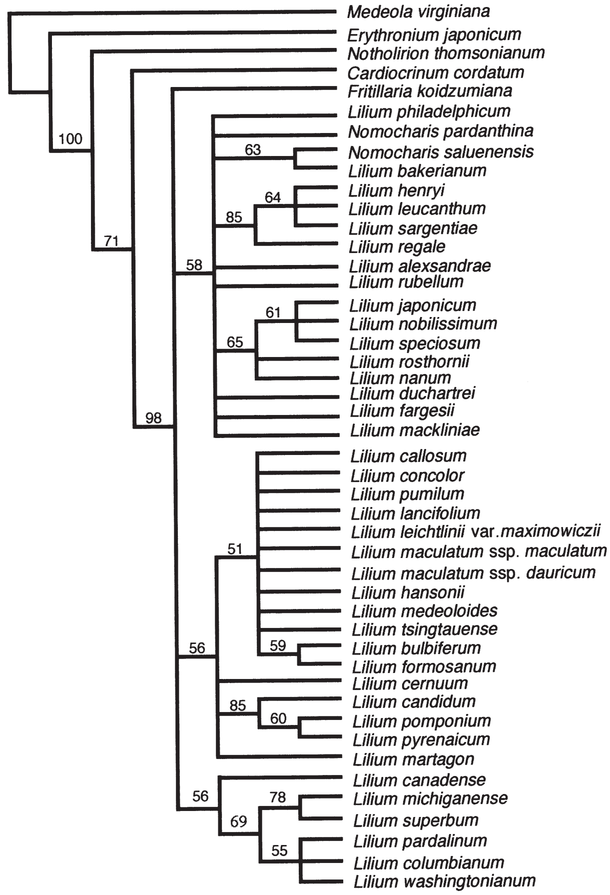

Fig. 5 The 50% majority-rule consensus tree obtained from the phylogenetic analysis of amino acid sequences (plus indels) of maturase (which encoded by *matK* gene) for 49 taxa of Liliaceae *sensu stricto*, using *Medeola* and *Erythronium* as outgroups (× 1000 replications). Figures above the branches are bootstrap values. The length of the shortest tree (L) was 223, a consistency index (CI) of 0.807, a retention index (RI) of 0.803, and a homoplasy index of 0.193.

Discussion

Phylogenetic position of Liliaceae sensu stricto

In the present study, molecular systematic analyses were first conducted on the Liliaceae *sensu stricto*, focusing on the following points.

First, the intergeneric phylogenetic relationships were analyzed for all genera included in the Liliaceae *sensu stricto* (*sensu* Takhtajan 1997), except for *Lloydia*, and *Rhinopetalum* which is confined to central Asia, using two molecular markers: *rbcL* and *matK* gene of *cp*DNA. The topologies obtained for the *rbcL* and *matK* genes of *cp*DNA obtained in this study were more or less identical (Figs 1 and 2), and were congruent with the taxonomic concept of Liliaceae *sensu stricto* recently proposed by Tamura (1998), i.e. Liliaceae *sensu stricto* is composed of three major subgroups, the first subgroup consisting of *Erythronium, Amana, Tulipa* and *Gagea*, the second consisting of *Lilium, Nomocharis, Fritillaria, Cardiocrinum* and *Notholirion* and the third somewhat distantly related to the former two, *Medeola* and *Clintonia* (Figs 1 and 2). Indeed, *Medeola* and *Clintonia* are referred to a subfamily in the Liliaceae (Tamura 1998) or separate families within the Liliales (Takhtajan 1987, 1997).

Second, the infrageneric relationships within *Lilium* and closely related genera, such as *Nomocharis, Cardiocrinum, Notholirion* and *Fritillaria* (Comber 1949; Liang 1980) were critically examined based on the phylogenetic analyses using *rbcL* and *matK* genes (Figs 3 and 4), although resolution by the *rbcL* gene was very limited due to low base substitution rates, as was expected by our earlier studies (Kato *et al*. 1995; Kazempour Osaloo & Kawano 1999; Kazempour Osaloo *et al*. 1999).

Phylogenetic relationships among Lilium *species revealed by* rbcL *and* matK *gene sequence data*

Based on the molecular analyses of the *rbcL* and *matK* genes, taxonomic schemes for the genus *Lilium* and allied genera were re-evaluated.

The *rbcL* gene has evolved very slowly and its phylogenetic resolution was thus very limited; the *matK* gene showed much higher sequence variation and divergence rates, including an unexpectedly high number of indels (insertion/deletion events). The phylogenetic tree obtained by the *matK* gene sequence data showed that *Lilium* consists of three distinct major clades (Fig. 4). Clade I consisted of a group mainly ranging in the Sino–Japanese floristic region (sections *Archaelilion* and *Sinomartagon*) and one species in eastern North America (section *Pseudolilium*), and also including *Nomocharis* species.

Most of the species in clade II comprise a widespread Eurasian group, ranging from the Far East to Europe, with species belonging to sections *Liriotypus, Martagon, Leucolirion, Sinomartagon* and *Daurolirion*, and extend from Japan to Manchuria and eastern Siberia via the Korean Peninsula and further southward to the Ryukyu Islands, Taiwan and possibly the Philippines. However, only *L. martagon* extends widely over northern Europe. On the other hand, several species, such as *L. candidum, L. pyrenaicum, L. pomponium* and *L. bulbiferum*, are more localized in the Mediterranean region (Feldmaier & McRae 1982).

Clade III consisted of only a North American group including the majority of section *Pseudolilium*, except for *L. philadelphicum*, which belonged to clade I (Fig. 4). *Fritillaria, Notholirion* and *Cardiocrinum* proved to be sisters to the major clade. A notable finding is that *Fritillaria* turned out to be a sister group of the major *Nomocharis–Lilium* clade, diverging at the basal position of the *matK* tree with a 100% bootstrap value (Fig. 4). However, *Cardiocrinum* and *Notholirion* were sisters to the remaining major clade (Fig. 4), although *Cardiocrinum* has been often included in *Lilium* (Comber 1949; Ohwi 1956).

The phylogenetic relationships obtained by these molecular analyses based on the *matK* gene sequences were very controversial, because most of the sectional classifications by earlier taxonomists (cf. Comber 1949; Liang 1980; for others see Table 1) were not in agreement with the molecular phylogenetic trees reconstructed in the present study (Fig. 4).

Major discrepancies are as follows.

1. Section *Pseudolirium*, a North American group, was split into two distantly related clades (Fig. 4). The phylogenetic position of *L. philadelphicum* is very puzzling. This species belonged to one of the subclades of clade I, but all six of the remaining species examined belonged to clade III (Fig. 4). In the present study, we also examined the *matK* sequences of a narrow-leaved variety of *L. philadelphicum* var. *andenum* (Nutt.) Ker (or *L. umbellatum* Pursh) (Fernald 1950) but both proved to have the same *matK* gene sequences. It should also be noted here that only *L. philadelphicum* has no cross ability with any other North American *Lilium* taxa (Lighty 1968). In this study, we included *L. mackliniae* for molecular analyses and it proved to belong to clade I, based on the *matK* gene tree (Fig. 4), although its sectional delimitation has yet to be determined. Recently, C-band patterns of somatic chromosomes of *L. mackliniae* were examined by Smyth *et al*. (1989), who place it tentatively in *Sinomartagon*, suggesting that this species should be placed in a new and separate section.
2. Two sections, *Leucolirion*, including *L. longiflorum* and *L. formosanum*, and *Liriotypus*, including a pair of species, *L. candidum* and *L. pomponium*, are situated in two subclades of clade II.
3. Section *Martagon* was split into two, belonging to two subclades (see Fig. 4). Four species, *L. hansonii, L. tsingtauense, L. medeoloides*, and *L. martagon*, constituted parts of different subclades of clade II, whereas two species, *L. sargenteae* and *L. regale*, constituted part of the subclades of clade I.
4. Section *Sinomartagon* was split into four distantly related clades: one large group of six species, *L. lancifolium, L. leichtlinii* v. *maximowiczii, L. cernuum, L. callosum, L. concolor* and *L. pumilum*, which constitute part of a distinct subclade of clade II, and six species, *L. bakerianum, L. henryi, L. nanum, L. rosthronii* and *L. duchartrei*, all of which are scattered in four different subclades of clade I. This fact indicates that section *Sinomartagon* consists of exceedingly heterogeneous groups. Indeed, based on the C-band patterns of chromosomes, Smyth *et al*. (1989) suggested that *L. henryi* (previously classed in sect. *Shinomartagon*) should be included in section *Leucolirion*, together with *L. regale* (see Fig. 4).
5. Two subspecies belonging to section *Daurolirion*, *L. maculatum* ssp. *maculatum* and ssp. *dauricum* belong to part of the subclade of clade II. We believe, however, that these subspecies represent independent species, judging from their very distinct morphological as well as life-history characters and different geographic ranges (Hara 1963; Hayashi & Kawano, unpublished data).
6. Two *Nomocharis* species belonged to two separate subclades of clade I, forming a pair with *L. bakerianum* (Fig. 4).

The amino acid topology obtained in the present study simply supported the phylogenetic relationships obtained by the *matK* and *rbcL* base sequence data (Fig. 5). The level of resolution was slightly lower than the analyses by *matK* gene base sequence data but was higher than those by the *rbcL* gene. As the genus *Lilium* comprises approximately 100 species and also because *Nomocharis* turned out to be very closely related and may be an ingroup taxon, more detailed phylogenetic analyses are needed based both on base sequence data and on translated amino acid compositions (Miyata 1998; Kazempour Osaloo & Kawano 1999).

A comparison of molecular data with morphological and life-history characters

In the present study, trends of divergence in several morphological and life-history traits of *Lilium* were critically examined (Table 6; Figs 6 and 7). In his classical paper of *Lilium* taxonomy, Comber (1949) selected the following traits as having diagnostic values for sectional definitions: (i) types of seed germination (hypogeal or epigeal; immediate or delayed); (ii) seed size (heavy or light); (iii) bulb characters (scales jointed or entire; erect, subrhizomatous, rhizomatous or stoloniferous; white or purple in color); (iv) stem characters (erect or stoloniform; one or somtimes two per bulb; stem root present or absent); (v) phyllotaxis (whorled or scattered); (vi) petiole (obvious, obscure or absent); (vii) floral shape (Turk's cap or trumpet); (viii) perianth segments (papillose or smooth); (ix) stigma (large or small); and (x) nectary (pubescent or glabrous).

In the present study, we have chosen the following eight traits for character scoring (Table 7). The character states of eight traits were overlaid on the molecular tree reconstructed based on the *matK* gene (Figs 6 and 7).

It is interesting to note that most of the characters chosen by Comber (1949), except those for bulb characters, exhibit remarkably convergent differentiations. Bulb characters are assumed to have differentiated between taxa of section *Pseudolirion* and those of the other six sections (Stout 1928; Comber 1949) (Fig. 6d). All North American taxa of section *Pseudolirion* possess stoloniferous or rhizomatous bulbs, except for *L. philadelphicum* and *L. catesbaei*, which have almost erect concentric bulbs which are characterisitc of all taxa in the Eurasian sections (Woodcock & Stearn 1950; Fox 1985). It should be noted here that *L. philadelphicum* has no or extremely low cross ability with any of the other North American taxa (Lighty 1960).

The patterns of divergence found in most of the other characters, e.g. types of germination (Fig. 6b), which have been used for the taxonomic delimitation of infrageneric groups are at present inexplicable simply in terms of phylogenetic implications and/or of any specific environmental constraint (Stout 1924; Barton 1936; Baranova 1974). For example, very closely related taxa, *L. maculatum* ssp. *maculatum* and ssp. *dauricum* of section *Daurolirion* (clade II in Fig. 4) show different germination types, the former being epigeal, whereas the latter hypogeal (Hayashi 1990). However, Baranova (1987) reported intermediate germination types from Caucasian lily species, *L. szovitsianum*, *L. polyphyllum* and so on. Therefore, this specific character seems not to reflect the phylogenetic constraint, although Comber (1949) and Lighty (1968) regarded this character to be of diagnostic value for sectional delimitation. Somewhat divergent trends can also be seen in the seed germination pattern, immediate or delayed (Comber 1949) (cf. Fig. 6b). It should be noted here that Comber (1949) regarded hypogeal and delayed germination (Fig. 6a,b), whorled leaves (Fig. 7b), jointed bulb scales (Fig. 6c,d), and large heavy seeds (Fig. 7a) as primitive characters in *Lilium*. However, the seed germination types in *Cardiocrinum* (a typical woodland element), *Nomocharis* (a meadow or woodland element) and *Fritillaria* (dry meadow, alpine-arctic meadow and woodland elements) are all epigeal (Hayashi, unpublished observation). Furthermore, *Cardiocinum* and *Notholirion* are both monocarpic perennials (Kawano 1975; Hayashi, unpublished observation), which are without doubt a derived life-history character. This fact suggests that contemporary *Cardiocinum* and *Notholirion* are not the ancestral members of the Liliaceae *sensu stricto* (see Fig. 4).

When all of the character states found in *Lilium* and allied genera such as *Fritillaria*, *Cardiocrinum* and *Notholirion* are considered, most of the key traits used by Comber (1949) are no doubt homoplacious and obviously reflect environmental constraints acting on patterns of differentiation. We should recall again that all character states found in contemporary species are the consequences of interactions between phylogenetic and environmental constraints (Kawano & Kato 1995). Floral characters have traditionally been emphasized as of key diagnostic value in most of earlier taxonomic studies of the genus *Lilium* (cf. Table 1). Indeed, in *Lilium*, sectional delimitation by Wilson (1925) was based on the flower shape and directional orientation of flowering (Table 1), i.e. the 'trumpet type' represented by species of section *Leucolirion* (e.g. *L. longiflorum*), the 'Turk's cap' type, nodding in bloom, represented by those of section *Martagon* (e.g. *L. martagon*) (Adams & Dress 1982), the 'bowl-shaped', blooming horizontally wide-open, represented by those of section *Archelirion* (e.g. *L. auratum*), and the 'wide-open cup-shaped' type, upright blooming, represented by those of section *Pseudolirilum* (e.g. *L. philadelphicum* and *L. maculatum*) (Comber 1949; Woodcock & Stearns 1950; Adams & Dress 1982). There is a high possibility of concerted evolution in relation to

Table 6 Character scoring for eight traits (modified after Hayashi, 1992)

Taxa	1 Germination type	2 Germination pattern	3 Seed weight	4 Bulb scales	5 Joint segment	6 Phyllotaxis	7 Petiole	8 Perianth
Lilium hansonii	0	1	0	0	1	1	0	1
L. martagon	0	1	0	0	0	1	1	1
L. medeoloides	0	0	0	0	0	1	1	1
L. tsingtauense	0	1	0	0	1	1	1	1
L. columbianum	0	1	0	3	1	2	1	1
L. washingtonianum	0	1	0	3	1	1	1	1
L. pardalinum	0	1	0	4	0	1	1	1
L. superbum	0	1	1	1	0	1	1	1
L. michiganense	0	1	1	1	0	1	1	1
L. canadense	0	1	1	1	0	2	1	1
L. philadelphicum	1	0	1	0	1	1	1	1
L. bulbiferum	0	1	0	0	1	0	1	0
L. candidum	1	0	0	0	1	0	1	1
L. pomponium	1	0	0	0	1	0	1	1
L. alexsandrae	0	1	0	0	1	0	0	1
L. japonicum	0	1	0	0	1	0	0	1
L. nobilissimum	0	0	0	0	1	0	0	1
L. rubellum	0	1	0	0	1	0	0	1
L. speciosum	0	0	0	0	1	0	0	1
L. callosum	1	0	1	0	1	0	1	0
L. cernuum	1	0	1	0	1	0	1	0
L. pumilum	1	0	1	0	1	2	1	0
L. concolor	1	0	1	0	1	0	1	0
L. henryi	1	0	0	0	1	0	1	0
L. duchartrei	1	0	1	0	1	0	1	0
L. lancifolium	1	0	1	0	1	0	1	0
L. leichtlinii var. *maximowiczii*	1	0	1	0	1	0	1	0
L. rosthornii	1	0	1	0	1	0	1	0
L. bakerianum	1	0	1	0	1	0	1	1
L. nanum	1	0	1	0	1	0	1	1
L. sargentiae	1	0	1	0	1	0	1	0
L. regale	1	0	0	0	1	0	1	1
L. formosanum	1	0	1	0	1	0	1	1
L. maculatum								
ssp. *maculatum*	1	0	1	0	1	0	1	0
ssp. *dauricum*	0	0	0	0	0	0	1	0
L. leucanthum	1	0	1	0	1	0	1	1
L. fargesii	1	0	1	0	1	0	1	1
L. mackliniae	1	0	1	0	1	0	1	0
L. pyrenaicum	1	0	0	0	1	0	1	1

1. Germination type: hypogeal (0); epigeal (1)
2. Germination pattern: immediate (0); delayed (1)
3. Seed weight: heavy (0); light (1)
4. Bulb: concentric (normal form) (0); stoloniferous (1); concentric bulb with stoloniferous stem (2); rhizomatous with large scales (3); sub-rhizomatous with small scales (4)
5. Joint segments: present (0); absent (1)
6. Phyllotaxis: scattered (0); whorled (1)
7. Petiole: present (0); absent (1)
8. Perianth segment: papillose (0); smooth (1)

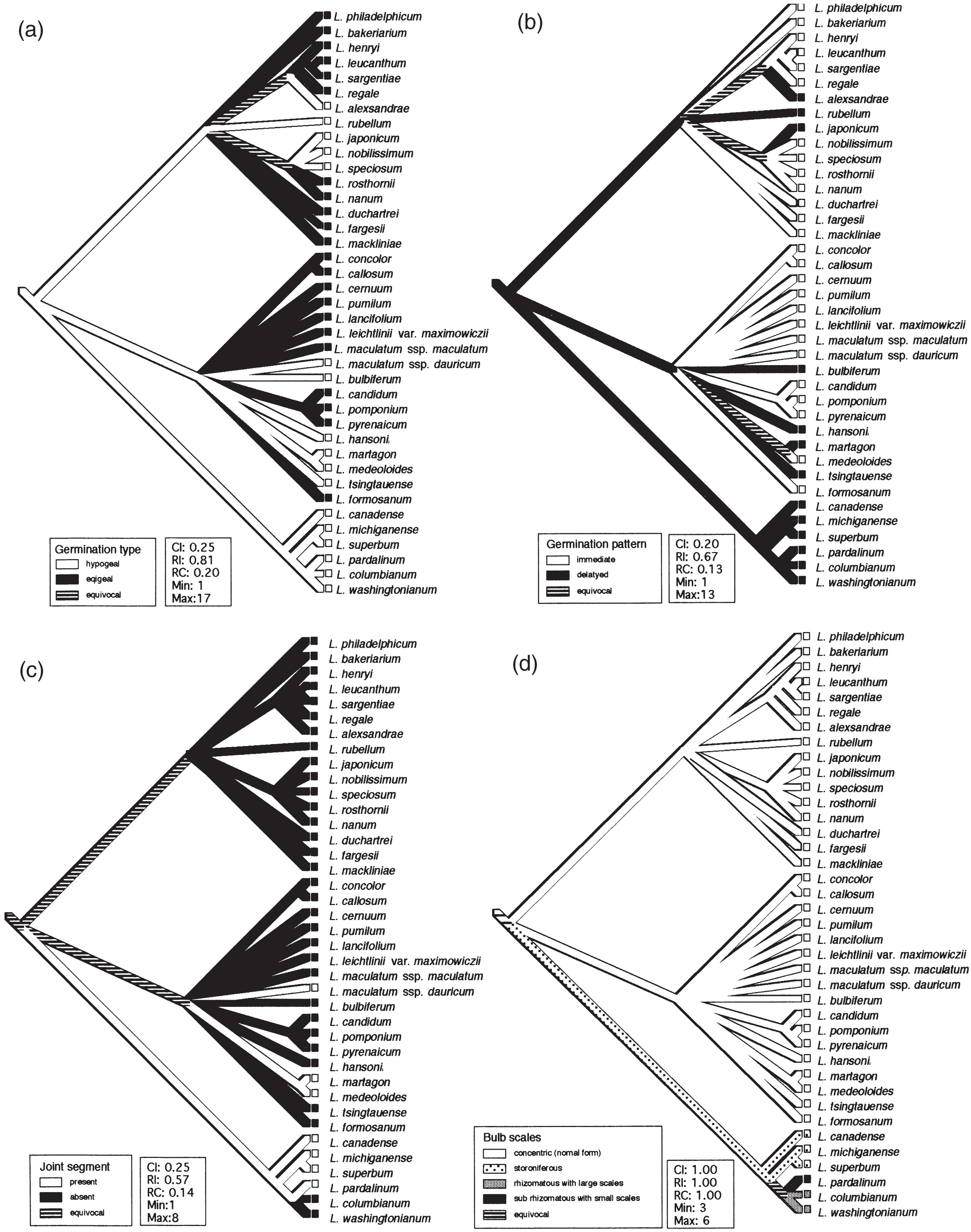

Fig. 6 Parsimoniously mapping of morphological characters onto the 50% majority-rule consensus tree of *matK* gene sequence of 39 *Lilium* taxa. Upper left (A), germination type of seeds; upper right (B), germination pattern of seed; lower left, joint segment of bulbs (C); and lower right (D), bulb scales (see Table 6).

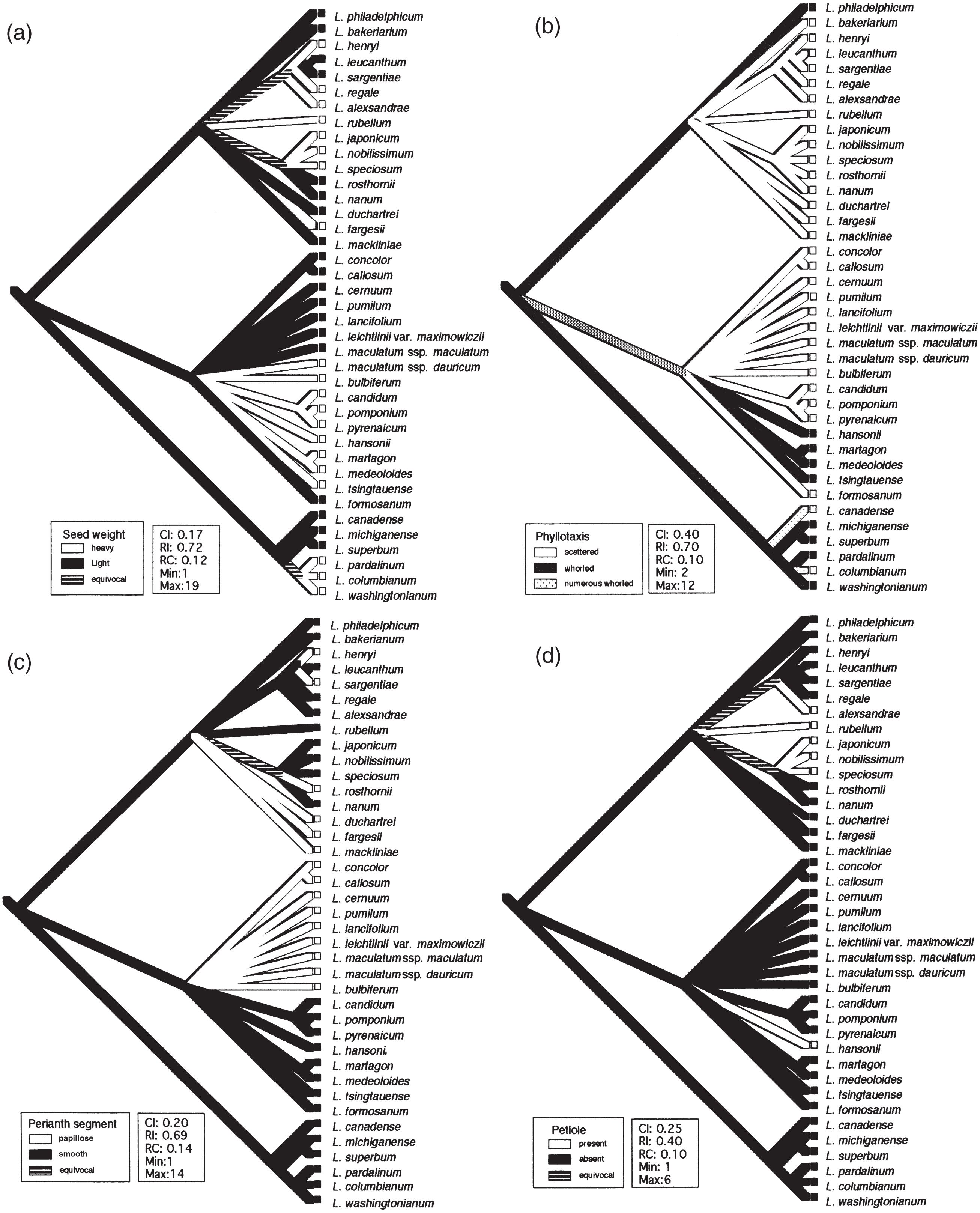

Fig. 7 Parsimoniously mapping of morphological characters onto the 50% majority-rule consensus tree of *matK* gene sequence of 39 *Lilium* taxa. Upper left (A), seed weight; upper right (B), phyllotaxis; lower left, perianth segment (C); and lower right (D), petiole (see Table 6).

floral types, timing of blooming, pigmentation, floral odors, and the kinds of pollinating agents – their body size, proboscis type and size, and their flower-visiting behaviors (Barth 1940; Grant & Grant 1968; Proctor & Yeo 1973).

Numerous recent findings on the intricate flower-pollinator networks suggest that differentiation of floral structures and functions in plants are tightly concerted with those of pollinators (primarily of insects) (Thien *et al*. 1998; Gottsberger 1999; Knudsen 1999; Raguso & Pichersky 1999; Williams & Whitten 1999). The possibility is high, therefore, that *Lilium* flowers have differentiated convergently in relation to pollinator specificity as a consequence of adaptive radiation (Wilson 1925; Comber 1949).

Conclusions

The results of molecular systematic analyses on the Liliaceae, including *Lilium*, *Nomocharis, Notholirion*, *Cardiocrinum* and *Fritillaria,* using *Medeola virginiana* and *Erythronium japonicum* as outgroups have provided new evidence concerning the systematic positions of these genera and also infrageneric delimitations within *Lilium*. First, it has long been believed that *Fritillaria* was only remotely related to *Lilium* among the four other genera of the Liliaceae, but the present result clearly showed that *Fritillaria* no doubt represents the closest relative to *Lilium*, whereas *Notholirion* and *Cardiocrinum* are sister groups to *Fritillaria* and *Lilium*, and most distantly related to *Lilium*.

It should also be noted here that *Cardiocrinum* has often been regarded as a member of the genus *Lilium* (Comber 1949; Ohwi 1956) but this does not hold true and there is no doubt that *Cardiocrinum* represents an independent group, perhaps representing one of the most primitive members of the Liliaceae.

In *Nomocharis*, two sections (five species in section *Ecristata* and three species in section *Nomocharis*) have been recognized (Sealy 1983; Liang 1984). In the present study we analyzed two species, each representing these two sections, but our results indicate that these two species belong to the ingroup taxa of *Lilium*. All previous infrageneric delimitations of *Lilium* by Comber (1949) and Liang (1980) (for others, see Table 1) were controversial and insupportable, suggesting the need for major revision. As far as the results of the molecular (Figs 1–5), morphological and life-history character analyses (Figs 6 and 7) are concerned, a further thorough study seems to be necessary of the infrageneric (especially sectional) delimitation of the genus *Lilium*.

For this paper, we studied only 49 taxa of *Lilium* and allied groups out of ca. 400 taxa in a narrowly defined Liliaceae, but important evidence and results concerning the phylogenetic relationships and systematic positions of *Lilium* and allied taxa have been revealed. Additional studies are now needed to cover the remaining groups and to elucidate the entire picture of the evolutionary–phylogenetic story of the Liliaceae *sensu stricto.*

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Note added after acceptance for publication

The results obtained in the present study using two molecular markers, *rbcL* and *matK* of *cp*DNA turned out to be very controversial as they contradicted the previous taxonomic concepts, especially at the infrageneric levels developed by Comber (1949) and Liang (1980). However, one most recent paper on the genus *Lilium* based on the sequence variations in the internal transcribed spacer (ITS) regions of 18S-25S nuclear ribosomal DNA by Nishikawa *et al*. (1999), which was published after the submission and acceptance of our paper, more or less supports earlier taxonomic treatments at the levels of section and subsection. However, we could not evaluate immediately the differences in phylogenetic trees reconstructed by *matK* gene of *cp*DNA by us and those for the ITS regions of ribosomal DNA by Nishikawa *et al*. (1999), and thus reserve our opinion on the differences in interpretation.

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