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MOLECULAR SYSTEMATICS OF THE GENUS *UVULARIA* AND RELATED TAXA BASED UPON *rbcL* GENE SEQUENCE DATA

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Abstract

To elucidate the phylogeny of the endemic North American genus *Uvularia* and related taxa, the chloroplast gene *rbc*L was sequenced in all the 5 species of the genus. The *rbc*L sequence data of 12 other genera viz., *Tricyrtis, Disporum, Prosartes, Streptopus, Clintonia, Polygonatum, Medeola, Gloriosa, Colchicum, Veratrum, Dioscorea* and *Scilla* representing different orders of Liliflorae were also analyzed to assess phylogenetic relationships among genera.

The two sections of *Uvularia* (*Uvularia* and *Oakesiela*) differed from each other by 14-27 substitutions (100d = 1.03 - 2.01 substitutions per site) and species within section *Uvularia* (*U. perfoliata* and *U. grandiflora*) by 14 substitutions (100d=1.11). Intra-sectional variation in *Oakesiella* was 8-18 substitutions i.e., 8 (100d=0.59, between *U. floridana* and *U. sessilifolia*) and 18 (100d = 1.33, between *U. sessilifolia* and *U. puberula*). Sequence data provided support for a monophyletic *Uvularia* comprising two subclades. One of these subclades is strongly supported and correspond to section *Uvularia*. A monophyletic group corresponding to section *Oakesiella* is also present in the shortest tree but is more weakly supported.

An analysis of *Uvularia rbcL* sequences together with other genera revealed that *Disporum* is the closest relative of the genus. The transfer of *Tricyrtis, Streptopus* and *Clintonia* to the *Uvulariaceae* from the Asparagales is discussed.

Introduction

Uvularia is a North American endemic genus with 5 species. The genus is distributed from southern Quebec to southern Ontario, Alabama, Arkansas and the eastern Dakotas (Wilbur, 1963). *Uvularia* species are perennial herbs which die every winter. They usually grow in deciduous forests or in well-drained uplands but also occasionally in flood plains, swampy forests or more rarely in coniferous woods.

The genus is traditionally placed in the tribe *Uvularieae* (Liliaceae) with *Disporum, Prosartes, Kreysigia, Streptopus, Schelhammera, Drymophila* and *Iphigenia* (Kunth, 1843). Rarely this genus has been placed in different tribes like Aspidistreae (Table 1). *Tricyrtis* and *Disporum* (East Asian genera) are typically considered to be close relatives of the genus (Wilbur, 1963; Takhtajan, 1987). Takhtajan (1987) placed *Uvularia* and *Disporum* in two different orders in family Melanthiaceae of order Liliales and family Convallariaceae of order Aspargales, respectively, yet in the text (Takhtajan 1987, p.302) he mentioned that these two genera are very closely related to each other both morphologically and karyologically. Dahlgren *et al.*, (1985) and Dahlgren (1989) recognized a distinct family Uvulariaceae which include two tribes Uvular-

Table 1. Previous classifications of *Uvularia* and related genera

Author (S)	Family	Tribe	Taxa analyzed presently	Genera not analyzed here
Baker (1875)	Asparagaceae	Convallarieae	Polyganatum	Convallaria
		Streptopeae	Steptopus,Clintonia,Disporum,Medeola	Prosartes
	Colchicaceae	Uvularieae	Uvularia, Gloriosa	Littonia
Bentham &	Liliaceae	Polygonateae	Polyganatum,Streptopus,Disporum?	Smilacina, Maianthemum
Hooker (1883)		Uvularieae	Uvularia,Tricyrtis,Disporum,Gloriosa	Littonia
		Medeoleae	Clintonia, Medeola	Scoliopus,Trillium,Paris
Krause,K (1930)	Liliaceae	Uvularieae	Uvularia,Gloriosa	Littonia, Sandersonia
		Polygonateae	Clintonia,Disporum,Streptopus	Mainthemum,Drymophila
			Polygonatum	Disporopsis,Tovaria
		Parideae	Medeola	Scoliopus,Paris,Trillium
Khokhryakov (1975)	Liliaceae	Colchiceae	Gloriosa,Tricyrtis	
		Aspidistreae	Clintonia,Disporum,Medeola	Smilacina,Maianthemum
			Polygonatum,Uvularia,Streptopus	
1)Dahlgren et al.(1985)	Convallariaceae	Polygonateae	Polygonatum	Smilacina,Maianthemum
	Uvulariaceae	Tricyrtideae	Tricyrtis	
		Uvularieae	UvulariaÇlintonia,Disporum,Streptopus,	Scoliopus (?)
		Glorioseae	Medeola, Gloriosa	Littonia, Scandersonia
2)Takhtajan(1987)	Melanthiaceae	Uvularieae	Uvularia	Kreysigia
		Scoliopeae		Scoliopus
		Tricyrtideae	Tricyrtis	
		Glorioseae	Gloriosa	Littonia,Sandersonia
	Medeolaceae	Medeoleae	Medeola	
	Convallariaceae	Polygonateae	Polyganatum,Clintonia,Disporum,Streptopus	Disporopsis,Prosartes

1)Dahlgren placed Convallariaceae in Asparagales and Uvulariaceae in Liliales.

2)Takhtajan placed Melanthiaceae and Medeolaceae in Liliales, and Convallariaceae in Asparagales.

* Our unpublished data.

ieae and Tricyrtideae. These names were conserved (Reveal & Hoogland, 1992) with *U. perfoliata* as its type species (Reveal, 1992).

Wilbur (1963) divided the genus into 2 sections: (i) Section *Oakesiella* which includes the sessiled-leaved species, *U. puberula* (=*U. pudica*), *U. sessilifolia* and *U. floridana*; and (ii) section *Uvularia* which includes the perfoliate leaved species, *U. grandiflora* and *U. perforliata*. In majority of morphological characters (Table 2) the two sections are well defined. However in some traits (stolons colonial hairiness and bracts) in which character distribution among species does not correspond to the placement of the species in the respective section. For many characters species of section *Oakesiella* seem closer to *U. perforllated* than to *U. grandiflora* of section *Uvularia*. Cytologically the major karyotype differences were observed between sections and among species; 2n= 14 for all species except *U. floridana* which has 2n = 12 (Anderson & Whitaker, 1934; Kawano & ltis, 1964; Utech, 1978). Five species of *Uvularia* viz., *U. perfoliata* L., *U. grandiflora* Smith, *U. puberula* Michx., *U. sessilifolia* L., and *U. floridana* Chapman are currently recognized (Britton & Brown, 1913; Wilbur, 1961; Rydberg, 1965; Radford *et al.*, 1978; Gleason & Cronquist, 1991).

The present report describes the sequencing of chloroplast gene encoding the large subunit of ribulose-1, 5- bisphosphate-carboxylase (*rbcL*) in all 5 species of the genus *Uvularia* to elucidate whether 1) The genus *Uvularia* is a monophyletic group? 2) What is the sister group of the genus *Uvularia?* 3) The placement of the genus in the higher order taxonomy, 4) Whether *rbc*L gene sequence data have enough power to give resolution in intra-generic relationships? and 5) Are molecular data congruence to the morphological accounts?

Materials and Methods

Plant material: The *rbc*L sequences of all the 5 species of the genus *Uvularia* were examined. Six genera viz., *Tricyrtis, Disporum, Prosartes, Streptopus, Clintonia* and *Polygonatum* were also analyzed because they are presumed to be close relatives of *Uvularia* (Dahlgren *et al.*, 1985; Shinwari *et al.*, 1994a,b,c). Sequences of *rbc*L for *Medeola, Gloriosa, Scilla, Dioscorea, Veratrum, Acorus* and *Colchicum* were analyzed to further confirm the inter-familial status of the *Uvulariaceae*. Voucher specimens of the plant materials (Table 3) are deposited in the herbaria of Kyoto University (KYO) and Carnegie Museum of Natural History (CM, North American taxa).

DNA extraction: Total genomic DNA was extracted from fresh or frozen leaves according to the method of Tai & Tanksley (1990), except that liquid nitrogen was used to assist in the grinding of plant tissue.

Polymerase chain reaction (PCR): The PCR was employed to amplify 1411bp of the *rbc*L gene using 2 primers that anneal to the 5' end (*rbc*LN':5'- ATGTCACCACAAACAGAAACT-3') and close to the 3' end of the *rbc*L coding region (DBRBAS2:5'-GCTTGAATTCGAATTTGATC-3'). To obtain the sequece of the 5' end of *rbc*L gene, PCR was conducted using an additional primer that anneals to the *atp*ß gene (*atp*ß 232 5' - CCGTCCGTAGCATCATAGC-3'), upstream from the *rbc*L gene (Table 4). The amplification reaction mixture (100 μl) contained 50-100ng of total DNA, 40 pmol of each primer, 0.2 mM each of dNTP, 50 mM KCl, 10 mM TrisCl pH

Table 2. Morphological differences among *Uvularia* species.

Species	Vegetative characters							Floral characters				
	Stolons colonial	Stems/Root[1]	Stem Pubescence	Branches	Leaves Base[2]	Leaves Hairs	Tepal Nect.	Bracts	Stigma Division	Ovary	Capsule	Seeds App endages
U. puberula	N	I-several stem	Y	+ + +	N	+	N	N	+ + +	sessile	acute	crest
U. sessilifolia	Y	solitary stem	N	+ + +	N	-	N	N	+	stipitate	acute(R)	crest
U. floridana	Y	solitary stem	N	+ + +	N	-	N	Y	+	sessile	acute(R)	crest
U. grandiflora	N	I-several stems	N	-(+)	Y	+	Y	Y	-	sessile	truncate	sac[3]
U. perforliata	Y	solitary stem	N	-(+)	Y	-	Y	Y	-	sessile	truncate	sac

[1]Junction from crown [2]Perfoliate [3]bladdery Nect. = Nectiferous R = with sharp ridges

Table 3. Sources of Plant materials.

Species	Localities	Collector (s)
Disporum sessile	Japan: Kyoto Pref: Ohmiya cho, Mt. Takano	Z.K. Shinwari
Tricyrtis affinis	Japan: Kyoto Pref.: Ohmiya-cho	Z.K. Shinwari
Uvularia grandiflora	USA: Wisconsin: Marathen Co. Forest	S.Kawano *et al.*,
U. floridana	USA; Florida: Gadsen Co.: Flat Creak	S.Kawano *et al.*,
U. perfloiata	USA: Arkansas:Garland Co.:HWY 270 Crys.Sp.	S.Kawano *et al.*,
U. pudica	USA: Vfrginia: Augusta Co.: George Washington National Forest	S.Kawano *et al.*,
U. sessilifolia	USA: Pennsylyania: Somerset Co.: Powdermill	S.Kawano *et al.*,
Clintonia borealis	USA: Wisconsin: Marathen Co. Forest	S. Kawano *et al.*,
Streptopus lanceolatus	USA: Wisconsin. Marathen Co. Forest	S. Kawano *et al.*,
Medeola virginiana	U.S.A.: Pennyslvania, Somerset Co.	S.Kawano *et al.*,
Gloriosa superba	Thailand: Loei, Ban Na Noi	H. Takahashi
Scilla scilloides	China:	S.Noda
Acorus calamus	Japan: Kyoto Pref., Kyoto University Botanical Garden	J. Katsuchi

8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100, (McPherson *et al.*, 1991) and 2.0 unit 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 niin, 72°C) was added. The amplified DNA was subjected to electrophoresis through 1% agarose gel and cut out of the gel. The DNA was purified by glass-milk extraction (GeneClean II, Bio101) and resuspended in 20 μl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The final yield was about 4 μg of DNA, enough for 8 sequencing reactions.

DNA sequencing: 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 manufacturer's instructions. Six primers were used for sequencing the sense-strand, and 10 primers were used for antisense-strand (Table 4). Both DNA strands were sequenced for all the species analyzed.

Phylogeny reconstruction: Two different methods were employed for phylogeny reconstruction, the maximum parsimony method (Fitch, 1977), and the neighbour-joining method (Saitou & Nei, 1987). Two different data sets were used. Firstly the relationships among 18 *rbc*L sequences of different orders of Liliflorae *sensu* Dahlgren *et al.*, (1985) were analyzed as OTUs to determine the higher order taxonomy of the genus. The *rbc*L sequence of *Acorus calamus* was used to root the tree. *Acorus* was demonstrated to be a sister group of all other Monocotyledons (Duvall *et al.*, 1993b). Secondly, relationships among six *rbc*L sequences were analyzed using *Disporum* sessile as an outgroup revealed by the first data set.

Table 4. PCR sequence primers used in the present study

Primer	Sequence	Location*	Strand
rbcLN'	5'-ATGTCACCACAAACAGAAACT-3'	1	sense
S 1	5'-AGGACGATGCTACCACATCG-3'	243	sense
S 2	5'-AAAACTTTCCAAGGCCC-3'	435	sense
S 3	5'-TTTATGCGTTGGAGAGACCG-3'	631	sense
S 4	5'-AATGCATGCAGTTATTG-3'	887	sense
S 5	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
RV5	5'-CCGTAGTTTGCGGATAA-3'	557	antisense
RV1	5'-TTGTAACGATCAAGACT-3'	242	antisense
RV4	5'-TCAGTCCACACAGTTGTCCA-3'	215	antisense
PX6	5'-GCATCGTCCTTTGTAACGA-3'	252	antisense
atpß232	5'-CCGTCCGTAGCATCATAGC-3'	atpß232	antisense

*Location of 5' end base of the primer is indicated with regard to site number of *rbc*L. gene. Design of Primers N'-TRRV1 is based on wheat and *Dioscorea rbc*L, atpß232 on wheat, rice and *Nicotiana* atpß, all others on Liliflorae's *rbc*L.

For the maximum parsimony method, a computer program PAUP (version 3.1.1, Swofford, 1993) was used. According to Albert *et al.*, (1993), both equal and differential weighting should give the same results, if the number of terminal taxa permits the detection of historically misleading changes in character state.

As a conservative approach, we applied 4 different weighting sets in our analysis. (1) the first second and third positions of the codons were weighted in the ratio of 3:3:1, and the transition/transversion probability of nucleotide substitution was set to 2:1, (2) the first, second and third positions of the codons were weighed in the ratio of 3:3:1, and the transition/transversion probability of nucleotide substitution was set to 1:1, (3) First, second and third positions of the codons were weighed in the ratio of 1:1:1, and the transition/transversion probability of nucleotide substitution was set to 2:1, (4) Applying equal weights for all the characters. First, second and third positions of the codons were weighted in the ratio of 1:1:1, and the transition/transversion probability of nucleotide substitution was set to 1:1.

Heuristic search option (random addition sequence with 10 replicates, TBR swapping algorithm) was used to find the most parsimonious tree. Bootstrapping with 100 replications was conducted to obtain estimates of support for monophyletic groups.

Kimura's (1981) (two parameter) estimates for the number of substitutions per site (Between sequences) were calculated using the DNADIST program (Phylip version 3.4, Felsenstein, 1992). The resulting distance matrix (Table 5) was then analyzed using the NEIGHBOUR program of Phylip (Saitou & Nei, 1987). The SEQBOOT programe of Phylip was used to put the bootstrap confidence value to each branch of the tree.

Table 5. The upper diagonal showing the base pair differences and the number of base substitutions per site given as 100xd which was calculated according to Kimura (1981) (lower diagonal).

	U.perfoliata	*U.grandiflora*	*U.sessilifolia*	*U.floridana*	*U.puberula*
Uvularia perfoliata	---	14	20	20	26
U. grandiflora	1.11	---	14	14	22
U. sessilifolia	1.55	1.03	---	8	18
U. floridana	1.55	1.03	0.59	---	18
U. puberula	2.01	1.63	1.33	1.33	---

Results

From the sequence data set, the actual number of base substitution was counted for all the 5 species of *Uvularia* and the number of substitution per site was calculated pairwisely by Kimura's (1981) two parameter method (Table 5). The two sections of *Uvularia* (*Uvularia* and *Oakesiella*) differed from each other by 14-27 substitutions (100d=1.03-2.01 substitutions per site) and species within section *Uvularia* (*U. perfoliata* and *U. grandiflora*) by 14 base substitutions (100d=1.11). Intra-sectional variation in *Oakesiella* was 8-18 substitutions, i.e., 8 (100d=0.59, between *U. floridana* and *U. sessilifolia*) and 18 (100d=1.33, between *U. sessilifolia* and *U. puberula*).

Phylogenetic analyses: The results of phylogenetic analyses of the *rbc*L sequences of the 18 taxa are shown in Fig.1, 2 and 3. The maximum parsimony (MP) method (Fig.1a) represents tree obtained by assigning equal weights for all the characters. Same tree (in principle) was obtained by all different weighting schemes. The neighbour-joining (NJ) method (Fig.1b) provides the same tree topology as the MP method. The study revealed that *Disporum* is the closest relative of the genus, as *Disporum* showed monophyly with *Uvularia* in 95 and 97% of the cases in MP and NJ methods, respectively. Both these taxa represent the Uvulariaceae *sensu* Dahlgren *et al.*, (1985).

In the analysis of maximum parsimony method, of second data set, all 4 combinations for the first, second and third positions of the codons as well as the transition/tranversion probabilities, gave similar tree topology. For each scheme a single most parsimonious tree was obtained. The single most parsimonious tree obtained by assigning the equal weight to the first, second and third position of codons is shown in Fig.2. L (Length of shortest tree (s) found) = 63, CI (Consistency Index) = 0.873, RI (Retention Index) = 0.579, RC (Rescaled Consistency Index) = 0.505 and HI (Homoplasy Index) = 0.127. Similar tree topologies (in principle) were given by the neighbour - joining method as well. In both trees, we can recognize at least two clades. *U. grandiflora* and *U. perfoliata* form a clade (hereafter indicated as the clade "A") and the remaining 3 species (*U. floridana*, *U. sessilifolia* and *U. puberula*) form a clade (hereafter as clade "B"). For clade "A", the monophyly of the species is strongly supported (bootstrap values are 81% and 84% in the two trees), and for clade "B", the

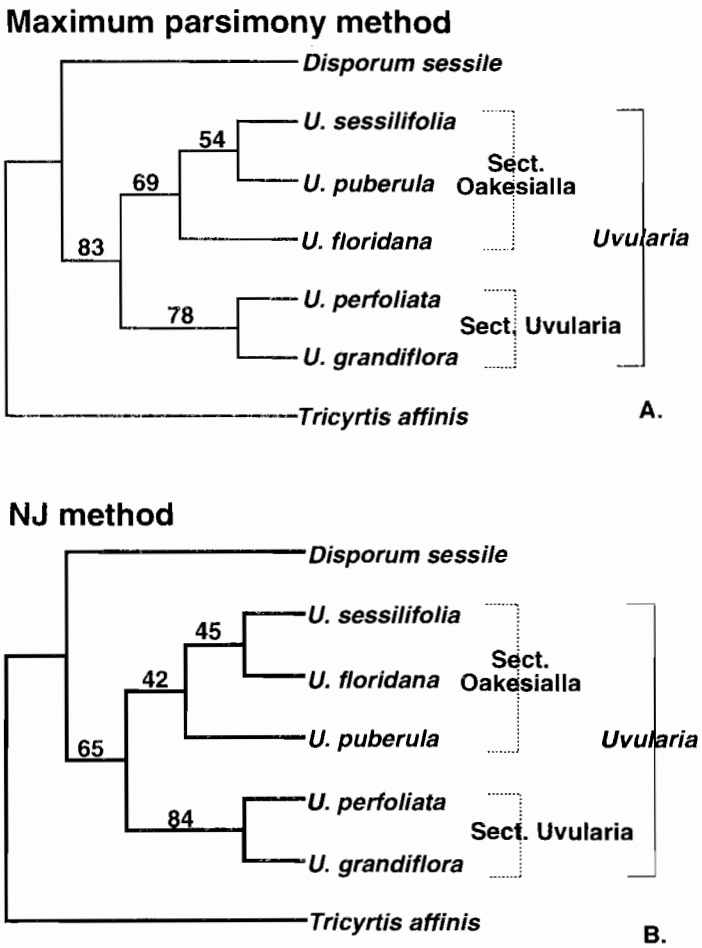

Fig.1. Phytogenetic tree of genus *Uvularia* using Tricyrtis affinis as an outgroup. Maximum parsimony method NJ 16 method.

monophyly was more weakly supported (69% bootstrap value in the MP; 42% in NJ). From these results, it is apparent that the monophyly of section *Uvularia* is strongly supported, but the presence of a clade corresponding section *Oakesiella* is more weakly supported.

The study revealed that Liliales *sensu* Dahlgren *et al.*, (1985) seems not to be a monophyletic group. The results obtained by the *rbc*L sequence data support the transfer of genera like *Prosartes, Streptopus* and *Clintonia* from Asparagales to Liliales.

Discussion

The result of the present study revealed that *Uvularia* is a monophyletic genus and is supported in 57 and 80% of the cases after 100 bootstrap replications in MP and NJ

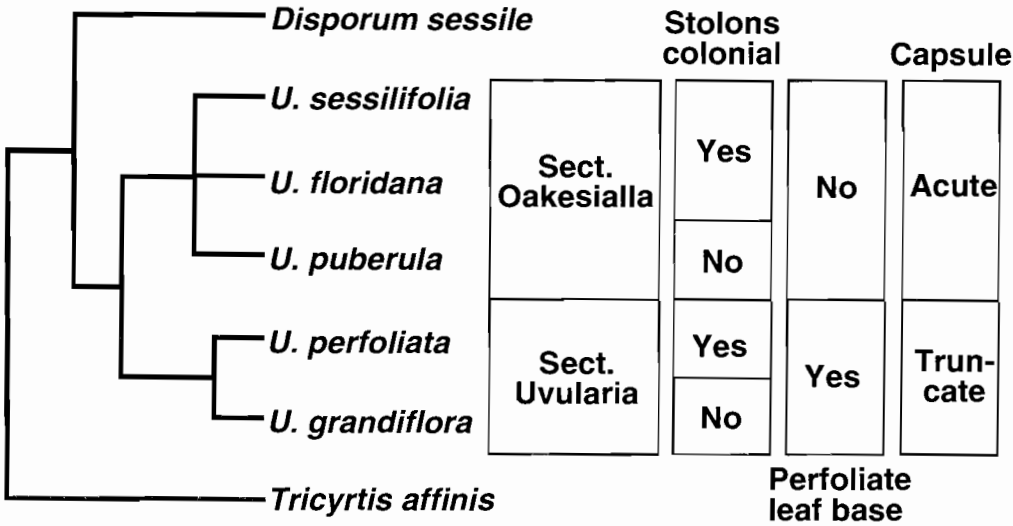

Fig.2. The single most parsimonious tree obtained by assigning equal weight to first 2nd, and third position Codon (See text for details).

methods, respectively. The genus have a close affinity to the Asiatic *Disporum* (Fig.1,2). The monophyly of the 2 genera (*Uvularia* and *Disporum*) was supported in 95-97% bootstrap probabilities. This view is concordant with those of Takhtajan (1987), Shinwari *et al.*, (1994a,b,c) and Fukuhara & Shinwari (1994).

The *rbc*L gene has been found useful for phylogenetic analyses of intergeneric and higher order relationships among Angiosperms (Doebley *et al.*, 1990; Giannasi *et al.*, 1992; Chase *et al.*, 1993; Duvall *et al.*, 1993a; Shinwari, 1995). This is a first report of *rbc*L sequence data, where all species of a small genus were analyzed. The monophyly of the section *Uvularia* which is characterized by its possession of perfoliate foliage is well supported with high bootstrap probability (42% in case of neighbour joining and 69% in maximum parsimony). Section *Oakesialla* which is characterized by sessile-leaves was rather weakly supported because of low bootstrap probability ratio (84% in case of neighbour joining and 81% in maximum parsimony). Morphologically (Wilbur, 1963) and karyologically (Utech, 1978) too, the monophyly of the section *Oakesialla* was not well supported because of somewhat intermediate position of *U. puberula*. Therefore, the data support Wilbur's (1963) classification of the genus into 2 sections. The present study revealed, that *rbc*L sequence data has sometimes weak resolution of phylogeny reconstruction among closely related species.

The molecular data is congruent with morphological accounts. For example the transfer of genera like *Prosartes, Streptopus* and *Clintonia* from Asparagales to Liliales (Dahlgren *et al.*, 1985) was supported by *rbc*L sequece data. Moreover, suggestion of autonomous family Tricyrtidaceae (Dahlgren & Clifford, 1982) was also supported by the present data.

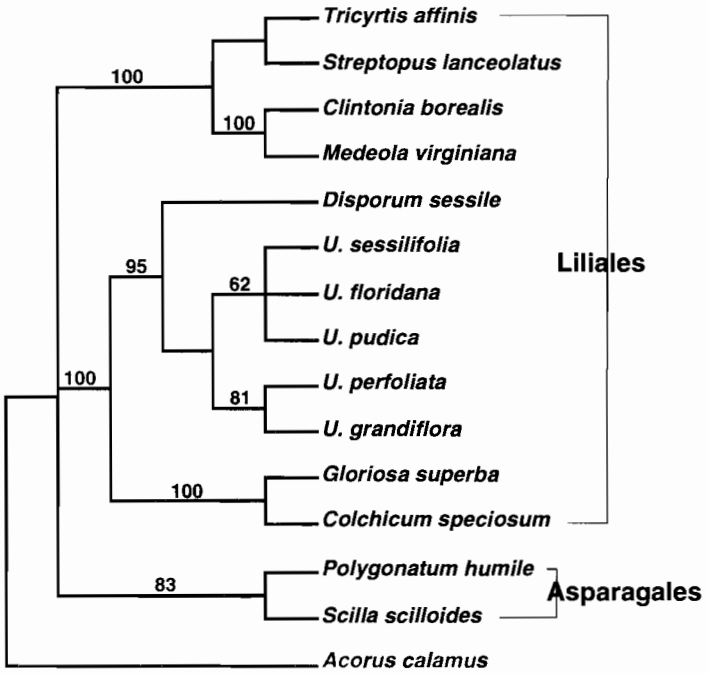

Fig.3. Consenses tree obtained by the maximum parsimony method, for higher order taxonomy of *Uvularia* and related taxa. Values on branches indicate the bootstrapping probabilities.

There is need for further studies in order to establish more objective systems of the group under consideration.

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