Plant Species Biol. **13** : 129–146, 1998

PLANT SPECIES BIOLOGY

Molecular Systematics of the Genus *Uvularia* and Selected Liliales Based upon *matK* and *rbcL* Gene Sequence Data

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Abstract **To elucidate the affinity and phylogeny of the endemic North American genus *Uvularia*, two chloroplast genes, *matK* and *rbcL*, were sequenced for all five species of the genus (*Uvularia floridana, U. grandifolia, U. perfoliata, U. puberula,* and *U. sessilifolia*) and four selected members of the Liliales (*Erythronium japonicum, Disporum sessile, Medeola virginiana,* and *Clintonia borealis*). Sequence data of both *matK* and *rbcL* genes support an *Uvularia* which consist of two clades, section *Oakesiella* and section *Uvularia*. Though sessile-leaved and associated with section *Oakesiella, U. puberula* exhibits several intermediate characteristics between the sections. However, the overall molecular results correspond to an earlier sub-grouping based upon gross morphology, karyology and ecological life history traits. These two cpDNA genes, notably *mat*K tree, proved to be informative in reaffirming relationships within *Uvularia*. Differentiation patterns among selected morphological, karyological and life history traits were also analyzed in comparison to the resulting molecular topologies.**

In comparison to the selected outgroups, *Disporum sessile* proved to be closely related to *Uvularia* in a narrowly defined Uvulariaceae — Uvulariea *sensu* Takhtajan (1997) or an expanded Colchicaceae with a "uvularioid line" *sensu* Nordenstam (1998). The outgroup taxa, *Erythronium, Medeola,* and *Clintonia,* associate as a well supported lineage within a narrowly defined Liliaceae. Comment is also made on the multiple origins of berry fruits in the Liliales.

Key words: **chloroplast DNA, *Clintonia, Disporum, Erythronium*, Liliaceae, Liliales, *matK, Medeola*, Medeolaceae, molecular systematics, *rbcL, Uvularia*, Uvulariaceae.**

Uvularia, a spring blooming genus of five species, is endemic to eastern North America. They commonly occur from southern Quebec and Ontario to Florida and Louisiana and westward to Manitoba, the Dakotas, Kansas and the Ozarkian uplands (Soper, 1952; Wilbur, 1963; Utech and Kawano, 1999). The species of *Uvularia* are either perennial or pseudo-annual herbs (Whigham, 1974; Kawano, 1985; Kawano et al., 1986; Kudoh et al., 1999) which die back every winter. Found mainly in mesic deciduous or well-drained upland forests, they also occur in swampy forests of alluvial bottomlands and more rarely, xeric coniferous woods.

During the earlier part of this century, the three sessile-leaved species of the genus were treated as *Oakesiella* (=*Oakesia* S. Watson) by Small (1913). Wilbur (1961, 1963) in revising the taxonomy of this Linnean genus recognized two sections in *Uvularia*: (1) Section *Oakesiella* (Small) Wilbur which includes the three sessile-leaved species, *U. puberula* Michx. (=*U. pudica* (Walter) Fernald; =*U. carolina* (J.F. Gmel.) Wilbur; =*U. nitida* (Britt.) Mackenzie), *U. sessilifolia* L. and *U. floridana* Chapman; and (2) Section *Uvularia* which includes the two perfoliate-leaved species, *U. grandiflora* J.E. Smith and *U. perfoliata* L.

The genus is based on the type of *Uvularia perfoliata* (Reveal, 1992) while the family name, Uvulariaceae A. Gray ex Kunth, is a proposed conserved name (Reveal and Hoogland, 1992). An excluded name, *Uvularia lanceolata* Aiton cited in Wilbur (1963), has become a new name for the North American *Streptopus roseus* Michx., *S. lanceolatus* (Aiton) Reveal (Reveal, 1993). Studies in morphological variation include works by Holm (1891), Anderson and Whitaker (1934), Fernald (1935), and Dietz (1952). More recent floristic treatments include those of Radford et al. (1964), Fernald (1970), Gleason and Cronquist (1991; see also Holmgren [1998]), and Utech and Kawano (1999).

Within eastern North America the species of *Uvularia*

exhibit geographical separation as well as ecological replacement. In section *Uvularia, U. grandiflora* is common on the western side of the Appalachian Mountains preferring calcareous soils, while *U. perfoliata* is more common on the eastern side in more acidic soils. In section *Oakesiella. U. sessilifolia* ranges widely throughout eastern North America, and *U. puberula* is restricted to the unglaciated, upper elevations of the southern Appalachians and adjoining Piedmont, while *U. floridana* is confined to several watershed of the outer Coastal

Table 1. Recent classification schemes for *Uvularia.*

Author	Order	Family	Subfamily andor Tribe	Genera (Examined in this study & Hayashi et al. (1997) in bold)
Dahlgren et al. (1985)	Liliales	Uvulariaceae (2 tribes)	Uvularieae	*Uvularia, Disporum, Prosartes, Clintonia* (*?*), *Kreysigia, Medeola*(*?*), *Schelhammera, Scoliopus* (*?*), *Streptopus*
	Liliales	Liliaceae	(Not indicataed)	*Erythronium, Tulipa, Gagea, Lloydia, Cardiocrinim, Lilium, Fritillaria, Nomocharis, Notholirion*
Takhtajan (1987)	Liliales	Melanthiaceae (2 subfamilies & 13 tribes)	Melanthioideae-Uvularieae	*Uvularia, Kreysigia, Schelhammer, Burchardia* (Reya)
	Liliales	Medeolaceae		*Medeola*
	Liliales	Liliaceae (3 tribes)	Tulipeae	*Erythronium, Tulipa*
	Melanthiales	Melanthiaceae	Lilieae	*Cardiocrinum, Lilium, Notholirion, Nomocharis, Fritillaria, Rhinopetalum*
			Melanthioideae-Scolipeae	*Scoliopus*
	Asparagales	Convallariaceae (2 subfamilies & 3 tribes)	Convallarioideae -Polygonateae	*Disporum, Clintonia, Disporopsis, Drymophila, Maianthemum, Oligobotrya, Polygonatum, Prosartes, Smilacina, Streptopus*
Takhtajan (1997)	Colchicales	Uvulariaceae (2 tribes)	Uvularieae	*Uvularia, Schelhammera* (*incl. Kreysigia*), *Kuntheria, Tripladenia*
			Streptopeae	*Disporum, Clintonia, Disporopsis, Prosartes, Streptopus*
	Colchicales	Scoliopaceae	(monotypic)	*Scoliopus*
	Liliales	Medeolaceae	(monotypic)	*Medeola*
	Liliales	Liliaceae (3 tribes)	Tulipeae	*Erythronium, Tulipa*
			Lilieae	*Cardiocrinum, Lilium, Notholirion, Nomocharis, Fritillaria, Rhinopetalum*
Nordenstam (1998) in Kubitzki	Liliales	Colchicaceae (2 lines suggested)	"Wurmbaeoid" line	*Colchicum* (*incl. Bulbocodium, Merender*), *Androcymbium, Baeometra, Burcharida, Camptorrhiza, Gloriosa, Hexacyrtis, Iphigenia, Littonia, Onixotis, Ornithoglossum, Sandersonia, Wurmbea*
			"*Uvularioid*" *line*	*Uvularia, Disporum, Schelhammera* (incl. *Kreysigia*), *Tripladenia, Kuntheria*
Tamura (1998a) in Kubitzki	Liliales	Calochortaceae		*Scoliopus, Tricyrtis, Calochortus, Streptopus, Prosartes*
Tamura (1998) in Kubitzki	Liliales	Liliaceae (2 subfamilies & 2 tribes)	Medeoloideae	*Clintonia, Medeola*
			Liliaceae-Tulipeae	*Erythronium, Tulipa, Gagea, Lloydia,*
			Lilioideae-Lilieae	*Cardiocrinum, Lilium, Fritillaria, Nomocharis, Northolirion*

(?) indicates monographing author questioned position.
Tamura (1998a)=Calochortaceae; Tamura (1998b)=Liliaceae.

Plain (Wilbur, 1963; Johnson, 1969). While the section members are allopatric throughout most of eastern North America, there are areas in the southern Appalachians, i.e., Rabun Bald, Georgia, where two or three species can co-occur.

Karyologically, major differences have been observed between the sections and among species. Counts of $2n=14$ have been reported for all species (Belling, 1925; Anderson and Whitaker, 1934; Kawano and Iltis, 1964; Utech, 1980; Therman and Denniston, 1984), except *U. floridana* which has a $2n=12$ complement (Utech, 1978a). A base number of $x=7$ characterizes the genus while the $x=6$ reported for *U. floridana* probably represents an derived aneuploid reduction for this narrowly restricted Coastal Plain species.

The four outgroup taxa selected for this study *(Erythronium japoncium, Disporum sessile, Medeola virginiana* and *Clintonia borealis*) have had a recent history of taxonomic shifting which is in large part due to the polyphyletic, berry-fruited elements in the non-monophyletic Uvulariacaeae of Dahlgren (Dahlgren and Clifford, 1982; Dahlgren et al., 1985). The fruits in *Erythronium* are loculicidal capsules (Utech and Kawano, 1975a) and typical of the Liliaceae *sensu stricto* (Takhtajan, 1997; Tamura, 1998a). Recent classifications of these outgroup taxa and *Uvularia* based on Dahlgren et al. (1985), Takhtajan (1987, 1997), Nordenstam (1998), and Tamura (1998a) are presented in Table 1.

It has been suggested from *rbcL* data (Shinwari et al., 1994a, 1994b) as well as from cytological (Utech and Kawano, 1975b; Tamura, 1995) and seed coat anatomy evidence (Fukuhara and Shinwari, 1994) that *Disporum* section *Disporum* (*D. sessile* and other species) is closely related to *Uvularia* and that *Medeola* and *Clintonia* are best treated in a narrowly defined Liliaceae (Tamura, 1998a).

The following questions were specifically addressed in this paper: (1) Is the genus *Uvularia* monophyletic? (2) Can molecular data resolve phylogenetic relationships among the five *Uvularia* species? (3) Are *matK* and *rbcL* sequence data congruent with morphological and karyological data regarding the relationships within *Uvularia?* (4) Can patterns of character differentiation be inferred based on the molecular topology? (5) Is the berry-fruited genus, *Disporum,* closely related to the capsule-fruited *Uvularia,* and are *Medeola* and *Clintonia* sister members of the Liliaceae?

In the present study, we used two molecular markers of the chloroplast DNA (cpDNA), *matK* and *rbcL*, to elucidate the affinities among all five *Uvularia* species and the outgroup taxa. *matK* is one of the cpDNA genes that encodes the maturase enzyme which splices the precursor of $tRNA^{Lys}$ (UUU) (Neuhaus and Link, 1987). According to Olmstead and Palmer (1994), among the some 20 chloroplast genes (>1 kbp in length) that are useful in molecular systematics, the *matK* gene is known to have the highest overall nucleotide substitution rate.

Therefore, due to its sufficient length (~1500 bp) and high substitution rate, the *matK* gene has become a valuable analytical tool in addressing systematic and evolutionary questions at various taxonomic levels, but especially at levels of closely related species (Steele and Vilgalys, 1994; Johnson and Soltis, 1994, 1995; Soltis et al., 1996; Liang and Hilu, 1996; Hilu and Liang, 1997).

Also in this study, the *cp*DNA gene, *rbcL*, was sequenced for all five *Uvularia* species and the outgroup taxa. However, the *rbcL* gene has been found most useful, in contrast to *matK*, for phylogenetic analyses of intergeneric, familial and/or higher order relationships among angiosperms (e.g., Doebley et al., 1990; Soltis et al., 1990, 1993; Wilson et al., 1990; Bousquet et al., 1992; Gaut et al., 1992; Giannasi et al., 1992; Rettig et al., 1992; Chase et al., 1993; Conti et al., 1993; Duvall et al., 1993a, 1993b; Qiu et al., 1993; Smith et al., 1993; Morgan and Soltis, 1993; Price and Palmer, 1993; Rodman et al., 1993; Olmstead et al., 1993; Xiang et al., 1993; Kron and Chase, 1993; Michaels et al., 1993; Shinwari et al., 1994a, 1994b; Kato et al., 1995; Kazempour Osaloo et al., 1999).

Notes: Recent higher order classification of *Uvularia* and related taxa studied.

Dahlgren et al. (1985)—Treatment of Colchicaceae largely follows Nordenstam (1982); 3 tribes recognized.

Nordenstam, B. 1982. A monograph of the genus *Ornithoglossum* (Liliaceae). Op. Bot. 64: 1–51. (*Ornithoglossum*)

Dahlgren et al. (1985)—The present circumscription of Liliaceae is supported also by Huber (1969) and Schultze (1980); it is a rather homogeneous family, the closest relatives of which are undoubtedly the Calochortaceae, Uvulariaceae and Colchicaceae p. 235; Whether also the genus *Medeola* should be treated in Uvulariaceae or Liliaceae is not yet fully clear (see Berg 1962a,b; Utech 1978a) p. 238.

Takhtajan (1997)—Uvulariaceae: Such somewhat intermediate genera as *Disporum, Prosartes, Streptopus* and *Clintonia* repeatedly changed their taxonomic position. p. 483. (*Prosartes* is recognized in list)

Takhtajan (1997)—Uvulariaceae: Accoring to Dahlgren et al. (1985: 140), the combination of attributes in *Disporum* (including *Prosartes*), *Streptopus* and *Clintonia,* namely the absence of oxalate raphides, presence of perigonal rather than septal nectaries, lack of parietall cell and other embryological characerts (mentioned in Björnstad 1970), suggests that they should be transferred to the Uvulariaceae, a suggestion we have followed. p. 483.

Takhtajan (1997)—Calochortaceae (monotypic): Calochortaceae are rather isolated within the order, but embryologically they are nearest to the Scoliopaceae (based on Berg (1962: 51).

Material and Methods

1. *Plant Material*

We examined the *matK* and *rbc*L sequences of all five *Uvularia* species and the four outgroup taxa (Table 2). Voucher specimens are deposited in the herbaria of Kyoto University (KYO) and Carnegie Museum of Natural History (CM). *matK* and *rbcL* sequences for all *Uvularia* and outgroup taxa used in this study have been registered with the DNA Data Bank of Japan (DDBJ).

2. *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 *atpβ* gene (*atpβ* 232 5′- CCGTCCGTAGCA-TCATAGC-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 mM each of dNTP, 50 mM KCl, 10 mM Tris HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100, (McPherson et al., 1991, 1995) and 2.0 units of Taq DNA polymerase (Wako Chemicals). Amplification was conducted in a DNA Thermal Cycler (Perkin Elmer Cetus) 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 (GeneClean II, Bio101) and resuspended in 20 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The final yield averaged about 4 mg of DNA, enough for eight sequencing reactions.

Table 2. Sources of plant materials.

Species	Localities	Collector(s)	DDBJ Accession Numbers
Uvularia grandiflora	USA: Wisconsin, Marathon Co., Forest	S. Kawano et al.	AB009950*; AB024395**
U. floridana	USA: Florida, Gadsen Co., Flat Creek	S. Kawano et al.	AB009949*; AB024396**
U. perfloiata	USA: Arkansas, Garland Co., Crystal Spring	S. Kawano et al.	AB009951*; AB024540**
U. puberula	USA: Virginia, Augusta Co., George Washington National Forest	S. Kawano et al.	AB009952*; AB024541**
U. sessilifolia	USA: Pennsylvania, Westmoreland Co., Powdermill	S. Kawano et al.	AB009948*; AB024397**
Amana edulis	Japan: Tokyo Metropolitan, Kiyose	Y. Iiizumi	AB024385*; AB024388**
Gagea lutea	Japan: Akita Pref., Kisagata-cho, Ohtake	Y. Horii	AB024389**
Cardiocrinum cordatum	Japan: Osaka Pref., Kannan-cho, Nikaharabe	K. Hayashi	AB024392**
Clintonia borealis	USA: Wisconsin, Marathon Co., Forest	S. Kawano et al.	D17372*; AB024542**
Disporum sessile	Japan: Kyoto Pref., Ohmiya-cho, Mt. Takano	Z.K. Shinwari	D17376*; AB024543**
Erythronium japonicum	Japan: Toyama Pref., Yatsuo	S. Kawano	D28156*; AB024387**
Fritillaria loidzumiana	Japan: Toyama Pref., Yatsuo-cho	K. Hayashi	AB024390**
Lilium bakerianum	China: Yunnan	Unknown	AB024544**
L. candidum	Palestine:	Cult. in Yurigahara	AB024545**
L. superbum	USA: Penn., Westmoorland Co., Donegal	K. Hayashi et al.	AB024546**
Medeola virginiana	USA: Pennsylvania, Somerset Co.	S. Kawano et al.	D28158*; AB024547**
Nomocharis saluenensis	China: Yunnan	Cult. in Yurigahara	AB024391**
Notholirion thomsonianum	Western Himalaya	Unknown	AB024393**
Scoliopus bigelovii	USA: California, Humboldt Co.	S. Kawano	D28162*; AB024394**
Tulipa turkestanica	Turkey	Unknown	AB024396**
Trillium underwoodii	USA: Florida, Gaden Co., Flat River.	M. Ohara et al.	AB017412**

Sequences registarered with the DNA Data Bank of Japan (DDBJ); DDBJ accession numbers are order for *rbcL* * and *matK* **, respectively.

Table 3. PCR sequence primers for *rbcL* gene used in the present study.

Primer	Sequence	Location*	Strand
*rbcL*N'	5'-ATGTCACCACAAACAGAAACT-3'	1	sense
S1	5'-AGGACGATGCTACCACATCG-3'	243	sense
S2	5'-AAAACTTTCCAAGGCCC-3'	435	sense
S3	5'-TTTATGCGTTGGAGAGACCG-3'	631	sense
S4	5'-AATGCATGCAGTTATTG-3'	887	sense
S5	5'-GGTATTCATGTTTGGCA-3'	1141	sense
DBRBAS2	5'-GCTTGAATTCGAATTTGATC-3'	1411	antisense
DBRBAS1	5'-TTACGAGCTTGTACACACGC-3'	1295	antisense
TRRV1	5'-TAGAGACCCAATCTTGAGTG-3'	1111	antisense
RV7	5'-ATATGCCAAACATGAATACC-3'	1160	antisense
RV6	5'-TGAGCCAAGCTAGTTATTTGC-3'	845	antisense
RV3	5'-GCTAAGTAGTCATGCAT-3'	812	antisense
RV5	5'-CCGTAGTTCTTTGCGGATAA-3'	557	antisense
RV1	5'-TTGTAACGATCAAGACT-3'	242	antisense
RV4	5'-TCAGTCCACACAGTTGTCCA-3'	215	antisense
PX6	5'-GCATCGTCCTTTGTAACGA-3'	252	antisense
*atpβ*232	5'-CCGTCCGTAGCATCATAGC-3'	*atpβ*232	antisense

* Location of 5' end base of the primer is indicated with regard to site number of *rbcL* gene. Design of Primers N'-TRRV1 is based on wheat and *Dioscorea rbcL*, *atpβ*232 on wheat, rice and *Nicotiana atpβ*, all others on Liliflorae's *rbcL*.

3. *DNA Extraction and Polymerase Chain Reaction for the* matK *Gene*

The total genomic DNA was extracted from silica gel-dried, fresh and/or frozen leaves using the CTAB method of Doyle and Doyle (1987) or that of Tai and Tanksley (1990), except that liquid nitrogen was used to assist in plant tissue grinding. The *matK* gene was amplified using the Taq polymerase (Toyobo) and primer pairs, *trnK*-3914FM (F_1) and *trnK*-2R (R_1) (Fig. 1; Table 4). For the PCR amplification, each reaction

Table 4. PCR sequence primers for *matK* gene 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 trnK* and *matK* gene (Sugita et al., 1985).

Primer	Sequence	Locations	Strands	Authors
F_1(*trnK*-3914FM)	5'-ATCTGGGTTGCTAACTCAATGG-3'	4– 19	sense	Johnson & Soltis, 1994
F_2(FF74)	5'-ATACCCTGTTCGGACCATATTG-3'	669– 689	sense	Yoshida & Hayashi, 1999
F_3(FL32)	5'-CCAAGAAATGCCTCCTGTC-3'	713– 732	sense	Yoshida & Hayashi, 1999
F_4(AF)	5'-CTATATCCACTTATCTTTCAGGAGT-3'	804– 828	sense	Ooi et al., 1995
F_5(BFM)	5'-TCAAAGGGTTTTTCAGTCATTGTGG-3'	1038–1062	sense	Hayashi, 1999
F_6(EF1)	5'-CCTTCAATACTGGATTCAAGATG-3'	1250–1270	sense	Yoshida & Murakami,1999
F_7(EF2)	5'-CTCATGAAGAAATGGAGATATTACC-3'	1638–1662	sense	Yoshida & Murakami, 1999
F_8(CF)	5'-TTGATCGATTTGGTCGGATATGTAG-3'	2057–2080	sense	Yoshida & Hayashi, 1999
R_1(*trnK*-2R)	5'-AACTAGTCGGATGGAGTAG-3'	2573–2554	antisense	Johnson & Soltis, 1994
R_2(8R)	5'-AAAGTTCTAGCACAAGAAAGTCGA-3'	2080–2057	antisense	Ooi et al., 1995
R_3(RM)	5'-CTACATATCCGACCAAATCGATCAA-3'	1990–1966	antisense	Hayashi, 1999
R_4(ER1)	5'-GGTAATATCTCCATTTCTTCATGAG-3'	1662–1638	ntisense	Yoshida & Murakami, 1999
R_5(ER2)	5'-CATCTTGAATCCAGTATTGAAGG-3'	1270–1250	antisense	Yoshida & Murakami, 1999
R_6(AR)	5'-CTGTTGATACATTCGA-3'	956– 941	antisense	Yoshida & Hayashi, 1999

The location was based on the starting position of *trnK*5'.

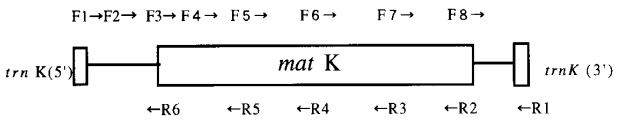

Fig. 1. Primer positions used for amplification and sequencing of *matK* gene of *cp*DNA.

mixture (100 μl) contained 54 μl of sterile water, 10 μl of 10 × Taq polymerase reaction buffer (Toyobo), 10 μl 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 Cetus) for 35 cycles. Each PCR cycle proceeded in the following manner: (1) 1 min at 94°C to denature the double-stranded template DNA; (2) 2 min at 50°C to anneal primers to single-stranded DNA; and (3) 3 min at 72°C to extent primers. The first cycle was preceded by an initial denaturation step of 2 min at 94°C, and a final extension at 72°C for 7 min following 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 dNTPs, the PCR product was electrophoresed in a 1% agarose gel (using 1x TAE as the gel buffer) stained with ethidium bromide and then excised with a scalpel under low wave length UV light. The gel slice containing the DNA fragment was transferred to a 1.5 ml microcentrifuge tube and the DNA was recovered from the agarose gel using the Gene Clean II Kit (Bio 101, Inc.) according to the manufacturer's instruction. The purified DNA was resuspended in 20 μl of sterile water.

4. *DNA Sequencing of the* matK *and* rbcL *Genes*

For sequencing the *matK* gene, purified double-stranded DNAs were then used in cycle sequencing reactions that were conducted using the Prism™ Dye Deoxy Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The cycle sequencing reaction mixture contained 80 ng of template DNA, 8 μl of terminator premix, 3.2 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. Reactions mixtures were subsequently stored at 4°C.

The primers used in this study were *trnK*-3914 FM (F_1), *matK*-FF74 (F_2), *matK*-FL32 (F_3), *matK*-AF (F_4), *matK*-BFM (F_5), *matK*-EF1 (F_6), and *matK*-EF2 (F_7) for the sense strand, and *trnK*-2R (R_1), *matK*-8R (R_2), matK-RM (R_3), *matK*-ER1 (R_4), *matK*-ER2(R_5), *matK*-AR (R_6), and for the antisense strand. The internal primers, *matK*-EF1, *matK*-EF2, *matK*-ER1, and *matK*-ER2, were designed based on the amplified region of primers, *trnK*-3914FM and *TrnK*-2R of Johnson and Soltis (1995). The location and base composition of each of the primers used in this study are given in Fig. 1 and Table 4.

Following the cycle sequencing, the reactions were purified using the Ethanol Precipitation Protocol 1 (according to the Perkin Elmer Corporation's instruction, revision A, August 1995) 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 of deionized foramide to one part of mixture of 25 mmol/L EDTA and blue dextrine) and analyzed in an ABI Prism™ 377 DNA Sequencer using 50% Long Ranger (a gel solution) run in 1x TBE buffer.

For sequencing the *rbcL* gene, the purified double-stranded PCR product was used as a template for direct sequencing with an auto-sequencer (ABI 373A) and Taq DyeDeoxy™ terminator cycle sequencing kit (ABI) according to the manufacturer's instructions. Six primers were used for sequencing the sense-strand, and ten primers were used for antisense-strand (Table 3). Both DNA strands of all five *Uvularia* species and the four outgroup taxa were sequenced and analyzed.

5. *Data Analysis*

The *matK* sequences were visually aligned with SeqEd (version 1.0.3, Applied Biosystems, Inc.). 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. We employed two different methods for phylogeny reconstruction, namely, the maximum parsimony (MP) method (Fitch, 1971, 1977) and the neighbor-joining (NJ) method (Saitou and Nei, 1987).

Phylogenetic analysis using the maximum parsimony method was performed with PAUP version 3.1.1 (Swofford, 1993). The most parsimonious trees were obtained using the heuristic search option involving 1000 replications of random addition sequence and tree-bisection-reconnection (TBR) branch-swapping. All characters were specified as unweighted. To obtain confidence limits for the various clades, bootstrap analysis (Felsenstein, 1985) was conducted. Bootstrap values with 1000 replication were calculated using the heuristic search option with TBR branch-swapping and simple addition sequence algorithms.

For the neighbor-joining (NJ) method, the computer program PHYLIP, version 3.57c (Felsenstein, 1995) was used. To obtain the neighbor-joining tree, the following procedures was followed. Kimura's (1981) two-parameter estimates of the number of nucleotide substitutions per site (between sequences) were calculated using the DNADIST program of PHYLIP. A transition/ transversion ratio of 1.0 was used. The resulting distance matrix was then analyzed by the NEIGHBOR program of PHYLIP to obtain the tree. The SEQBOOT program of PHYLIP (1000 replicates) was used to

assign the bootstrap confidence value to each branch of the tree.

Results

1. *Number of base substitustions within the* rbcL *and* matK *genes*

The sequencings of *rbc*L (1380 bp) and *matK* (1620 bp) genes of *cp*DNA were conducted for the five *Uvularia* and four outgroup taxa (Table 2). From these sequence data sets, the actual numbers of base substitustions were counted and the numbers of substitustions per site calculated pairwisely using Kimura's (1981) two parameters method (Tables 5 and 6).

The two sections of *Uvularia* (*Uvularia* and *Oakesiella*) differed from each other by 4–16 substitutitons in *rbcL* gene (100xd=0.29–1.16 substitustions per site) and 3–20 substitustions in *matK* gene (100xd=0.23–1.52). Intra-sectional variation of *rbcL* gene among the species within section *Uvularia* (*U. perfoliata* and *U. grandiflora*) differed by 4 base substitustions (100xd=0.29). Intra-sectional variation in *Oakesiella* was 4–9 substitustions, i. e., 4 (100xd=0.29, between *U. floridana* and *U. sessilifolia*) and 9 (100xd=0.65, between *U. sessilifolia* and *U. puberula*).

Intra-sectional variation of *matK* gene in *Oakesiella* was 8–9 substitustions, i. e., 8 (100xd=0.54, between *U. floridana* and *U. sessilifolia*) and 9 (100xd=0.59, between *U. sessilifolia* and *U. puberula*), and intra-sectional variation in *Uvularia* was 3 substitustions (100xd=1.15, between *U. perfoliata* and *U. grandifolia*). Among these base substitustions, we found 18 variable site changes in number of 1380 bp of *rbcL* gene sequenced, of which 11 bp (61.11%) were informative site changes; while in 1380 bp of *matK* gene (except for *U. floridana* in which 54 bp from 5′ upstream were not readable), 23 variable site changes were noted, of which 12 bp (52.17%) were informative site changes.

Table 5. Pairwise divergence of *rbcL* sequences from five *Uvularia* and four outgroup taxa. Values in upper right half of the matrix are Kimura's(1980) two parameter distance. The actual number of divergence sites appears in the lower half of the matrix.

	E. japonicum	*D. sessile*	*U. puberula*	*U. floridana*	*U. grandiflora*	*U. perfoliata*	*U. sessilifolia*	*M. virginiana*	*C. borealis*
Erythronium japonicum	—	7.92	7.51	7.10	7.26	7.03	7.10	2.87	2.49
Disporum sessile	104	—	2.05	1.83	2.20	2.05	2.12	8.49	7.68
Uvularia puberula	99	28	—	0.51	1.16	1.02	0.65	7.81	7.74
U. floridana	94	25	7	—	0.80	0.65	0.29	7.41	7.17
U. grandiflora	96	30	16	11	—	0.29	0.94	7.57	7.49
U. perfoliata	93	28	14	9	4	—	0.80	7.33	7.25
U. sessilifolia	94	29	9	4	13	11	—	7.41	7.17
Medeola virginiana	39	111	103	98	100	97	98	—	1.53
Clintonia borealis	34	101	102	95	100	96	95	21	—

Table 6. Pairwise divergence of *matK* sequences from five *Uvularia* and four outfroup taxa. Values in upper right half of the matrix are Kimura's (1980) two parameter distance. The actual number of divergence sites appears in the lower half of the matrix.

	E. japonicum	*D. sessile*	*U. puberula*	*U. floridana*	*U. grandiflora*	*U. perfoliata*	*U. sessilifolia*	*M. virginiana*	*C. borealis*
Erythronium japonicum	—	21.68	21.54	21.80	21.44	16.71	21.62	10.53	5.840
Disporum sessile	269	—	1.850	1.640	1.440	1.520	1.780	16.58	16.70
Uvularia. puberula	277	27	—	0.89	0.92	1.15	0.59	16.6	16.61
U. floridana	270	24	13	—	1.31	1.52	0.54	16.32	16.6
U. grandiflora	276	22	15	20	—	1.15	0.99	16.49	16.41
U. perfoliata	193	20	16	20	3	—	0.23	16.56	16.42
U. sessilifolia	278	26	9	8	15	16	—	16.33	16.79
Medeola virginiana	148	222	230	219	231	200	227	—	3.57
Clintonia borealis	74	183	190	180	189	189	191	54	—

2. *Phylogenetic Analyses Based upon* matK*and* rbcL *Gene*

Results of the phylogenetic analyses of the *rbcL* and *matK* sequences for the five *Uvularia* taxa are shown in Figs. 2–7 using *Erythronium japonicum, Disporum sessile, Medeola virginiana* and *Clintonia borealis* as outgroup species. The maximum parsimony (MP) trees for both *rbcL* and *matK* genes (Figs. 2 and 4) were obtained by assigning equal weights for all the characters. The neighbor-joining (NJ) method (Figs. 3 and 5) provided the same tree topologies for both genes as the MP method. The maximum parsimony (MP) and neighbor-joining (NJ) trees for *rbcL*+*matK* genes were also obtained (Figs. 6 and 7).

For the *rbc*L gene L (length of shortest tree(s) found) =165, CI (consistency index) =0.915, RI (retention index) =0.923, RC (rescaled consistency index) =0.844 and HI (homoplasy index) =0.085, and for the *matK* gene, L (length of shortest tree(s) found) =401, CI (consistency index) =0.922, RI (retention index) = 0.927, RC (rescaled consistency index) =0.867 and HI (homoplasy index) =0.078.

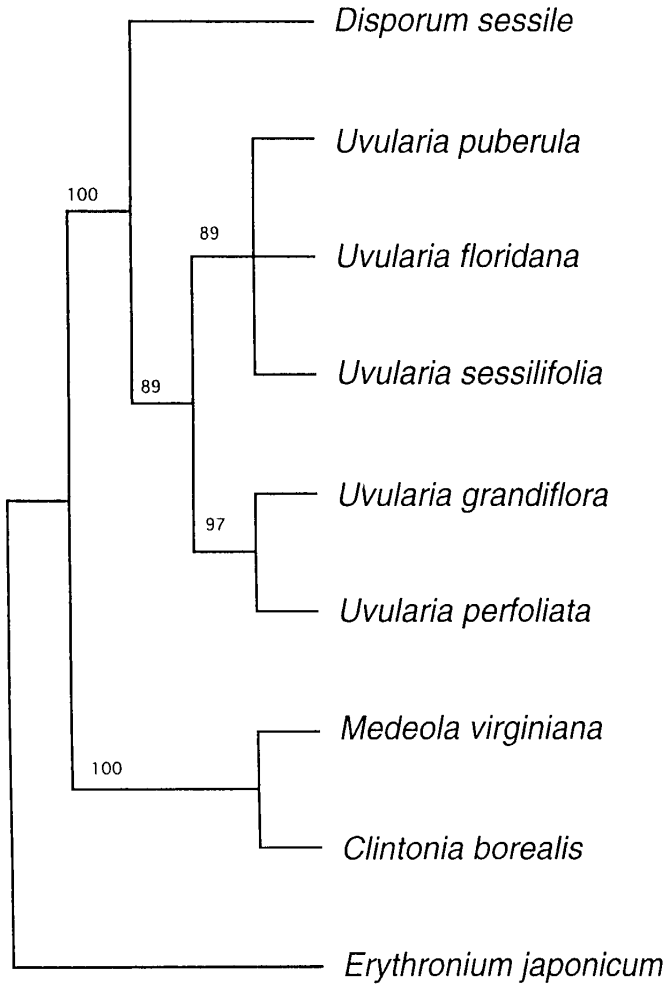

Fig. 2. The 50% majority-rule consensus tree constructed from the phylogenetic analysis of *rbcL* gene sequences of *Uvularia* and outgroup taxa (1,000 bootstrap replicates). Figures above branches are bootstrap values.

In trees for each gene as well as their combination, we can recognize two distinct clades. *U. grandiflora* and *U. perfoliata* form a clade which we have called the section *Uvularia* clade. The remaining three species (*U. floridana, U. sessilifolia* and *U. puberula*) also form a clade which we have called the section *Oakesiella* clade. For both sections and clades, i.e., *Uvularia* and *Oakesiella*, their monophylies are supported for both genes with bootstrap values of 89% and 97% in the *rbcL* tree and 85% and 99% in the *matK* tree, and, and 97% and 100% for combined tree of *rbcL*+*matK* genes. As expected, there was a slightly higher resolution of among species affinities using the *matK* gene than the *rbcL* gene, just as seen in the inter-specific relationships within section *Oakesiella* (Fig. 4). An equally high resolution for inter-sectional as well as inter-specific relationships within the genus *Uvularia* was obtained in the combined tree of *rbcL* and *matK* genes (Fig. 6).

The NJ trees for both *rbcL* and *matK* genes, and for combined data of *rbcL*+*matK* genes were also constructed. It is interesting to note here that the resolution of the NJ trees based upon the *matK* gene and also the combined *rbcL*+*matK* genes for the phylogeny of five *Uvularia* species, using *Disporum sessile*, was less as sharp as the topology obtained by the MP method (Figs. 5 and 7). The reason for this is very clear, if we

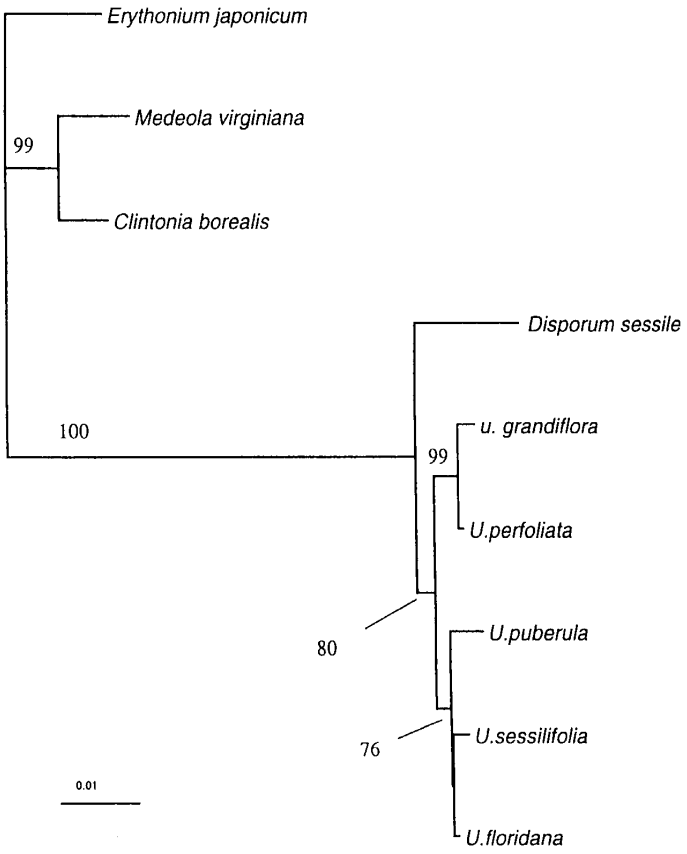

Fig. 3. The neighbor-joining (NJ) tree constructed from the phylogenetic analysis of *rbcL* sequences of *Uvularia* and outgroup taxa.

Table 7. Indels unique to *matK* gene of the genus *Uvularia*.

Indels Taxa bp	394–399	399–404	637–642	844–849
Erythronium japonicum	–	–	–	–
Disporum sessile	–	–	ACTCCG	CTTATA
Medeola virginiana	GAAGAA	–	CAGAAT	ATTATA
Clintonia borealis	–	TCTATT	ACTCTG	ATTATA
Uvularia puberula	–	–	ACTCCG	CTTATA
Uvularia floridana	–	–	ACTCCG	CTTATA
Uvularia grandiflora	–	–	ACTCCG	CTTCTA
Uvularia perfoliata	–	–	ACTCCG	CTTCTA
Uvularia sessilifolia	–	–	ACTCCG	CTTATA

examine the presence or absence of indels (Table 7).

No indels were found in *rbcL* gene of all higher plants so far examined, but in case of the *matK* gene indels have been recorded from various higher plant taxa (Johnson and Soltis, 1995). In all five *Uvularia* species sequenced in this study, two common insertions were discovered at 637–642 bp (insertion-1) and 844–849bp (insertion-2), i.e., insertion-1, ACTCCG, is shared with *Disporum sessile*, which was used as an outgroup species. But, a slightly different insertion was also found in *Clintonia borealis* (ACTCTG), but entirely different in *Medeola virginiana* (CAGAAT) used as outgroups; this insertion was, however, completely lacking in *Erythronium japonicum* (Table 7).

Three *Uvularia* species of Section *Oakesiella* (*U. puberula, U. floridana*, and *U. sessilifolia*) share insertion-2 (CTTATA) with *Disporum sessile*, an outgroup

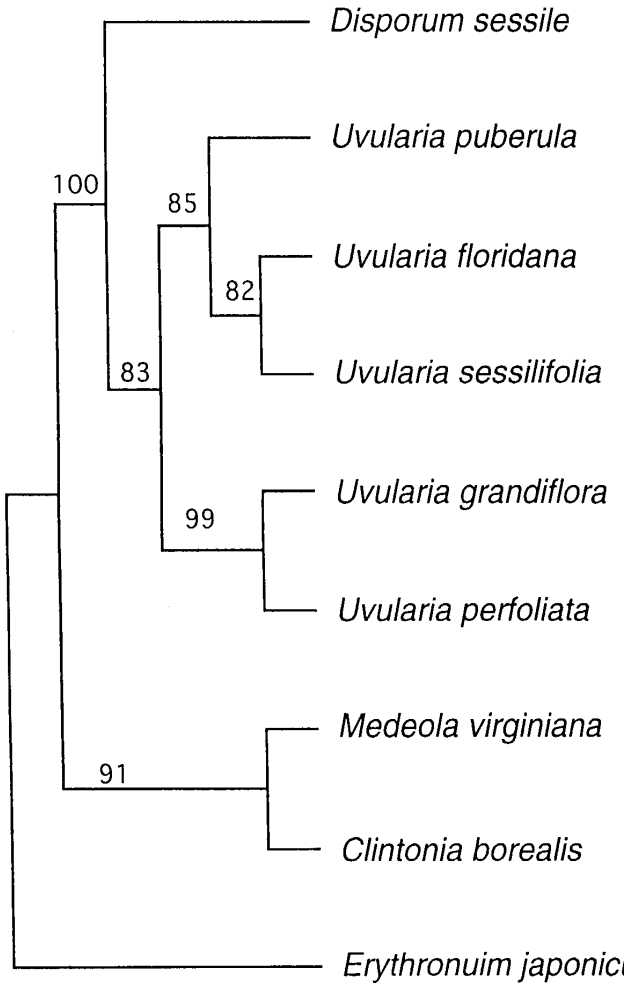

Fig. 4. The 50% majority-rule consensus tree constructed from the phylogenetic analysis of *matK* sequences of *Uvularia* and outgroup taxa (× 1,000 replications). Figures above branches are bootstrap values.

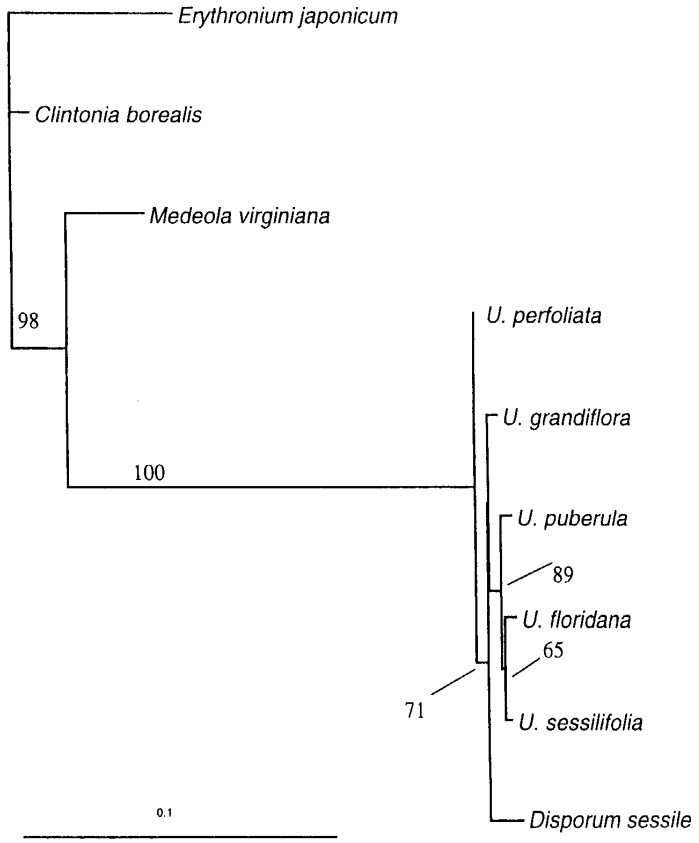

Fig. 5. The neighbor-joining (NJ) tree constructed from the phylogenetic analysis of *matK* sequences of *Uvularia* and outgroup taxa (× 1,000 replications). Figures above branches are bootstrap values.

taxon. Two species of Section *Uvularia, U. perfoliata* and *U. grandiflora*, however, possess a slightly different insertion (CTTCTA) from *Disporum sessile* (CTTATA). Three outgroup taxa have entirely different indels, i.e., *Medeola virginiana* and *Clintonia borealis* have GAAGAA at 394–399 bp and TCTATT of 399–404 bp, respectively, but it is lacking in *Erythronium japonicum*.

In the NJ tree of *matK* gene (Fig. 5) and of combined *rbcL*+*matK* genes (Fig. 7), these two insertions will not be incorporated in the construction of the topology.

Discussion

1. *Monophyly and Intergeneric Affinities within* Uvularia

The present study has shown that *Uvularia* is a monophyletic genus having bootstrap values of 89% and 80% using the MP and NJ methods for *rbcL*, respectively (Figs. 2 and 3). Furthermore, the *matK* gene sequence data provided a finer, but parallel resolution of the interspecific relationships in the genus than the *rbcL* gene. From the *matK* results, the monophyly of section *Uvularia,* whose species are characterized by perfoliate foliage, is well supported with high bootstrap probability, 99% for the MP method (Fig. 4). Section *Oakesiella,* which is characterized by sessile-leaved taxa, was strongly supported by high bootstrap probability ratio, 85% for MP and 89% for NJ (Figs. 4 and 5). *Uvularia puberula* is a sister to *U. floridana* and *U. sessilifolia*.

The results of both *rbcL* and *matK* gene sequence data showed similar topologies except for the NJ tree based on the *matK* data (Fig. 5). The phylogenetic relationships among five *Uvularia* taxa were more firmly confirmed by the topology obtained based upon the combined *matK* and *rbcL* data sets, i.e., 98% for MP (Fig. 6), but resolution by NJ for *Disporum* and *Uvularia* was not clear (Fig. 7).

2. *Differentiation of Morphological Characters and Life History Traits in* Uvularia

The molecular data are congruent with morphological accounts of the two sections (Table 8; Fig. 8) and its

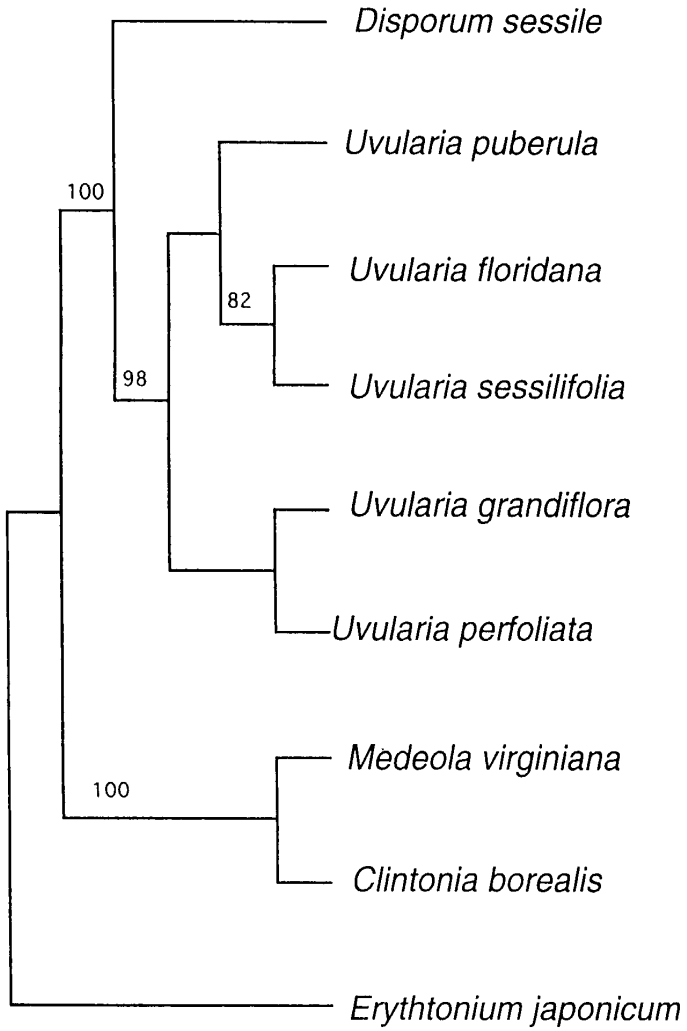

Fig. 6. The 50% majority-rule consensus tree constructed from the phylogenetic analysis of *matK*+*rbcL* sequences of *Uvularia* and outgroup taxa (× 1,000 replications). Numbers above branches are bootstrap values.

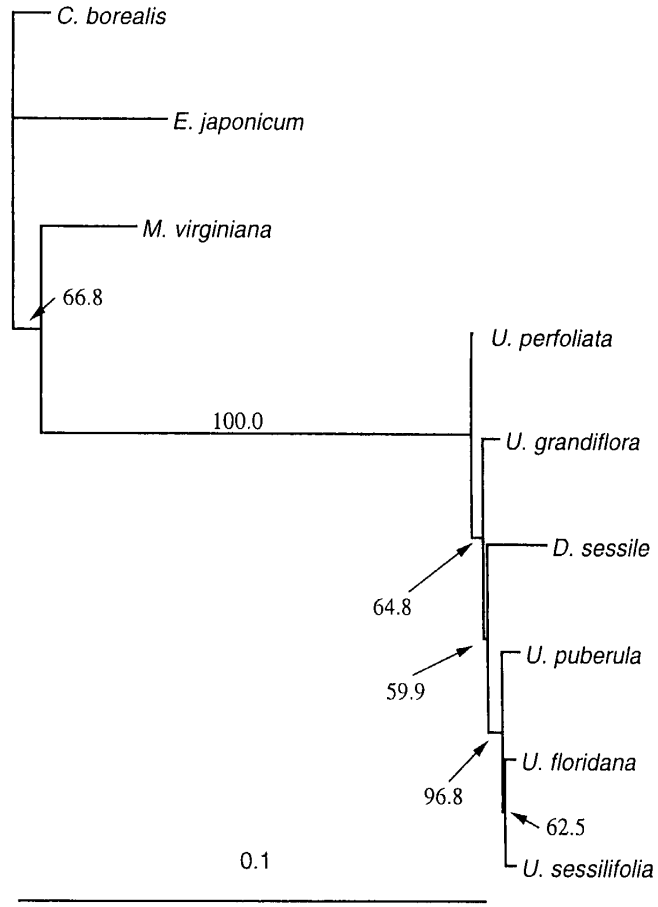

Fig. 7. The neighbor-joining (NJ) tree constructed from the phylogenetic analysis of *matK*+*rbcL* sequences of *Uvularia* and outgrouup taxa (× 1,000 replications). Numbers above branches are bootstrap values.

Table 8. Comparison of morphological characaters in *Uvularia.*

Species (Section)[1]		Vegetative Characters				Floral characters						
	Chromosome number (2*n*)	Stolons and/or rhizomes; Population character	Stems and upper X-section; Hairiness	Leaf attachment; Surface; Margins	Floral bracts; Flowers per stem	Tepal color; Length; Inner tepal surface	Stamen; Anther; Connectives lengths (mm)	Style; Stigmatic lobe Lengths (mm)	Ovary base	Locular opening and Level of ovule insertion[8]	Capsule shape; Inner wall surface; Dehiscences	Seed aril
U. puberula (*Oakesiella*)	14[2]	Stolons absent; rhizomes 0.5-l cm with clusters of fleshy roots; clumped	1-several stems angled above; puberulent above, notably at nodes	Sessile; puberulent on veins below; margins papillose	Absent; 1–3 per stem	Greenish to pale yellow; 10–25 mm; smooth	Stamens 6.5–17 mm; anthers 5–12 mm; connectives 0.6–0.8 mm	Styles 8–14 mm; stigmatic lobes 4–6 mm	Sessile to subsessile, triquetrous	Ovules at lowest level of locular opening	Broadly ellipisoid, sharply 3-winged; smooth, tardily dehiscent	Crested
U. sessiliolia (*Oakesiella*)	14[2]	Stolons present[6]; rhizomes elongate, 10–15 cm, fibrous roots; colonial	Solitary stems[7], angled above; glabrous	Sessile; glabrous: margins minutely denticulate	Absent; 1 per stem	Pale straw-yellow, 13–25 mm; smooth	Stamens 8–15 mm; anthers 5–12 mm; connectives 0.3–0.5 mm	Styles 10–15 mm (weakly 3-cleft) stigmatic lobes 1–2 mm	Subsessile to stipitate (stipe 0.8–1.2 mm)	Ovules at lowest level of locular opening	Ellipisoid, sharply 3-winged: smooth, tardily dehiscent (fruiting stripe)	Crested
U. floridana (*Oakesiella*)	12[3]	Stolons present[6] rhizomes elongate, 10–15 cm, fibrous roots; colonial	Solitary stems[7], angled above; glabrous	Sessile; glabrous; margins papillose	Present (sessile, ovate); 1 per stem	Pale whitish yellow, 20–30 mm; smooth	Stamens 10–15 mm; anthers 5–10 mm; connectives 0.5–20 mm	Styles 10–15 mm; stigmatic lobes 3–5 mm	Sessile to subsessile	Ovules at lowest level of locular opening	rhomboid, sharply 3-winged, apical rostrate beak; smooth; tardily dehiscent	Crested
U. grandiflora (*Uvularia*)	14[2,4]	Stolons absent; rhizomes short with clusters of fleshy roots; clumped	1-several stems, rounded above; glabrous	Perfoliate; white pubescent on veins below; margins smooth	Present (perfoliate); 1–3 (-4) per stem	Golden yellow; 25–50 mm; smooth	Stamens 10–25 mm; anthers 12–18 mm; connectives 0.3–0.7 mm	Styles 8–12 mm; stigmatic lobes 2–5 mm	Sessile	Ovules above lowest level of locular opening	Obovoid to ob-pyramidal, summit truncate with 3 rounded lobes; densely pebbled; dehiscent	Membranous
U. perfoliata (*Uvularia*)	14[2,4,5]	Stolons present[6]; rhizomes short; colonial	Solitary stems[7], rounded above; glaucous	Perfoliate; glaucous below; margins smooth	Present (perfoliate); 1 per stem	Straw-yellow; 20–35 mm; orange papillose	Stamens 10–17 mm; anthers 6–10 mm; connectives 0.9–1.1 mm	Styles 8–10 mm; stigmatic lobes 3–5 mm	Sessile	Ovules above lowest level of locular opening	Obovoid truncate, 3-lobed with 2 rostrate beaks per lobe; densely pebbled; dehiscent	Membranous

[1] Wilber (1963); [2] Kawano and Iltis (1964); [3] Utech (1978a); [4] Utech (1980); [5] Therman and Denniston (1984); [6] Stolon present = pseudo-annual (Kawano, 1985; Kawano et al., 1986; Kudoh et al., 1999); [7] Solitary stem = stirct or 1-branched; [8] Sterling (1977).

taxonomic circumscription (Wilbur, 1963) in which *U. puberula* occupies a somewhat intermediate position.

For most morphological characters, the two sections which represent the perfoliate-leaved species (*Uvularia*) and the sessile-leaved species (*Oakesiella*) respectively, are well defined (Table 8; Fig. 8). The flowers in *Uvularia* are solitary per branch, terminal, but appearing axillary with pendent peduncles. The tepals are distinct, imbriate, nectiferous and promptly deciduous. Sterling (1977) reported an uniquely triparite dorsal bundle within the carpellary vasculature of *Uvularia* as well as trivenous tepellary median bundles. Interestingly, the staminal vasculature was monovenous. The tricarpellate, syncarpous pistils in *Uvularia* lack septal glands, although all species have perigonal tepal nectaries. Sterling (1977) also documented a sectional difference in the levels of carpellary opening and ovule insertion. Carpellary sutures were open at the level of the lowermost ovular insertion in *U. floridana*, *U. puberula* (=*U. pudica*; =*U. carolina*) and *U. sessilifolia*, and above that in *U. grandiflora* and *U. perfoliata*. The lack of crystals or raphides in *Uvularia* reported by Goldblatt et al. (1984) confirmed similar observations by Huber (1969), Gibbs (1974), and Sterling (1977). The absence of septal glands and raphides and the presence of perigonal nectaries characterize, in part, a narrowly defined Liliaceae (Takhtajan, 1997; Tamura, 1998a) or Colchicaeae (Takhtajan, 1997; Nordenstam, 1998). The presence of arils on *Uvularia* seeds (Table 8, Fig. 8c) suggests zoochory and in particular, ants (Thompson, 1981). Major differences in the clustered versus colonial root systems in *U. per-*

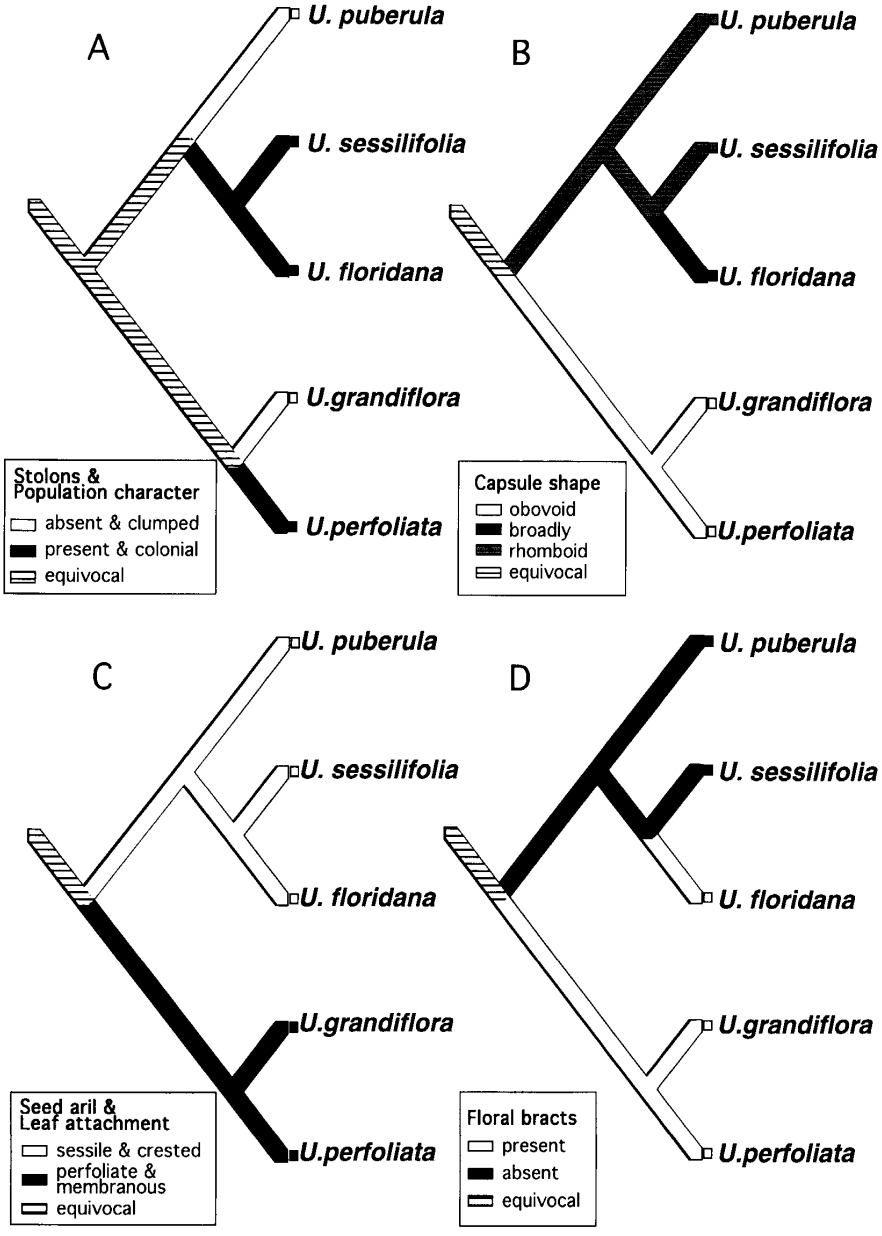

Fig. 8. Parsimoniously mapping of morphological characters onto the 50% majority-rule consensus tree of *matK* gene sequences of five *Uvularia* species (shown in Fig. 4). Upper left, stolons and population character; upper right, capsule shape; lower left, seed aril and leaf attachment; lower left, floral bract.

Table 9. Character scoring for six morphlogical and life history traits of *Uvularia* species.

Taxa	1 Stolons	2 Population character	3 Leaf attachment	4 Floral bracts	5 Capsule shape	6 Seed aril
U. puberula	0	0	0	1	1	0
U. sessilifolia	1	1	0	1	1	0
U. floridana	1	1	0	0	2	0
U. grandiflora	0	0	1	0	0	1
U. perfoliata	1	1	1	0	0	1

1. Stolons: absent (0); present (1)
2. Population character: clumped (0); colonial (1)
3. Leaf attachment: sessile (0); perfoliate (1)
4. Floral bracts: present (0); absent (1)
5. Capsule shape: obovoid (0); broadly (1); rhomboid (2)
6. Seed aril: crested (0); membranous (1)

foliata and *U. sessilifolia* were compared and illustrated by Holm (1891).

The molecular data are also congruent with the karyological accounts as well. The two species of section *Uvularia* have nearly similar karyotypes based on chromosomal size and karyotypic symmetry while more karyotypic variation occurs among the three species in section *Oakesiella* (Kawano and Iltis, 1964; Utech, 1978a, 1980; Therman and Denniston, 1984; Table 8). The karyotype of *U. puberula* is intermediate in size and symmetry between members of the two sections (Utech, 1978a) and processes a mid-sized telocentric chromosomal pair which is unique within the genus. A base number of $x=7$ characterizes the genus while the $x=6$ report for *U. floridana* probably represents a derived aneuploid reduction.

Additional structural heterozygosity in *Uvularia*, particularly *U. grandiflora*, was suggested by Belling (1926) who documented meiotic chromosome rings. Analogous rings in the pseudo-annual, Japanese *Disporum smilacinum* (Section *Disporum*) by Utech and Kawano (1977) was related to its range wide pollen infertility and reduced seed set. Such structural genetic mechanisms represent parallel, if not analogous, phylogenetic constraints operating in both taxa.

Several morphological as well as life history traits, such as presence or absence of floral bracts, seed arils, petioles, stolons, and capsule shape, were overlaid on the molecular tree constructed based on the *matK* sequence data (Tables 8 and 9; Figs. 4 and 8). From these dendrograms, we could readily see the interactions in the evolutionary divergence between phylogenetic constraints and environmental constraints. The distributions of some morphological characters such as capsule shape, seed aril and leaf attachment correspond to the sectional placement of the species, representing the lineage groups (Fig. 8B, C and D), but stoloniferous root systems and unique clonal structures differentiated in *U. sessilifolia* and *U. floridana*, and *U. perfoliata*, which belong to different clades (Fig. 8A) reflect the consequences of convergent differentiations in the life history traits under strong environmental constraints (Kawano et al., 1986; Kawano et al., 1992). The associations of the following characters, i.e., solitary stems with a single flower per stem, colonial stolons (pseudo-annual habit) and low seed productions occur in both sections — *U. sessilifolia* and *U. floridana* in section *Oakesiella* and *U. perfoliata* in section *Uvularia* (Table 8). Tall multi-stemmed clumped growth form is unique only in *U. grandiflora*.

A somewhat similar character association is also seen in Asiatic *Disporum* species. *Disporum smilacinum*, the same section as *D. sessile*, shares a common pseudo-annual habit with those clonal species of *Uvularia* (Kawano, 1985, 1987). All these characteristic life history traits and their unique associations found in *Uvularia* reflect the interactions between environmental constraints vs. phylogenetic constraints (Kawano et al., 1992; Hayashi et al., unpubl.obs.; Hayashi and Kawano, 1999; Kazempour Osaloo et al., 1999; Kazempour Osaloo and Kawano, 1999; Kawano and Kazempour Osaloo, unpubl. obs. and in preparation).

3. *Phylogenetic Position of* Uvularia

The present study has revealed that *Uvularia* is a monophyletic genus and that *Disporum sessile,* a representative of *Disporum* section *Disporum,* from northeastern Asia is a close ally, while *Erythronium japonicum, Medeola virginiana* and *Clintonia borealis* are not closely related to them, *but to each other* (Fig. 6). The broad scale, *rbc*L molecular studies of Chase et al. (1993, 1995) and Duvall et al. (1993) have associated *Medeola* with *Lilium*.

The four outgroup taxa selected for this study *(Erythronium japoncium, Medeola virginiana, Clintonia borealis,* and *Disporum sessile*) have had a recent history of taxonomic shifting due in large part to the lat-

ter three having polyphyletic, berry fruits (Utech, 1981). The fruits in *Erythronium* are loculicidal capsules (Utech and Kawano, 1975a) which are typical of the Liliaceae *sensu stricto*). Historically, several of these genera have at one time or another been linked with variously circumscribed tribes, the Parideae, Polygonateae or Uvularieae. Recent classification of these outgroup taxa (Dahlgren et al., 1985; Takhtajan, 1987, 1997; Nordenstam, 1998; Tamura, 1998a) are summarized in Table 1.

As shown in Fig. 9, the results of our recent molecular analyses based on the *matK* gene sequences (Hayashi and Kawano, 1999; Hayashi et al., unpubl. obs.) clearly demonstrate that *Clintonia* and *Medeola* belong to the same lineage of the Liliaceae-Medeoloideae (Tamura, 1998b), while *Erythronium* is a member of the Liliaceae-Lilioideae-Tulipeae (Tamura, 1998b; Hayashi and Kawano, 1999). *Erythronium* has been assigned for a long unquestioned time to a narrowly defined Liliaceae, tribe *Tulipeae. Medeola,* on the other hand, has long been associated with the

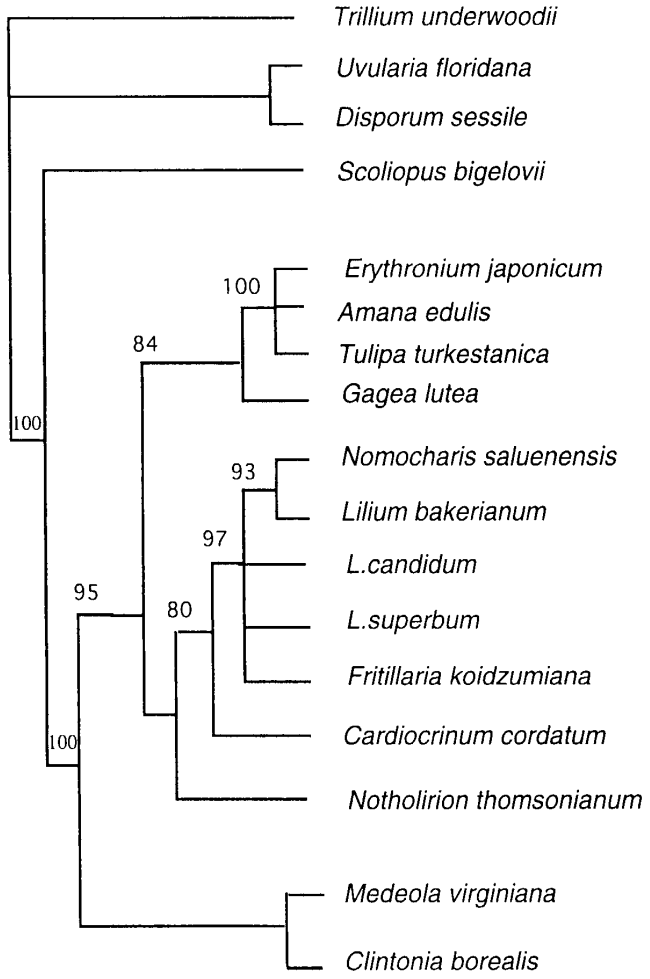

Fig. 9. The 50% majority-rule consensus tree obtained from the phylogenetic analysis of *matK* gene sequences for the members of Liliaceae *s. str.* and other genera belonging to different families, using *Trillium* as outgroup (×2,000 replications). Figures above the branches are bootstrap values.

Englerian (Krause, 1930; Melchior, 1964) and Hutchinsonian (Hutchinson, 1934, 1959, 1973) Parideae or more recently, Trilliaceae which was, however, questioned by Berg (1962a, 1962b) and Utech (1978b). Takhtajan created a monotypic family, Medeolaceae (1987), for this genus placing it next to a strictly defined Liliaceae and Liliales (1997). There is palynological as well as other support for the association of *Medeola* with the Liliaceae. Kosenko (1991) documented general pollen similarities between *Lilium* and *Medeola* as well as *Tulipa* and *Erythronium* which supports earlier work on *Erythronium* (Takahashi, 1987) and *Medeola* (Takahashi, 1984) as well as *Clintonia* (Takahashi and Sohma, 1982). Both *Disporum* and *Uvularia* exhibit simultaneous microsporogenesis (Rudall et al., 1997). Tamura (1998a, b) included *Medeola* and *Clintonia*, another berry fruited member of the Polygonateae, in a new subfamily Medeoloideae within a conservatively defined Liliaceae. Takhtajan (1997) maintained *Clintonia* in his Uvulariaceae-Streptopeae with *Disporum, Disporopsis, Prosartes,* and *Streptopus* following Dahlgren and others' (1985) suggestion that they be so transferred and grouped.

The Asian, berry fruited *Disporum* (*Disporum* section *Disporum*), also a historical Polygonateae cohort, has shifted as well due to conflicting characters and its non-supported association with the North American *Prosartes (Disporum* section *Prosartes*) (Hara, 1988; Tamura et al., 1992; Shinwari et al., 1994a, 1994b; Fukuhara and Shinwari, 1994; Utech et al., 1995; Tamura, 1995; Chase et al., 1995). Furthermore, it has been suggested from cytological data (Tamura et al., 1992; Tamura, 1995), palynology (Takahashi and Sohma, 1980) and from seed coat anatomy (Fukuhara and Shinwari, 1994) that *Disporum* section *Disporum* (*D. sessile* and others) is related to *Uvularia* and not to *Prosartes. Prosartes* and *Streptopus*, also berry fruited genera of the Englerian Polygonatae, do however share numerous characters (Shinwari et al., 1994a, 1994b; Fukuhara and Shinwari, 1994; Tamura, 1995). Tamura (1998b) associated *Prosartes* and *Streptopus* in the Calochortaceae along with *Calochortus, Tricyrtis* and *Scoliopus*.

The Melanthiaceae of Takhtajan (1997) is now defined in a much narrower sense (Goldblatt, 1995; Zomlefer, 1997; Tamura, 1998c). For many botanists (Baker, 1875, 1880; Bentham, 1880; Baillon, 1894; Krause, 1930; Hutchinson, 1934, 1959, 1973; Melchior, 1964; Huber, 1969), the Uvularieae historically also embraced members of the Glorioseae, a tribe of the Wurmbaeoideae. Buxbaum (1925) used the Uvularieae in a very restricted sense to include only the genera *Buchardia, Kreysigia, Schelhammera*, and *Uvularia*. Many of these genera are found the warmer and temperate zones of the southern hemisphere. *Disporum* and *Uvularia* are temperate northern hemisphere outliers.

Further molecular studies using *Uvularia* as a nomenclatural as well as genetic marker are now needed in order to establish family cohorts from the southern hemisphere within either a narrowly defined Uvulariaceae — Uvulariae which includes *Disporum* (Tahjatajan, 1997) or an expanded Colchicaceacae with "uvularioid" and "wurmbaeoid" lines (Nordenstam, 1982, 1998).

3. *Multiple Origins of the Berry Fruit in the Liliales*

That the berry fruit has had multiple origins in the Liliales is now apparent. *Disporum* (berry) and *Uvularia* (locucidal capsule) are now placed in the Colchicaceae (Nordenstam in Kubitzki, 1998). *Medeola* (berry) and *Clintonia* (berry) in the Liliaceae *sensu stricto* whose members have primarily locucidal capsules (Tamura, 1998b). *Prosartes* (berry) and *Streptopus* (berry) are now in the Calochortaceae with septicidal capsuled members (Tamura, 1998a). That many of these berry fruited species co-occur in the temperate forests of the northern hemisphere should not be viewed as an example of adaptive radiation from a common berry fruited ancestor, but rather one of convergent evolution from various diverse lineages. The selective "bottle-neck" of these black to blue and orange to red berries is no doubt related to bird-dispersal in a woodland environment whose history goes back to the temperate Tertiary forests. In contrast to berries and bird-dispersal, the capsule fruited species whose seeds have arils or elaiosomes and relate to ant-dispersal (Thompson, 1981).

Acknowledgments Financial support for the present study by the Japanese Government via Grant-in-Aids for International Scientific Research (Field Research) Nos. 01041055, 05041090, and 08041143 from Monbusho (Ministry of Education, Science and Culture) to Shoichi Kawano, corresponding author, is most gratefully acknowledged.

Our thanks are also due to Toru Terachi of Kyoto Sangyo University for providing *rbcL* sequencing primers. We extend our thanks to Ryohei Terauchi and Zabta Khan Shinwari for their assistance in the earlier phases of this study. Collection of North American materials was facilitated by many of our colleagues, Doug Coleman, Minoru Tamura, Akira Hiratsuka, Hideki Takasu, Masashi Ohara, and Hiroshi Kudoh, for which we extend our gratitude.

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Received December 20, 1998. Accepted March 1, 1999.