J. Plant Res. 112: 35-49, 1999

Journal of Plant Research

Molecular Systematics of Trilliaceae I. Phylogenetic Analyses of *Trillium* Using *matK* Gene Sequences

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Comparative DNA sequencing of the chloroplast gene *matK* was conducted using 41 *Trillium* taxa and two outgroup taxa (*Veratrum maackii* and *Helonias bullata*). A total of 1608 base pairs were analyzed and compared., and then there were 61 variable (36 informative) sites among *Trillium* species. Fifteen insertion/deletion events (indels) of six or fifteen base pairs were also detected. Phylogenetic analyses of the sequence data revealed that the subgenus *Phyllantherum* (sessile-flowered species) forms a distinct monophyletic group, whereas the subgenus *Trillium* (pedicellate-flowered species) does not form a monophyletic group, and is composed of three distinct groups and three basally located species in the tree: (1) the *Erectum* group, (2) the *Grandiflorum* group, and (3) the *Pusillum* group and (4) the three species, including *T. govanianum*, *T. undulatum*, and *T. rivale*. *T. rivale* appears to be the most basally diverged and a very specialized taxon among the ingroup members. Our *matK* data indicated that the closest relative of the subgenus *Phyllantherum* is the *Grandiflorum* group. The results are concordant with the results of the RFLP analysis of *cp*DNA and also more or less with those of the cladistic analysis of morphological characters.

Key words: Arcto-Tertiary element — *cp*DNA — *matK* gene — Phylogeny — Trilliaceae — *Trillium*

The genus *Trillium* (Trilliaceae) contains ca. 48 related species in temperate eastern Asia and eastern North America, as well as western North America (Samejima and Samejima 1987, Soukup 1980, Fukuda *et al*. 1996, Zomlefer 1996, Case and Case 1997). The majority of *Trillium* species are associated with the ancient Arcto-Tertiary deciduous forests, which have persisted with dramatic changes in geographical ranges since the early Tertiary period in the northern hemisphere but especially in the North American continent during the Pleistocene Ice Age (Li 1952, Axelrod 1966, Delcourt and Delcourt 1981, 1983, Tiffney 1985). Today, each species of *Trillium* is restricted to one of three geographical areas—eastern Asia, western and eastern North America. All 38 North American species are diploid ($2n=10$), except for the rare triploids (Darlington and Shaw 1959). In contrast, only one of the ten Asian species, *T. camschatcense* Ker-Gawler (= *T. kamtschaticum* Pallas), is diploid. The remaining species are allopolyploids showing a complex polyploid series of $2n=15, 20, 30$ with combinations of different genomes (Haga 1937, 1951, Haga *et al*. 1974, Kurabayashi 1958).

The genus consists of two subgenera: one comprises the pedicellate-flowered (erect and declinate) species (subgenus *Trillium*), while the other includes the sessile-flowered species (subgenus *Phyllantherum* Raf.). The pedicellate members are distributed in both eastern Asia and North America, whereas all the sessile species are limited to North America, occurring in both the western and eastern United States (Freeman 1975, Samejima and Samejima 1987, Case and Case 1997). These *Trillium* species have long been studied from various standpoints including comparative morphology (Gates 1917, Berg 1958, Freeman 1975, Ihara and Ihara 1978, Samejima and Samejima 1987), cytogenetics and karyology (Bailey 1951, 1954, Haga and Kurabayashi 1954, Dyer 1964a, b, c, Utech 1980), speciation at the intra- and inter-population levels (Kurabayashi 1958, Haga *et al*. 1974, Fukuda and Channell 1975, Fukuda and Grant 1980, Fukuda and Itou 1996), pollen morphology (Takahashi 1982, 1983, Wei 1995), life history characteristics and population biology (Kawano *et al*. 1986, 1992, Kawano 1994, Ohara and Kawano 1986a, b, Ohara and Utech 1986, 1988, Ohara 1989, Ohara *et al*. 1990), molecular systematics and evolutionary consideration based on chloroplast DNA (*cp*DNA) variation (Kato *et al*. 1995a, b) and cladistic analyses of morphological characters (Kawano and Kato 1995). All of these earlier studies were critically reviewed by Zomlefer (1996).

In the present study, the chloroplast gene *matK* that encodes the maturase for splicing the precursor of the $\mathrm{tRNA^{Lys}}$ (UUU) (Neuhaus and Link 1987) was sequenced to provide molecular characters for inferring phylogenetic relationships. According to Olmstead and Palmer (1994), among 20 chloroplast genes (>1 kbp in length) that are useful in

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molecular systematics, the *matK* gene is known to have the highest overall nucleotide substitution rate. Therefore, the sufficient size (~1,500 bp) and the high rate of substitutions makes the gene a valuable source of information for addressing systematic and evolutionary questions at a range of taxonomic levels, from closely related species to the generic, familial, and even supra-familial levels (see Steele and Vilgalys 1994, Johnson and Soltis 1994, 1995, Soltis *et al.* 1996, Liang and Hilu 1996, Hilu and Liang 1997).

The objectives of this study are to clarify more rigorously the inter- and intra-subgeneric relationships in *Trillium* (using 41 *Trillium* and 2 outgroup taxa) based upon the sequence data of the *matK* gene and also to compare the results of these molecular data with those of both cladistic analyses of morphological characters (Kawano and Kato 1995) and RFLP analyses of *cp*DNA (Kato *et al.* 1995a). An overall attempt will also be made to shed light upon the evolutionary history of this very interesting genus of Arcto-Tertiary origin.

Materials and Methods

Plant samples

Forty-one taxa of *Trillium* were sampled and analyzed in the present study (Table 1). The proportion of species sampled within each subgenus are 19/25 (subgenus *Trillium*) and 22/23 (subgenus *Phyllantherum*). *Veratrum maackii* Regel (Melanthiaceae) and *Helonias bullata* L. (Heloniadaceae) (Takhtajan 1997) were used as outgroups for the following reasons. First, the choice of *Paris and Daiswa* as outgroups for both molecular (Kato *et al.* 1995a) and morphological studies (Kawano and Kato 1995) of *Trillium* posed a question, because the results thus far obtained suggest that they may be possible ingroup taxa (*i.e.*, within *Trillium*) in studies of both *rbcL* (Kato *et al.* 1995b) and *matK* as well as ITS region of nrDNA in the Trilliaceae (Kazempour Osaloo and Kawano 1999). Second, several molecular analyses (Kato *et al.* 1995b, Davis 1995, Chase *et al.* 1995, Kawano *et al.* unpubl. data on the Liliiflorae sensu Dahlgren *et al.* 1985) suggested an unexpected relationship of the Trilliaceae with certain members of the Melanthiaceae.

Voucher specimens of the plants analyzed are deposited in the herbaria of Kyoto University (KYO) and the Carnegie Museum of Natural History (CM). All *Trillium* and outgroup *matK* sequences used in this study were registered in the DNA Data Bank of Japan (DDBJ) (Table 1).

DNA extraction

Total genomic DNA was extracted from silica gel-dried leaves using the modified CTAB method of Doyle and Doyle (1987). In many cases, the same DNAs used in recent *cp*DNA restriction site analysis (Kato *et al.* 1995a) were used in the present study to generate *matK* sequences (Table 1).

Polymerase chain reaction (PCR)

The *matK* gene was amplified using the Taq polymerase (Toyobo) and primer pair, *trn*K-FF74 and *trn*K-2R (Fig. 1). 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 mM $MgCl_2$, 16 μl of 1.25 mM dNTPs (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 (20-50 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 extend primers. The first cycle was preceded by an initial denaturation step of 2 min at 94 C, and a final extention 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 dNTPs, 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 UV light with a scalpel. 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.

DNA sequencing

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). 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 sec at 96 C, annealing for 15 sec at 50 C, and extension for 4 min at 60 C; reactions were then held at 4 C. The following sequencing primers were used: *trn*K-FF74, *mat*K-AF, *mat*K-BF, *mat*K-TF1, *mat*K-TF2 and *mat*K-CF for sense strand, *trn*K-2R, *mat*K-8R, *mat*K-TR1, *mat*K-TR2, and *mat*K-AR for antisense strand. The internal primers *mat*K-TF1, *mat*K-TF2, *mat*K-TR1, and *mat*K-TR2, were designed based on the amplified region of primers, *mat*K-BF and *mat*K-R of Ooi *et al.* (1995) for the two distantly related species of *Trillium*, *i.e.*, *T. camschatcense* and *T. sessile*. The location and base composition of each of the primers used in this study are given in Table 2.

Following the cycle sequencing, the reactions were purified using the Ethanol Precipitation Protocol 1 (according to the Perkin Elmer Corporation's instruction protocol, revision A, August 1995) to remove unincorporated dye terminators and then completely dried in a vaccum. The reaction pellets were resuspended in 6 μl of loading buffer [five parts of deionized foramide to one part of a mixture of 25 mmol/l EDTA and blue dextran] and analyzed in an ABI Prism TM_{377} DNA Sequencer using 50% Long Ranger (a gel solution) run in 1× TBE buffer.

Data analysis

The *matK* sequences were visually aligned with SeqEd (version 1.0.3, Applied Biosystems, Inc.); the few insertion/

Table 1. Taxa included in the *matK* sequencing analyses

	Taxa	Locality	Collector(s)	DDBJ accession number
Eastern Asiatic species :				
1.	*T. apetalon* Makino	Japan : Toyama, Ehcu-miyazaki	S. Yoshida *et al.*	AB017378
2.	*T. camschatcense* Ker-Gawler	Japan : Hokkaido, Samani-cho	H. Kato*†	AB017379
3.	*T. govanianum* Waliich ex Royle	Bhutan : Himalayas, Shin-gonpa	S. Umezawa	AB017391
4.	*T. smallii* Maximowicz	Japan : Hokkaido, Kojima Island	S. Kawano *et al.*	AB017408
5.	*T. tschonoskii* Maximowicz	Japan : Hokkaido, Hakodate-yama	S. Kawano *et al.*	AB017411
Eastern North American pedicellate-flowered species :				
6.	*T. catesbaei* Elliott	U.S.A. : Cult. in Knoxville garden	J. Freshour	AB017380
7.	*T. cernuum* L.	U.S.A. : Wisconsin, Iron Co.	S. Kawano *et al.**	AB017381
8.	*T. erectum* L.	U.S.A. : Pennsylvania, Westmoreland Co.	S. Kawano *et al.**	AB017388
9.	*T. flexipes* Rafin.	U.S.A. : Illinois, Jackson Co.	M. Ohara *et al.*	AB017389
10.	*T. grandiflorum* (Michaux) Salisb.	U.S.A. : Pennsylvania, Westmoreland Co.	S. Kawano *et al.**	AB017392
11.	*T. nivale* Riddell	U.S.A. : Pennsylvania, Westmoreland Co.	F.H. Utech	AB017398
12.	*T. pusillum* Michaux	U.S.A. : North Carolina, Sokes Co.	S. Kawano *et al.*	AB017401
13.	*T. rugelii* Rendle	Japan : Cult. in Bot. Gard. of Hokkaido Univ.	Unknown*	AB017405
14.	*T. simile* Gleason	U.S.A. : Tennessee, Porters Creek, Great Smoky Mountains	S. Kawano	AB017407
15.	*T. sulcatum* Patrick	U.S.A. : North Carolina, Burke Co.	S. Kawano *et al.**	AB017410
16.	*T. undulatum* Willdenow	U.S.A. : Pennsylvania, Westmoreland Co.	S. Kawano *et al.**	AB017413
17.	*T. vaseyi* Haribson	Sweden : Cult. in Götenburg Bot. Gard.	Unknown	AB017414
Western North American pedicellate-flowered species :				
18.	*T. ovatum* Pursh	U.S.A. : California, Del Norte Co.	S. Kawano *et al.**	AB017399
19a.	*T. rivale* S. Watson	U.S.A. : Califomia, Del Norte Co.	S. Kawano *et al.**	AB017404
19b.	*T. rivale* S. Watson	U.S.A. : Oregon, Takilma, Siskiyou Nat. For.	K. Hayashi *et al.*	AB017404
19c.	*T. rivale* S. Watson	U.S.A. : Oregon, Josephine Co. Eight Dollar Rd.	S. Kawano et al.	AB017404
Eastern North Americal sessile-flowered species :				
20.	*T. cuneatum* Rafin.	U.S.A. : Georgia, Rabun Co.	M. Ohara *et al.*	AB017384
21.	*T. decipiens* Freeman	U.S.A. : Florida, Jackson Co.	M. Ohara *et al.**	AB017385
22.	*T. decumbens* Harbison	U.S.A. : Alabama, Shelby Co., Cahaba River	M. Ohara *et al.*	AB017386
23.	*T. discolor* Wray ex Hooker	U.S.A. : South Carolina, McCormick Co.	M. Ohara *et al.*	AB017387
24.	*T. foetidissimum* Freeman	U.S.A. : Louisiana, West Feliciana Parish	M. Ohara *et al.**	AB017390
25.	*T. lancifolium* Rafin.	U.S.A. : South Carolina, McCormick Co.	M. Ohara *et al.**	AB017394
26.	*T. ludovicianum* Harbison	U.S.A. : Louisiana, Rapides Parish, Alexandria	M. Ohara *et al.*	AB017395
27.	*T. luteum* (Muhl.) Haribson	U.S.A. : North Carolina, Graham Co.	M. Ohara *et al.**	AB017396
28.	*T. maculatum* Rafin.	U.S.A. : Georgia, Early Co., Dry Creek	M. Ohara *et al.**	AB017397
29.	*T. recurvatum* Beck	U.S.A. : Arcansas, Newton Co.	M. Ohara *et al.**	AB017402
30.	*T. reliquum* Freeman	U.S.A. : Georgia, Columbia Co.	M. Ohara *et al.**	AB017403
31.	*T. sessile* L.	U.S.A. : Pennsylvania ; Westmoreland Co.	S. Kawano *et al.**†	AB017406
32.	*T. stamineum* Harbison	U.S.A. : Mississippi, Lauderdale Co., Kewanee	M. Ohara *et al.**	AB017409
33.	*T. underwoodii* Small	U.S.A. : Florida, Gaden Co., Flat River	M. Ohara *et al.*	AB017412
34.	*T. viride* Deck	U.S.A. : Missouri, Washington Co.	M. Ohara *et al.**	AB017415
35.	*T. viridescens* Nuttall	U.S.A. : Arkansas, Washington Co.	S. Kawano *et al.**	AB017416
Western North American sessile-flowered species :				
36.	*T. albidum* Freeman	U.S.A. : California, Sonoma Co.	M. Ohara *et al.**	AB017376
37.	*T. angustipetalum* (Torrey) Freeman	U.S.A. : California, Stanislaus Nat. For.	S. Kawano *et al.*	AB017377
38.	*T. chloropetalum* (Torrey) Howell	U.S.A. : California, Santa Cruz Co.	M. Ohara *et al.**	AB017382
39.	*T. chloropetalum* var. *giganteum* (Hooker & Arnot) Munz	U.S.A. : California, Santa Cruz Co.	S. Kawano *et al.*	AB017383
40.	*T. kurabayashii* Freeman	U.S.A. : California, Del Norte Co.	M. Ohara *et al.**	AB017393
41.	*T. petiolatum* Pursh	U.S.A. : Washington, Chelan Co.	M. Ohara *et al.**	AB017400
Outgroup taxa :				
42.	*Helonias bullata* L.	U.S.A. : Virginia, Augusta Co., Blue Ridge	S. Kawano *et al.*	AB017375
43.	*Veratrum maackii* Regel	Japan : Gifu, Nyukawa-mura	H. Kato	AB017417

* and † designate DNA samples used previously for RFLP analysis of *cp*DNA (see Kato *et al.* 1995a) and *rbcL* sequencing (see Kato *et al.* 1995b), respectively.

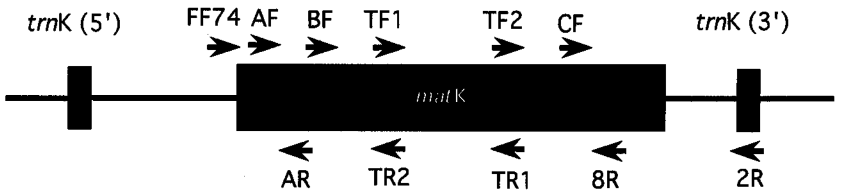

Fig. 1. Relative position of the PCR amplification and sequencing primers used in this study. Arrows indicate the direction of strand synthesis. Boxed areas represent coding region.

Table 2. Location and base composition of amplification and sequencing primers used in this study

Primer	5′ sequence 3′	Location*	Designed by
trnK-FF74	ATACCCTGTTCGGACCATATTG	676-697	Yoshida and Hayashi unpubl.
matK-AF	CTATATCCACTTATCTTTCAGGAGT	804-828	Ooi *et al.* 1995
matK-BF	TCAGAAGGTTTTGCAGTCATTGTGG	1059-1083	Ooi *et al.* 1995
matK-TF1	TAGTTCAAATCCTTCAATGCTGG	1252-1274	Kazempour Osaloo and Murakami**
matK-TF2	TGTACTAATAAATCCTTCGGCGG	1793-1815	Kazempour Osaloo and Murakami**
matK-CF	TTGACCGATTTGGGCGTATATGCAG	1966-1990	Yoshida and Hayashi unpubl.
trnK-2R	AACTAGTCGGATGGAGTAG	2573-2555	Steele and Vilgalys 1994
matK-8R	AAAGTTCTAGCACAAGAAAGTCGA	2104-2081	Ooi *et al.* 1995
matK-TR1	CCGCCGAAGGATTTATTAGTACA	1815-1793	Kazempour Osaloo and Murakami**
matK-TR2	CCAGCATTGAAGGATTTGAACTA	1274-1252	Kazempour Osaloo and Murakami**
matK-AR	CTGTTGATACATTCGA	956-941	Yoshida and Hayashi unpubl.

* Location indicates the start and end nucleotide positions relative to the tobacco sequence (Sugita *et al.* 1985).

** These primers were designed in this study.

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: maximum parsimony (Fitch 1971, 1977) and neighbor-joining (Saitou and Nei 1987).

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, a bootstrap analysis (Felsenstein 1985) was conducted. Bootstrap values with 1,000 replications were calculated using the heuristic search option (with TBR branch-swapping and simple addition sequence algorithms).

For the neighbor-joining method, the computer program PHYLIP, version 3.57 c (Felsenstein, 1995), was used. To obtain the consensus neighbor-joining tree, the following procedures were conducted: Kimuras (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 (1,000 replicates) was used to assign a bootstrap confidence value to each branch of the tree.

Results

We analyzed the *matK* sequences for 41 taxa of *Trillium* and for two outgroup taxa. The alignment of 43 entire *matK* sequences provided a matrix of 1,608 base pairs (bp). Among the ingroup taxa, variable positions are more frequent in the 5′ half of the gene than in the 3′ half, with 54% of all variable sites occurring between positions 1 and 778. Of the 61 variable sites, 36 (59%) are phylogenetically informative. Among trilliums, *T. rivale*, recorded the highest autapomorphic nucleotide sites (14 bp). *T. govanianum*, *T. undulatum* and *T. pusillum* had 8, 4, and 2 autapomorphic nucleotide sites, respectively. The *matK* sequence divergence from pairwise comparisons of *Trillium* species ranged from 0 to 1.82% (Table 3). Fifteen insertion and deletion events (indels) of 6 or 15 bp were detected. Of these 15 indels, eight (six 6 bp insertions, and two 6 bp deletions) are unique to and shared by two or more *Trillium* species (Table 4). These indels were used to construct the phylogenetic trees. It is noteworthy that within *Trillium*, there is a higher number of nucleotide substitutions (49 bp) among the pedicellate-flowered species than among the sessile-flowered species (12 bp).

Phylogenetic analysis of the *matK* sequences (excluding indels) resulted in four equally parsimonious trees of 304 steps with a consistency index (CI) of 0.924 (0.819 excluding uninformative characters) and a retention index (RI) of 0.916. Parsimony analysis of *matK* sequences plus indels (coded as presence/absence characters) produced three most parsimonious trees of 321 steps with a CI of 0.916 (0.806 excluding uninformative characters) and an RI of 0.913.

The strict consensus of the resulting trees based on both analyses exhibited nearly the same topology (Fig. 2). The topology of the tree reconstructed by the neighbor-joining method (Fig. 3) was similar to that of the strict consensus of the most parsimonious trees. The tree topologies shown in Figs. 2 and 3 give informative pictures of the infrageneric groups of *Trillium, i.e.*, four major groups and three basally diverged species that can be recognized within the genus *Trillium*.

1) The *Sessile* group (subgenus *Phyllantherum*), consisting of subgroups *Recurvatum* (including *Trillium recurvatum* and *T. lancifolium*), *Albidum* (including 13 species, together with the former subgroup making a clade with more than 85% bootstrap value), and *Decipiens* (including *T. decipiens, T. underwoodii, T. decumbens, T. reliquum*, and *T. discolor*, with more than 65% bootstrap value) as well as two species *T. stamineum* and *T. petiolatum*. Monophyly of the group was supported by more than 80% of the bootstrap value in both the most parsimonious and neighbor-joining trees.

2) The *Grandiflorum* group (supported by a 98% bootstrap value) is represented by *Trillium grandiflorum, T. nivale, T. simile*, and *T. ovatum*. This group is most likely a sister to the *Sessile* clade.

3) The *Pusillum* group includes only *Trillium pusillum*, which is a sister taxon to the *Grandiflorum-Sessile* clade.

4) The *Erectum* group (with more than 65% bootstrap value), consists of subgroups *Camschatcense* (including eastern Asian species, *Trillium camschatcense, T. apetalon, T. smallii*, and *T. tschonoskii*, with more than 75% bootstrap value), and *Cernuum* (including eastern North American species, with more than 80% bootstrap value). The latter subgroup is, in turn, composed of two subclades: one includes *T. erectum, T. sulcatum, T. cernuum, T. flexipes*, and the other comprises *T. rugelii, T. catesbaei, and T. vaseyi*. The Erectum group is clustered basal to the *Grandiflorum-Sessile* and the *T. pusillum* clade.

5) The three morphologically and geographically diverse species, *Trillium govanianum* of the Himalayas, *T. undulatum* of eastern North America, and *T. rivale* of western North America, diverge at the base of the tree. However, attention should be given to the fact that *T. rivale* and the majority of the pedicellate species share Indel I (Fig. 2 and Table 4), with the exception of *T. govanianum* and *T. undulatum*.

Discussion

The phylogenetic analysis of the entire (1,608 bp or 1,608 plus 15 indels) *matK* sequence for 43 taxa provided a robust infrageneric-level phylogeny for *Trillium* (Figs. 2 and 3). The results of the present study were compared to two other data sets: RFLP analyses of *cp*DNA (Kato *et al.* 1995a) and cladistic analyses of 22 gross morphological characters and one floral fragrance character (Kawano and Kato 1995). It is significant that all three trees resulting from the above-mentioned data sets (Fig. 8 in Kato *et al.* 1995a, Figs. 1 and 2 in Kawano and Kato 1995, cf. Figs. 2 and 3) are highly concordant in general relationships within *Trillium*.

Monophyly and phylogenetic affinities of subgenus Phyllantherum

All these three analyses provide strong support for the monophyly of the subgenus *Phyllantherum* (*Sessile* group). Thus, there is good support for recognizing the subgenus *Phyllantherum* as a distinct lineage. All these data sets also support the viewpoints of Berg (1958), who, based on morphological similarities (*e.g.*, in flower construction) between species of the *Sessile* group, suggested that the group is monophyletic. Freeman (1975) suggested on a morphological and distributional basis that this subgenus can be further divided into three groups: I (two species, *Trillium lancifolium* and *T. recurvatum*), II (the *T. sessile* group including seven species: *T. decipiens, T. decumbens, T. discolor, T. reliquum, T. sessile, T. stamineum* and *T. underwoodii*), and III (the *T. maculatum* group including 13 species: *T. albidum, T. angustipetalum, T. chloropetalum, T. cuneatum, T. foetidissimum, T. gracile, T. kurabayashii, T. ludovicianum, T. luteum, T. maculatum, T. petiolatum, T. viride*, and *T. viridescens*).

All three data sets and pollen characters (Takahashi 1982, Wei 1995) confirm that two species of Group I, namely, *Trillium recurvatum* and *T. lancifolium*, which are characterized by basally recurved sepals, strongly incurved anthers, slender, elongated rhizomes, and echinate-type exine of the pollen, make a monophyletic group consisting of only a pair of species. However, these data sets, in particular molecular data, do not support the delimitation of either the *T. sessile* or the *T. maculatum* groups of Freeman (1975) (Figs. 2 and 3). Indeed, groups II and III *sensu* Freeman (1975) comprise very heterogeneous species assemblages in their gross morphology (Kawano and Kato 1995).

The present *matK* gene analysis shows that the four sessile-flowered species, *Trillium decipiens, T. reliquum, T. decumbens* and *T. underwoodii* form a single clade, which partially coincides with the previous results obtained based on the RFLP analysis of *cp*DNA, in which only two species, *T. decipiens* and *T. reliquum* were examined (Kato *et al.* 1995). All of these four species shared Indel III (Fig. 2). The results of cladistic analysis of morphological characters also support more or less the above results (Kawano and Kato 1995).

Both the *matK* and cladistic analyses of morphological characters confirm that *Trillium petiolatum* is an exceedingly specialized species which is distantly related to the remaining members of the *Sessile* group. This species possesses a short above-ground stem with long petiolated leaves at the ground surface and a unique corrugate-type exine of pollen (Takahashi 1982) and grows in xeric rocky habitats (Kawano *et al.* 1992).

The results obtained thus far clearly show that the phylogenetic tree based on *matK* sequences agrees with the RFLP analysis of the *cp*DNA tree in supporting a close relationship between the *Grandiflorum* and *Sessile* groups. The close affinity between these two groups suggests that the *Sessile* group may have evolved from common ancestors of the *Grandiflorum* group. In light of the past geological succession (Chaney 1944, Axelrod 1966) and more recent

Table 3. Base pair differences (above diagonal) and number of base substitutions per site (below diagonal),

	T. cam.	*T. sma.*	*T. ape.*	*T. ere.*	*T. cer.*	*T. rug.*	*T. vas.*	*T. gra.*	*T. niv.*	*T. sim.*	*T. ova.*	*T. pus.*
*T. camschatcense*1)	—	0	1	4	4	4	4	13	13	14	10	9
T. smallii	0.00	—	1	4	4	4	4	13	13	14	10	9
T. apetalon	0.06	0.06	—	5	5	5	5	14	14	15	11	8
*T. erectum*2)	0.26	0.26	0.32	—	0	2	2	15	15	16	12	11
T. cernuum	0.26	0.26	0.32	0.00	—	2	2	15	15	16	12	11
*T. rugelii*3)	0.26	0.26	0.32	0.13	0.13	—	0	13	13	14	10	9
T. vaseyi	0.26	0.26	0.32	0.13	0.13	0.00	—	13	13	14	10	9
T. grandiflorum	0.84	0.84	0.91	0.97	0.97	0.84	0.84	—	0	1	3	12
T. nivale	0.84	0.84	0.91	0.97	0.97	0.84	0.84	0.00	—	1	3	12
T. simile	0.91	0.91	0.97	1.04	1.04	0.91	0.91	0.06	0.06	—	4	13
T. ovatum	0.65	0.65	0.71	0.78	0.78	0.65	0.65	0.19	0.19	0.26	—	9
T. pusillum	0.58	0.58	0.52	0.71	0.71	0.58	0.58	0.78	0.78	0.84	0.58	—
T. recurvatum	0.71	0.71	0.78	0.84	0.84	0.71	0.71	0.78	0.78	0.84	0.58	0.65
T. lancifolium	0.71	0.71	0.78	0.84	0.84	0.71	0.71	0.78	0.78	0.84	0.58	0.65
*T. sessile*4)	0.65	0.65	0.71	0.78	0.78	0.65	0.65	0.71	0.71	0.78	0.52	0.59
T. albidum	0.65	0.65	0.71	0.78	0.78	0.65	0.65	0.71	0.71	0.78	0.52	0.59
T. maculatum	0.71	0.71	0.78	0.84	0.84	0.71	0.71	0.78	0.78	0.84	0.58	0.65
T. luteum	0.71	0.71	0.78	0.84	0.84	0.71	0.71	0.78	0.78	0.84	0.58	0.65
*T. decipiens*5)	0.71	0.71	0.78	0.85	0.85	0.71	0.71	0.78	0.78	0.85	0.58	0.52
T. decumbens	0.71	0.71	0.78	0.85	0.85	0.71	0.71	0.78	0.78	0.85	0.58	0.52
T. reliquum	0.85	0.85	0.91	0.98	0.98	0.85	0.85	0.91	0.91	0.98	0.72	0.65
T. discolor	0.71	0.71	0.78	0.84	0.84	0.78	0.78	0.84	0.84	0.91	0.65	0.59
T. stamineum	0.58	0.58	0.65	0.71	0.71	0.58	0.58	0.65	0.65	0.71	0.45	0.52
T. petiolatum	0.59	0.59	0.65	0.72	0.72	0.59	0.59	0.65	0.65	0.72	0.45	0.52
T. govanianum	0.98	0.98	0.98	1.11	1.11	1.11	1.11	1.3	1.30	1.37	1.24	1.05
T. undulatum	0.65	0.65	0.65	0.78	0.78	0.78	0.78	1.17	1.17	1.24	0.98	0.92
T. rivale	1.30	1.30	1.36	1.56	1.56	1.56	1.56	1.76	1.76	1.82	1.56	1.63

The italic numbers denote that the same sequence is present in the following species, respectively.

1) *T. tschonoskii*

2) *T. sulcatum* and *T. flexipes*

3) *T. catesbaei*

4) *T. cuneatum, T. ludovicianum, T. viride, T. viridescens, T. foetidissimum, T. angustipetalum, T. kurabayashii, T. chloropetalum* var. *chloropetalum*, and *T. chloropetalum* var. *giganteum*

5) *T. underwoodii*

Quaternary vegetational changes (Davis 1983, Delcourt and Delcourt 1981), one of the centers of speciation for the sessile-flowered group could have been in the Coastal Plain and adjacent areas. The modern ranges of the eastern sessile-flowered species (Samejima and Samejima 1987, Case and Case 1997) are centered in several areas, *i.e.* Coastal Plain, Ozarkian uplands, southern Applachians and Cumberland Plateau (Samejima and Samejima 1987, Kawano 1994).

The very close similarities in gross morphology, *cp*DNA, *matK* and ITS (Kazempour Osaloo and Kawano 1999) sequence variation among all the sessile-flowered species and their geographical as well as ecological ranges suggest a relatively recent divergence of this group.

Phylogenetic affinities, and evolutionary divergence of subgenus Trillium

It is noteworthy that both cladistic analysis of morphological characters and molecular (*i.e.*, *cp*DNA and *matK* sequences) data suggest that the subgenus *Trillium* (pedicellate-flowered species) has diverged several times in its evolutionary history, resulting in at least two or three major lineages and three basally diverged species that are well differentiated and separated phylogenetically. In contrast to the subgenus *Phyllantherum*, members of the subgenus *Trillium* are highly variable in both gross morphology and *cp*DNA restriction site patterns as well as *matK* gene sequences. This is consistent with the hypothesis which suggests that at least several pedicellate-flowered species within subgenus *Trillium* have had more ancient origins than sessile-flowered species (Freeman 1975, Kawano and Kato 1995). However, speciation is still taking place in the pedicellate group through hybridization and polyploidization among some Asiatic species (Haga 1937, 1951, Kurabayashi 1958) as well as North American species (Case and Case 1997).

Since all of the species in the genera *Paris, Daiswa*, and *Kinugasa* that are related to *Trillium* share a common chargiven as 100×d, calculated according to Kimura (1981)

T. rec.	*T. lan.*	*T. ses.*	*T. alb.*	*T. mac.*	*T. Lut.*	*T. des.*	*T. dec.*	*T. rel.*	*T. dis.*	*T. sta.*	*T. pet.*	*T. gov.*	*T. und.*	*T. riv.*
11	11	10	10	11	11	11	11	13	11	9	9	15	10	20
11	11	10	10	11	11	11	11	13	11	9	9	15	10	20
12	12	11	11	12	12	12	12	14	12	10	10	15	10	21
13	13	12	12	13	13	13	13	15	13	11	11	17	12	24
13	13	12	12	13	13	13	13	15	13	11	11	17	12	24
11	11	10	10	11	11	11	11	13	12	9	9	17	12	24
11	11	10	10	11	11	11	11	13	12	9	9	17	12	24
12	12	11	11	12	12	12	12	14	13	10	10	20	18	27
12	12	11	11	12	12	12	12	14	13	10	10	20	18	27
13	13	12	12	13	13	13	13	15	14	11	11	21	19	28
9	9	8	8	9	9	9	9	11	10	7	7	19	15	24
10	10	9	9	10	10	8	8	10	9	8	8	16	14	25
—	0	1	1	2	2	6	6	8	7	4	4	20	17	27
0.00	—	1	1	2	2	6	6	8	7	4	4	20	17	27
0.06	0.06	—	0	1	1	5	5	7	6	3	3	19	16	26
0.06	0.06	0.00	—	1	1	5	5	7	6	3	3	19	16	26
0.13	0.13	0.06	0.06	—	0	6	6	8	7	4	4	20	17	27
0.13	0.13	0.06	0.06	0.00	—	6	6	8	7	4	4	20	17	27
0.39	0.39	0.32	0.32	0.39	0.39	—	0	2	3	4	4	20	17	27
0.39	0.39	0.32	0.32	0.39	0.39	0.00	—	2	3	4	4	20	17	27
0.52	0.52	0.45	0.45	0.52	0.52	0.13	0.13	—	5	6	4	20	19	27
0.45	0.45	0.39	0.39	0.45	0.45	0.19	0.19	0.32	—	5	5	20	17	27
0.26	0.26	0.19	0.19	0.26	0.26	0.26	0.26	0.39	0.32	—	2	18	15	25
0.26	0.26	0.19	0.19	0.26	0.26	0.26	0.26	0.26	0.32	0.13	—	18	15	25
1.30	0.30	1.24	1.24	1.30	1.30	1.30	1.30	1.31	1.30	1.17	1.18	—	17	27
1.11	1.11	1.04	1.04	1.11	1.11	1.11	1.11	1.24	1.11	0.98	0.98	1.11	—	23
1.77	1.77	1.70	1.70	1.77	1.77	1.77	1.77	1.77	1.77	1.63	1.64	1.77	1.50	—

acter- a single pedicellate flower-, and furthermore the taxa of the above genera diverge at the basal position of the tree (Kazempour Osaloo and Kawano 1999). It is presumed that ancestral species of the genus *Trillium* were represented by pedicellate-flowered members. Indeed, we know now that *Paris, Daiswa*, and *Kinugasa* share a number of site changes in the *matK* gene of *cp*DNA and ITS, and because of this these genera are clustered basally at the in-group positions in the tree for the genus *Trillium* (further detailed discussions in Kazempour Osaloo and Kawano 1999).

Available evidence also suggest that *Trillium undulatum, T. govanianum* and *T. rivale* in particular had earlier divergence from ancient ancestors of the pedicellate *Trillium*, since they are always basally situated in the trees (Kato *et al.* 1995a, Kawano and Kato 1995). The gross morphology of *T. undulatum* is characterized by undulated petals possessing a showy, reddish purple painted base, definitely petiolated leaves, a slender ellipsoid ovary with a thin but conspicuously three-parted stigma and a brilliant scarlet red berry and seeds with an oily elaiosome (ornithochory plus myrmechochory) (Ihara and Ihara 1978, Kawano *et al.* 1992, Kawano 1994, Kawano and Kato 1995) as well as pollen with verrucate-type exine (Takahashi 1982).

Trillium rivale, like *T. undulatum*, is a peculiar species that is characterized by long petioles, an exceedingly elongated peduncle at the fruiting stage which reaches the ground, an ovary with a short but thick style and a three-parted stigma, and a characteristic chromosome *D* (which has a short arm with a thread-like elongation between metaphase and anaphase) (Warmke 1937, Samejima and Samejima 1987).

The *matK* sequence patterns of *Trillium undulatum* (2*X*), *T rivale* (2*X*) (Warmke 1937, Bailey, 1954, Fukuda *et al.* 1968, Samejima and Samejima 1987), and *T. govanianum* (4*X*) (Haga and Watanabe 1966) (see following discussion) show greater variability than the remaining *Trillium* species, *i.e.* synapomorphic site changes with and without homoplasy as well as autoapomorphies (Fig. 2), when compared to the remaining trilliums and even related genera. As a whole, *T.*

Table 4. Characteristics of the 15 insertion/deletion events (indels) inferred in the multiple alignment of *matK* sequences in 41 taxa of *Trillium* relative to the reference taxon, *Veratum maackii*

Indel	RN	Size (bp)	Phylogenetically Informative	Sequence region*	Taxa
I	15	6	yes	CAA------GGA ...TTACAA...	*Erectum*, and *Grandiflorum* groups, *T. pusillum* and *T. rivale*
II	45	6	no	CAACAACACTTC ...------...	*T. pusillum*
III	114	6	yes	AAT------GGT ...ATAAATA..	*T. decipiens, T. underwoodii,* *T. decumbens*, and *T. reliquum*
IV	147	6	no	TTT------GGT G.GGGTTTA...	All *Trillium* species
V	336	15	no	CCA---------------TTA ...TTCTCGATGCGATTAG..	All *Trillium* species
VI	354	6	no	GAAAAAGAAATA ...------...	All *Trillium* species
VII	363	6	yes	ATA------AAA ...CAAATA...	*T. erectum, T. sulcatum, T. cernuum,* *T. flexipes, T. rugelii, T. catesbaei*, and *T. vaseyi*
VIII	570	6	yes	AAT------TTT ...TTAAAT..G	*T. erectum, T. sulcatum, T. cernuum,* *T. flexipes, T. rugelii, T. catesbaei*, and *T. vaseyi*
IX	573	6	yes	TTT------TTA ..GAATTTT...	*T. camschatcense, T. apetalon,* *T. tschonoskii*, and *T. smallii*
X	588	6	yes	AAACCTATTTAC ..T------.C.	*T. reliquum*, and *T. petiolatum*
XI	771	6	no	GTT------GTG .A.ATAGAT...	*T. maculatum*
VII	771	6	no	GTTAGAATGGTG .A.------...	All *Trillium* species
XIII	807	6	no	CTATCCCTAGGG ...------T..	All *Trillium* species
XIV	1,458	6	no	TTTCCTTTTACT ...------C..	All *Trillium* species
XV	1,533	15	no	AAT---------------TAG ...TCTTTAGATTGGTCA.GA	All *Trillium* species

* Dash represents missing base; dot indicates that the same nucleotide present in the reference taxon is also present in the species containing the indel. RN=reference nucleotide (the position of the last nucleotide preceding the indel in unaligned sequences of the reference taxon).

rivale has the highest variability in its *matK* and ITS sequences relative to other members of the Trilliaceae (Kazempour Osaloo and Kawano 1999). This taxon diverges at the base of both *matK* and ITS trees.

Gates (1917) postulated that *Trillium rivale* was possibly derived from *T. ovatum* through a dwarf mutation, and a somewhat similar viewpoint was presented by Fukuda *et al.* (1968) based on the results of differential chromosome staining of both *T. ovatum* and *T. rivale* using a cold treatment. However, as pointed out above, the results of the present molecular analysis do not indicate a direct phylogenetic relationship between these two species. We are not, thus, certain at present whether or not *T. rivale* represents one of the true ancestral species of the genus *Trillium*.

All three of these data sets show that *Trillium apetalon, T. smallii, T. tschonoskii*, and *T. camschatcense* of eastern Asia form a distinct clade, the *Camschatcense* subgroup. Therefore, these findings are completely in agreement with various lines of evidence, including gross morphology (Samejima and Samejima 1987), palynology (Takahashi 1983), chromosome morphology and genome constitutions (Haga 1937, Kurabayashi *et al.* 1958, Kurabayashi 1958), flavonoid chemistry (Yoshitama *et al.* 1997), and life history traits (Ohara 1989, Kawano *et al.* 1992, Kawano 1994) and suggests a close relationship among the members of the *Camschatcense* subgroup.

The results of both molecular analyses and cladistic analysis of morphological charaters, moreover, show that the members of the *Camschatcense* subgroup and *Trillium erectum, T. sulcatum, T. cernuum, T. flexipes, T. rugelii, T. catesbaei*, and *T. vaseyi* of eastern North America (the *Cernuum* subgroup) form a distinct clade, the *Erectum* group, although the tree topologies obtained are not exactly the same; in the *matK* tree, the group supported by moderate bootstrap value (>65%). The close affinities between the *Cernuum* and *Camschatcense* subgroups have also been confirmed by cross-pollination tests (Haga and Channell 1982), pollen morphology (Takahashi 1982, 1983), life history characteristics (Ohara 1989, Kawano *et al.* 1992, Kawano 1994), chromosome banding patterns (Fukuda 1990a) and isozyme analyses (Fukuda 1990b).

It is presumed that the members of the *Cernuum* and the *Camschatcense* subgroups may have originated from a

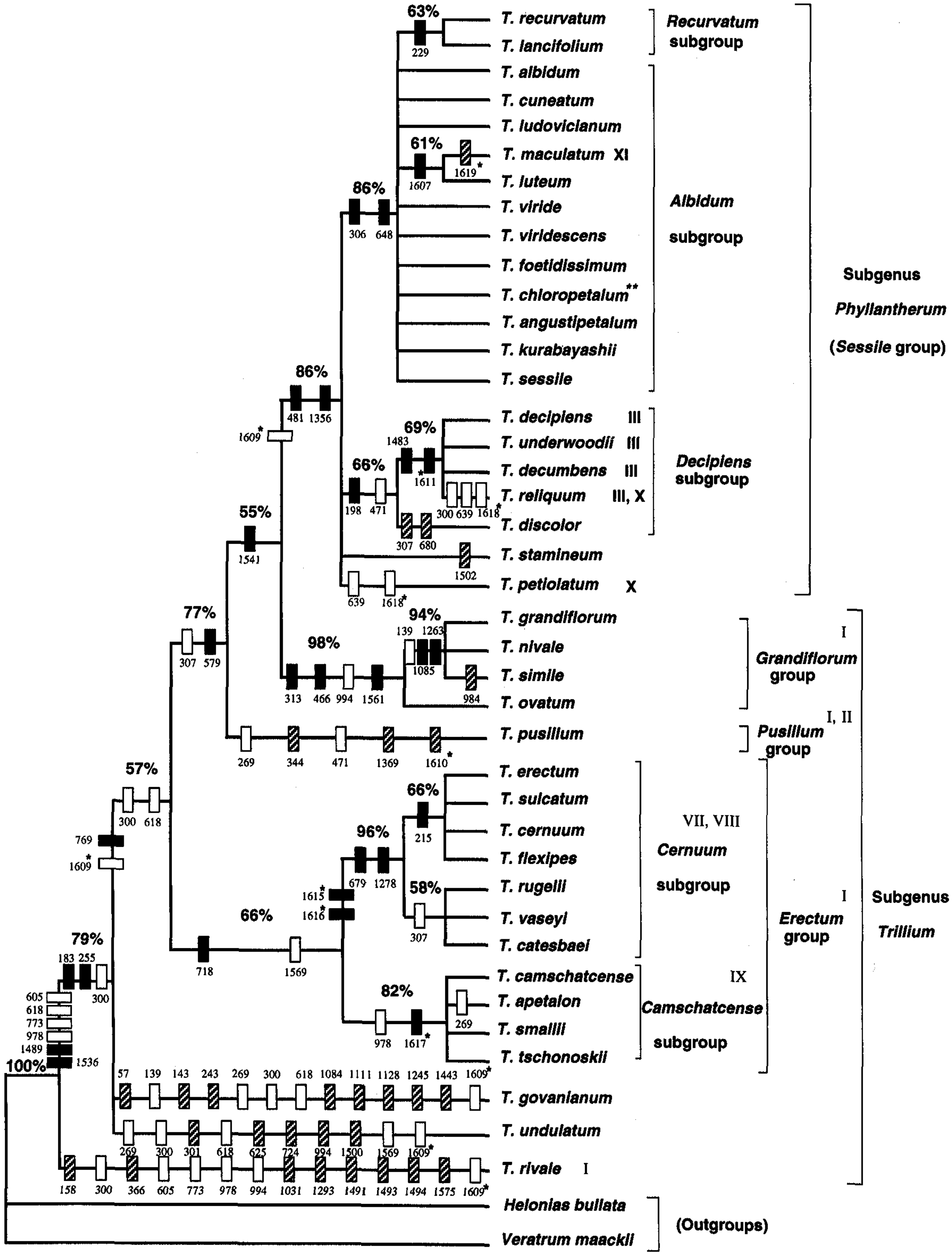

Fig. 2. Strict consensus of the three most parsimonious trees resulting from phylogenetic analysis of *matK* sequences plus indels (scored as binary characters) for *Trillium* and outgroup taxa. Percentages above the branches are bootstrap values. Open and black bars represent informative nucleotide site changes with and without homoplasy, respectively. Hatched bars represent autapomorphic nucleotide sites changes. *Bars represent indel characters. Indels are also depicted in roman numerals (shown in Table 4) for the taxa in which they occur. **Two varieties of *T. chloropetalum*, viz., var. *chloropetalum* and var. *giganteum* were analysed.

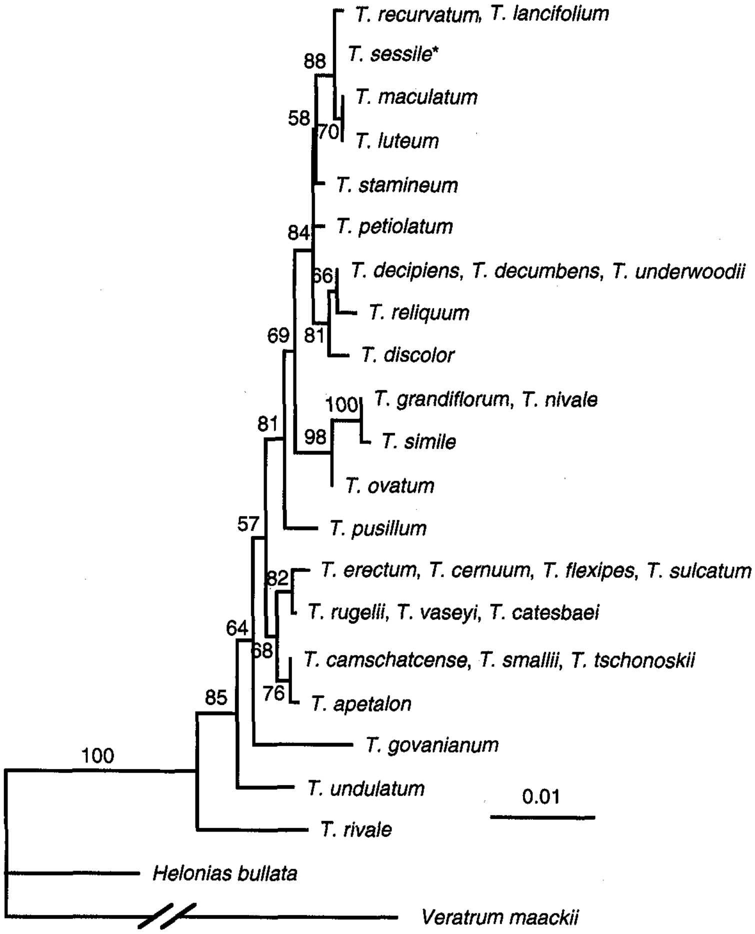

Fig. 3. Neighbor-joining distance tree resulting from phylogenetic analysis of *matK* sequences for *Trillium* and outgroup taxa. Percentages at internodes are bootstrap values. Scale indicates the base substitution per site. * *T. albidum, T. angustipetalum, T. chloropetalum* var. *chloropetalum, T. chloropetalum* var. *giganteum, T. cuneatum, T. foetidissimum, T. kurabayashii, T. ludovicianum, T. viride* and *T. viridescens*

common ancestor which once had a continuous geographical range from eastern North America to northeastern Asia across the Bering Sea before the Pleistocene glaciations (Fukuda 1990b), upon which the members of the *Camschatcense* subgroup further differentiated via polyploidization. Indeed, *Trillium erectum* and *T. camtschatcense* are still compatible and produced F1 seeds by artificial hybridization, although the F1 plants were completely sterile in pollen and seed-setting (Haga and Channell 1982). Furthermore, there are obviously some missing links among the currently existing species of the *Camschatcense* subgroup- *i.e.*, *T. camtschatcense* is the only diploid species with the K1K1 genome among Asiatic species, while *T. tschonoskii* and *T. apetalon* are tetraploids with different genomes, K2K2TT and SSUU respectively (Kurabayashi 1958).

Our *matK* analysis shows that *Trillium grandiflorum, T. nivale, T. simile* and *T. ovatum* form a very well-supported monophyletic group (the *Grandilorum* group), whereas this clade was not revealed by the cladistic analyses of morphological characters, a result that probably reflects the higher homoplasy in morphological characters, notably extreme specialization of various morphological characters in *T. nivale*. Case and Case (1997) recognized *T. simile* Gleason as an independent species in their taxonomic treatment, although it was once regarded as a variety of *T. vaseyi*, or a form of *T. erectum* var. *vaseyi*.

They referred *Trillium simile* to the *T. erectum* group, but the results of the present molecular analysis clearly indicate that this species doubtless belongs to the *Grandiflorum* group. This species has very showy large creamy white petals similar to *T. grandiflorum*, but a dark purple-black ovary (Case and Case 1997, Kawano unpubl. obs.). Palynological evidence also shows that the *Grandilorum* group, which has a spinulate exine ornamentation, is a distinct group (Takahashi 1982). In the *matK* tree, *T. nivale, T. simile*, and *T. grandiflorum* form a clade (with more than 93% bootstrap value) within the *Grandiflorum* group, whereas in the cladistic analysis of morphological characters, *T. grandiflorum* and *T. ovatum* form a clade that is basally positioned to the major pedicellate groups. The close affinity between *T. nivale* and *T. grandiflorum* was also noted by Bailey (1954) and Ihara and Ihara (1982) based on similarity of the karyotypes. Our *matK* data show that *T. pusillum* is a sister group to the *Grandiflorum-Sessile* clade, whereas in the cladistic analysis of morphological characters it forms a clade with *T. nivale*, which is outside the large major clade. This probably indicates homoplasy in the morphological criteria chosen for the analysis. *T. pusillum* comprises six geographical varieties, each with very localized ranges (Cabe 1995, Cabe and Werth 1995, Case and Case 1997), in which two forms, a pedicellate-flowered form (var. *pusillum*) and a sessile-flowered form (var. *virginanum*) have been known as representatives. Unlike the members of the *Grandiforum* group, *T. pusillum* is characterized by a connated portion at the base of the carpel prolongation, making a remarkable common style and stigmata, and by pollen exine with radially striated granules.

Based on the gross morphology and chromosome data, North American pedicellate-flowered *Trillium* species have been regarded as constituting two subgroups (Utech 1980, Samejima and Samejima 1987): the *Erectum* subgroup (six species, *T. erectum, T. sulcatum, T. cernuum, T. flexipes, T. rugelii*, and *T. vaseyi*) and the *Grandiflorum* subgroup (eight species, *T. grandiflorum, T. nivale, T. ovatum, T. persistens, T. catesbaei, T. pusillum, T. undulatum*, and *T. rivale*). As was mentioned above, our earlier RFLP analysis of *cp*DNA (Kato *et al.* 1995a) and the present molecular analysis support the monophyly of the *Erectum* subgroup, but the results show that the *Grandiflorum* subgroup (sensu Utech 1980, Samejima and Samejima 1987) is quite heterogeneous and does not form a single clade.

The phyolgenetic position of Trillium govanianum

It is now clear from the *matK* data that *Trillium govanianum* together with *T. undulatum* constitutes a sister group to the remaining groups, which seems to reflect more rigorously their true phylogenetic positions. These two species also have many characters in common, such as their very distinctive floral characters and pollen grains with a gemmate exine sculpture (Takahashi 1983 and pers. comm.), suggesting their close affinities.

On the other hand, the affinities and systematic treatment of *Trillium govanianum* with its very distinctive floral morphology, characteristic pollen grains (which is similar to those of *P. tetraphylla* and *K. japonica* as well as to exine sculpture of *T. undulatum* [Takahashi pers. comm.]), and karyotype, have long been considered enigmatic (Haga and Watanabe 1966, Takahashi 1983, 1984, Hara *et al.* 1978, Samejima and Samejima 1987, Case and Case 1997). Based on cytogenetic studies, Haga and Watanabe (1966) concluded that *T. govanianum* is an allotetraploid species ($2n=20$) composed of two different genomes (characterized by two pairs of satellited C chromosomes), neither of which are to be found in the present-day Japanese and North American species. Noltie (1994) and Case and Case (1997), following Hara *et al.* (1978), have resurrected *Trillidium* Kunth as a distinct genus, and treated the Himalayan species as *Trillidium govanianum* (Don) Kunth. Takahashi (1983), in agreement with Hara *et al.* (1978), suggested, on the basis of the isolated features (monosulcate) in pollen morphology of *T. govanianum*, that the species should be excluded from *Trillium*. However, topologies obtained for both *matK* (Figs. 2 and 3) and ITS (Kazempour Osaloo and Kawano 1999) analyses are not concordant with this palynological evidence.

The results of *matK* gene analyses (also RFLP in Kato *et al.* 1995a, and ITS in Kazempour Osaloo and Kawano 1999) show that *Trillium rivale, T. undulatum*, and *T. govanianum* may perhaps be ancestral to the remaining members of the *Trillium* species, although cladistic analyses of morphological characters do not conform precisely to the molecular analyses (Kawano and Kato 1995).

Ecological radiations in Trillium *habitats*

The modes of ecological radiations in the habitats of the pedicellate group of the genus *Trillium* should be noted here in more detail. The majority of the *Trillium* species are typical elements of the temperate deciduous forest floor (Samejima and Samejima 1987, Kawano 1994, Case and Case 1997). However, *T. undulatum* and *T. govanianum* are members of the northern hardwoods forests transitional to boreal forests or subalpine coniferous forests, which obviously represent a derived ecological condition in the genus *Trillium*.

The habitat of *Trillium rivale* needs a more detailed description. It is a highly specialized endemic species and occurs in exceedingly dry rocky serpentine habitats with good drainage at the northern California-Oregon border (Fukuda *et al.* 1968, Kawano *et al.* 1992, Case and Case 1997), where a number of other local endemics or edaphic ecotypes are also known (Kruckeberg 1954, Whittaker 1954, Proctor and Woodell 1975, Hickman 1993). There are known numerous cases of serpentine ecotypes or endemics that have differentiated from more wide-spread non-serpentine species (Kruckeberg 1951, 1954). The origin of *T. rivale* is likewise assumed to be strongly related to this extremely specialized edaphic condition in the serpentine habitats (Walker 1954, Raven 1964), but its phylogenetic position in the molecular tree constructed based upon the *matK* gene sequences is still inexplicable in terms of presently available information. The possession of the distinctly petiolate leaves of *T. rivale* is only a morphological character shared in common with *T. undulatum* and *T. govanianum*, both of which are also basally located together in the *matK* and ITS (data not presented here) trees.

Raven (1964) argues in reference to the results of a number of experimental studies (Kruckberg 1951, 1954, 1969a, b, Bradshaw 1952, Nixon and McMillan 1964) that plants which occupy unusual soil types such as serpentine almost inevitably induces genetically distinct variation under catastrophic selection (Lewis 1962). Indeed we do find an exceedingly high edaphic endemism in serpentine habitats (Mason 1946a, b, Kruckeberg 1951, 1954, 1969a, b, Ohba 1968, Brooks 1987). The precise eco-evolutionary status of *Trillium rivale* requires additional study.

Phylogenetic significance of matK *indels*

Several indels present in *Trillium* (Table 4) were used to infer phylogeny in this genus, and indeed this evidence further supported the phylogenetic interpretation of the tree topology.

Indel I is present in all members of both the *Erectum* and the *Grandiflorum* groups as well as in *Trillium pusillum* and *T. rivale*. This indel is an insertion and suggests probably multiple origins of the taxa. However, an alternative explanation is also possible because of the fact that indel I is shared by all the pedicellate species except for the two species, *T. undulatum* (2*X*) and *T. govanianum* (4*X*). If we assume three steps of evolutionary changes from the proto-*Trillium* ancestors which had this insertion, it is possible to have this topology consisting of four clades (the *Grandiflorum* group, *T. pusillum*, the *Erectum* group, and *T. rivale*) with indel I, as seen in Fig. 2. We can further assume that all descendant pedicellate species once shared this insertion, but it may have been lost again in the ancestors of *T. undulatum* and *T. govanianum*, although it is not directly traceable at present. Indeed, the number of base pair differences between *T. rivale* and the pedicellate species belonging to the *Erectum* group is much less than with the remaining groups of the pedicellate species (the *Grandiflorum* group) and the *Sessile* group, suggesting their much closer affinities (Table 3).

Indel II is unique to *Trillium pusillum*, a peculiar taxon that perhaps diverged much earlier than the *Grandiflorum* group. Indel III is present in *T. decipiens, T. underwoodii, T. decumbens*, and *T. reliquum*. These four species, together with *T. discolor*, form a monophyletic subgroup (the *Decipiens* subgroup) within the *Sessile* group. Thus, the present interpretation is that this indel did not occur in the ancestor of the *Decipiens* subgroup but appeared later on and crrently shared in the above four species. Indel X was detected only in *T. reliquum* and *T. petiolatum*. This indel is a deletion but its origin is inexplicable at present.

Indel XI is unique to *Trillium maculatum*, a member of the *Albidum* subgroup. The presence of this indel and also of the stop codon TAA (which also exists in *T. luteum*) instead of TGA (which is present in remaining *Trillium* and its relatives) in *T. maculatum* is very unique to this typical coastal plain species.

Significantly, all members of the *Cernuum* subgroup (from eastern North America) (see Fig. 2) possess indels VII and VIII, two insertions that were not detected elsewhere in the genus. Hence, these indels apparently arose only once. It is noteworthy that indels VII and VIII are absent, however, from all members of the *Camschatcense* subgroup (from northeastern Asia), taxa that constitute the sister clade to the *Cernuum* subgroup and that with the latter constitute the *Erectum* group. Likewise, indel IX is unique to all members of the *Camschatcense* subgroup and apparently also arose only once. Hence, this indel further supports the monophyly of the subgroup.

The results of the present analysis based on *matK* gene sequencing data provide more convincing evidence as to the phylogenetic relationships among the 41 *Trillium* taxa examined.

The present series of studies was accomplished with the aid of many of our colleagues, to whom we wish to extend our gratitude: to Noriaki Murakami, Hidetoshi Kato, Kazuhiko Hayashi, and Seiji Yoshida for their technical advice on sequencing and discussions during the course of this study; to Tatsundo Fukuhara and Magnus Liden for providing cultivated material of *Trillium vaseyi* from Göteborg, Sweden; to Susan Farmer and Jean Freshour, Knoxville, Tennessee, for their kindness in providing cultivated materials of *Trillium catesbaei* and *T. simile*, and also for information on the native localities of these species. Our cordial thanks are also due to Bill Grant for his thorough review of an earlier draft of the present paper. The present study was financially supported by the following grants: Grant-in-Aids for Scientific Research on Prioirty Areas No. 04264102, International Scientific Research (Field Research) No. 01041055, No. 05041090; No. 08041143 to Shoichi Kawano (corresponding author) from Monbusho (Ministry of Education, Science, and Culture) of the Japanese Government.

References

Axelrod, D.I. 1966. Origin of deciduous and evergreen habitats in temperate forests. Evolution **20**: 1-15.

Bailey, P.C. 1951. A study of the chromosome morphology of some species of *Trillium.* Bull. Torrey Bot. Club **78**: 324-330.

Bailey, P.C. 1954. Further study of the chromosome morphology of some species of *Trillium.* Bull. Torrey Bot. Club **81**: 68-75.

Berg, R.Y. 1958. Seed dispersal, morphology, and phylogeny of *Trillium*. Skr. Nor. Vidensk Akad. Oslo **1**: 1-36.

Bradshaw, A.D. 1952. Populations of *Agrostis tenuis* resistant to lead and zinc poisoning. Nature **169**: 1098.

Brooks, R.R. 1987. Serpentine and its Vegetation. Croom Helm Ltd., London.

Cabe, P. R. 1995. The *Trillium pusillum* Michaux (Liliaceae) complex in Virginia. I. Morphological investigations. Castanea **60**: 1-14.

Cabe, P.R. and Werth, C. 1995. The *Trillium pusillum* Michaux (Liliaceae) complex in Virginia. II. Isozyme evidence. Castanea **60**: 15-29.

Case, F.W. Jr. and Case, R.B. 1997. Trilliums. Timber Press, Portland, Oregon, U.S.A.

Chaney, R.W. 1944. Pliocene floras of California and Oregon. Carnegie Inst. Washington Publ. 553

Chase, M.W., Duvall, M.R., Hills, H.G., Conran, J.G., Cox., A.V., Eguiarte, L.E., Harwell, J., fay, M.F., Caddick, L.R., Cameron, K.M. and Hoot, S. 1995. Molecular phylogenetics of Lilianae. *In* P.J. Rudall, P.J. Cribb, D.F. Cutler, and C.J. Humphries, eds., Monocotyledons: Systematics and Evolution, Royal Botanical Gardens, Kew, pp. 109-137.

Dahlgren, R.M.T., Clifford, H.T. and Yeo, P.F. 1985. The Families of the Monocotyledones, Springer-Verlag, Berlin.

Darlington, C.D. and Shaw, G.W. 1959. Parallel polymorphism in the heterochromatin of *Trillium* species. Heredity **13**: 89-121.

Davis, J.I. 1995. A phylogenetic structure for the monocotyledons, as inferred from chloroplst DNA restriction site variation, and a comparison of measures of clade support. Syst. Bot. **20**: 503-527.

Davis, M.B. 1983. Quaternary history of deciduous forests of eastern North America and Europe. Ann. Missouri Bot. Gard. **70**: 550-563.

Delcourt, P.A. and Delcourt, H.R. 1981. Vegetation maps for eastern North America: 40,000 yr B.P. to the present. *In* R.C. Romans, ed., Geobotany **II**. Plenum Press, N.Y. and London.

Delcourt, P.A. and Delcourt, H.R. 1983. Late-Quaternary vegetational dynamics and community stability reconsidered. Quartern. Res. **19**: 265-271.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11-15.

Dyer, A.F. 1964a. Heterochromatin in American and Japanese species of *Trillium*. I. Fusion of chromocentres and distribution of H-segments. Cytologia **29**: 155-170.

Dyer, A.F. 1964b. Heterochromatin in American and Japanese species of *Trillium*. II. The behavior of H-segments. Cytologia **29**: 171-190.

Dyer, A.F. 1964c. Heterochromatin in American and Japanese species of *Trillium*. III. Chiasma frequency and distribution and the effect on it of heterochromatin. Cytologia **29**: 263-279.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39**: 783-791.

Felsenstein, J. 1995. PHYLIP (Phylogeny Inference Package) Version 3.57c, Univ. of Washington, Seattle.

Fitch, W.M. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. Syst. Zool. **20**: 406-416.

Fitch, W.M. 1977. On the problem of discovering the most parsimonious tree. Amer. Naturalist **111**: 223-257.

Freeman, J.D. 1975. Revision of *Trillium* subgenus *Phyllantherum* (Liliaceae). Brittonia **27**: 1-62.

Fukuda, I. 1990a. Chromosome differentiation among North American and Japanese *Trillium* species. *In* J.F. Crow and N. Takahata, eds., The Population Biology of Genes and Molecules, Baifukan (Publisher) Ltd., Tokyo, pp. 139-150.

Fukuda, I. 1990b Evolutionary relationships between the Asian and North American *Trillium* species. Sci. Rep. Tokyo Woman's Christian Univ. **87-89**: 1023-1029.

Fukuda, I. and Channell, R.B. 1975. Distribution and evolutionary significance of chromosome variation in *Trillium ovatum*. Evolution **29**: 257-267.

Fukuda, I. and Grant, W.F. 1980. Chromosome variation and evolution in *Trillium grandiflorum*. Canad. J. Genet. Cytol. **22**: 81-91.

Fukuda, I. and Itou, M. 1996. Genetic mechanisms of *Trillium* speciation in Japan. Genes Genet. Syst. **71**: 430 (abstract).

Fukuda, I., Freeman, J.D. and Itou, M. 1996. *Trillium channellii*, sp. nov. (Trilliaceae), in Japan, and *T. camschatcense* Ker Gawler, correct name for the Asiatic diploid *Trillium*. Novon **6**: 164-171.

Fukuda, I., Wiley, L. and Channell, R.B. 1968. A comparative study of *Trillium ovatum* and *Trillium rivale*. Sci. Rep. Tokyo Womens Christian Univ. 50 Years Anniversary Special Issue, 49-58.

Gates, R.R. 1917. A systematic study of the North American genus *Trillium*, its variability, and its relation to *Paris and Medeola*. Ann. Missouri Bot. Gard. **4**: 43-93.

Haga, T. 1937. Genome and polyploidy in the genus *Trillium* I. Chromosome affinity between the genomes. Jap. J. Genet. **13**: 135-145.

Haga, T. 1951. Genome and polyploidy in the Genus *Trillium* III. Origin of the polyploid species. Cytologia **16**: 243-258.

Haga, T. and Kurabayashi, M. 1954. Genome and polyploidy in the genus *Trillium*. V. Chromosomal variation in natural populations of *Trillium kamtschaticum* Pall. Mem. Fac. Sci. Kyushu Univ. Ser. E. Biol. **1**: 159-185.

Haga, T. and Watanabe, H. 1966. Chromosomes of a Himalayan *Trillium* in relation to the Japanese and American species. Proc. Jap. Acad. **42**: 160-164.

Haga, T., Watanabe, H. and Kanazawa, H. 1974. Hybridizations in natural populations of Japanese *Trilliums*. Jap. J. Genet. **49**: 399-412.

Haga, T. and Channell, R.B. 1982. Three species groups in American *Trillium* as revealed by compatibility with an Asiatic species. Bot. Mag. (Tokyo) **95**: 77-80.

Hara, H., Stearn, W.T. and Williams, L.H. 1978. An enumeration of the flowering plants of Nepal. Vol. 1. Trustees of British Museum, London.

Hickman, J.C. 1993. The Jepson Manual: Higher Plants of California. Univ. California Press, Berkeley.

Hilu, K.W. and Liang, H. 1997. The *matK* gene: sequence variation and application in plant systematics. Amer. J. Bot. **84**: 830-839.

Ihara, M. and Ihara, K. 1978. A biosystematics study on the pedicellate-flowered species of the North American *Trillium*. (1) Geographical distribution of major groups and their gynoecium norms. J. Geobot. **25**: 139-172.

Ihara, K. and Ihara, M. 1982. A biosystematics study on the pedicellate-flowered species of the North Ameican *Trillium*. (2). An interspecific relationship shown by karyotypes and mode of microsporogenesis. J. Phytogeogr. Taxon. **30**: 74-82.

Johnson, L.A. and Soltis, D.E. 1994. *matK* DNA sequences and phylogenetic reconstruction in Saxifragaceae *s. str*. Syst. Bot. **19**: 143-156.

Johnson, L.A. and Soltis, D.E. 1995. Phylogenetic inference in Saxifragaceae *sensu stricto* and *Gilia* (Poleminiaceae) using *matK* sequences. Ann. Missouri. Bot. Gard. **82**: 149-175.

Kato, H., Kawano, S., Terauchi, R., Ohara, M. and Utech, F.H. 1995a. Evolutionary biology of *Trillium* and related genera (Trilliaceae) I. Restriction site mapping and variation of chloroplast DNA and its systematic implications. Pl. Spec. Biol. **10**: 17-29.

Kato, H., Terauchi, R., Utech, F.H and Kawano, S. 1995b. Molecular systematics of the Trilliaceae *sensu lato* as inferred from *rbcL* sequence data. Mol. Phyl. Evol. **4**: 184-193.

Kawano, S. 1994. World *Trillium*: Life History and Evolution (in Japanese). World Pl. Monogr. Ser. **1**, 96 pp.

Kawano, S. and Kato, H. 1995. Evolutionary biology of

Trillium and related genera (Trilliaceae) II. Cladistic analyses on gross morphological characters, and phylogeny and evolution of the genus *Trillium*. Pl. Spec. Biol. **10**: 169-183.

Kawano, S., Ohara, M. and Utech, F.H. 1986. Life history studies on the genus *Trillium* (Liliaceae) II. Reproductive biology and survivorship of four eastern North American species. Pl. Spec. Biol. **1**: 47-58.

Kawano, S., Ohara, M. and Utech, F.H. 1992. Life history studies on the genus *Trillium* (Liliaceae) VI. Life history characteristics of three western North American species and their evolutionary-ecological implications. Pl. Spec. Biol. **7**: 21-36.

Kazempour Osaloo, S. and Kawano, S. 1999. Molecular systematics of Trilliaceae II. Phylogenetic analyses of *Trillium* and its allies using sequences of *rbcL* and *matK* genes of *cp*DNA and internal transcibed spacers (ITS) of 18S-26S nrDNA. Pl. Spec. Biol. **14** (in press).

Kimura, M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. Proc. Natl. Acad. Sci. USA **78**: 454-458.

Kurabayashi, M. 1958. Evolution and variation in Japanese species of *Trillium*. Evolution **12**: 286-310.

Kurabayashi, M., Saho, T., Hiraizumi, Y. and Samejima, K. 1958. Evolution and variation in *Trillium* X. Homological relationships among different genomes of Japanese *Trillium*. Cytologia **23**: 349-355.

Kruckeberg, A.R. 1951. Intraspecific variability in the response of certain native plant species to serpentine soil. Amer. J. Bot. **38**: 208-419.

Kruckeberg, A.R. 1954. The ecology of serpentine soils III. Plant species in relation to serpentine soils. Ecology **35**: 267-274.

Kruckeberg, A.R. 1969a. Soil diversity and the distribution of plants, with examples from western North America. Madroño **20**: 129-154.

Kruckeberg, A.R. 1969b. Plant life on serpentine and other ferroganisian rocks in northwestern North America. Syesis **2**: 15-114.

Lewis, H. 1962. Catastrophic selection as a factor in speciation. Evolution **16**: 257-271.

Li, H.L. 1952. Floristic relationships between eastern Asia and eastern North America. Trans. Amer. Phil. Soc. **42**: 371-429.

Liang, H. and Hilu, K.W. 1996. Application of the *matK* gene sequences to grass systematics. Can. J. Bot. **74**: 125-134.

Mason, H.L. 1946a. The edaphic factor in narrow endemism. I. The nature of environmental influences. Madroño **8**: 209-226.

Mason, H.L. 1946b. The edaphic factor in narrow endemism. II. The geographic occurrence of plants of highly restricted patterns of distribution. Madroño **8**: 209-226.

Neuhaus, H. and Link, G. 1987. The chloroplast tRNA^{LYS} (UUU) gene from mustard. Curr. Genet. **11**: 251-257.

Nixon, E.S. and MacMillan, C. 1964. The role of soil in the distribution of four grass species in Texas. Amer. Midl. Naturalist **71**: 114-140.

Noltie, H.J. 1994. Flora of Bhutan. **3**(1): 20-21. Royal Botanic Garden, Edinburgh.

Ohara, M. 1989. Life history evolution in the genus *Trillium*. Pl. Spec. Biol. **4**: 1-28.

Ohara, M. and Kawano, S. 1986a. Life history studies on the genus *Trillium* (Liliaceae) I. Reproductive biology of four Japanese species. Pl. Spec. Biol. **1**: 35-45.

Ohara, M. and Kawano, S. 1986b. Life history studies on the genus *Trillium* (Liliaceae). IV. Stage class structures and spatial distribution of four Japanese species. Pl. Spec. Biol. **1**: 147-161.

Ohara, M., Kawano, S. and Utech, F.H. 1990. Differentiation patterns of reproductive systems in the genus *Trillium*. Pl. Spec. Biol. **5**: 73-81.

Ohara, M. and Utech, F.H. 1986. Life history studies on the genus *Trillium* (Liliaceae) III. Reproductive biology of six sessile-flowered species occurring in the southeastern United States with special reference to vegetative reproduction. Pl. Spec. Biol. **1**: 135-145.

Ohara, M. and Utech, F.H. 1988. Life history studies on the genus *Trillium* (Liliaceae) V. Reproductive biology and survivorship of three declinate-flowered species. Pl. Spec. Biol. **3**: 35-41.

Ohba, T. 1968. Über die Serpentin-Pflanzengesellschaften der alpinen Stufe Japans. Bull. Kanagawa Pref. Museum **1**: 37-64.

Olmstead, R.G. and Palmer, J.D. 1994. Chloroplast DNA systematics: A review of methods and data analysis. Amer. J. Bot. **81**: 1205-1124.

Ooi, K., Endo, Y., Yokoyama, J. and Murakami, N. 1995. Useful primer designs to amplify DNA fragment of the plastid gene *matK* from angiosperm plants. J. Jap. Bot. **70**: 328-33

Proctor, J. and Woodell, S.R.J. 1975. The Ecology of Serpentine Soils. *In* A. Macfadyen, ed., Advances in Ecological Research, Vol. 9, Academic Press, London.

Raven, P.H. 1964. Catastrophic selection and edaphic endemism. Evolution **18**: 336-338.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406-425.

Samejima, K. and Samejima, J. 1987. *Trillium* Genus. Hokkaido Univ. Press, Sapporo, pp. 238

Soltis D.E., Kuzoff, R.K., Conti, E., Gornall, R. and Ferguson, K. 1996. *matK* and *rbcL* gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. Amer. J. Bot. **83**: 371-382.

Soukup, V.G. 1980. A new Trillium from the northwestern United States. Brittonia **32**: 330-333.

Steele, K.P. and Vilgalys, R. 1994. Phylogenetic analyses of Poleminiaceae using nucleotide sequences of the plastid gene *matK*. Syst. Bot. **19**: 126-142.

Sugita, M., Shinozaki, K. and Sugiura, M. 1985. Tobacco chloroplast tRNA^{LYS} (UUU) gene contains a 2.5 kilobase pairs intron: An open reading frame and a conserved boundary sequence in the intron. Proc. Natl. Acad. Sci. USA **82**: 3557-3561.

Swofford, D.L. 1993. PAUP: Phylogenetic Analysis Using Parsimony, version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign.

Takahashi, M. 1982. Pollen morphology in North American species of *Trillium*. Amer. J. Bot. **69**: 1185-1195.

Takahashi, M. 1983. Pollen morphology in Asiatic species of *Trillium*. Bot. Mag. (Tokyo) **96**: 377-384.

Takahashi, M. 1984. Pollen morphology in *Paris* and its related genera. Bot. Mag. (Tokyo) **97**: 233-245.

Tiffney, B.H. 1985. Perspective on the origin of the floristic similarity between eastern Asia and eastern North America. J. Arnold Arbor. **66**: 73-94.

Utech, F.H. 1980. Chromosome atlas of the vascular plants of western Pennsylvania, I. Ann. Carnegie Mus. **49**: 265-305.

Walker, R.B. 1954. The ecology of serpentine soils II. Factors affecting plant growth on serpentine soils. Ecology **35**: 259-266.

Warmke, H.E. 1937. Cytology of the pacific coast *Trilliums*. Amer. J. Bot. **24**: 376-383.

Wei, Z.-X. 1995. Pollen morphology of *Trillium*. Acta Bot. Yunn. *17*: 317-324.

Whittaker, R.H. The ecology of serpentine soils IV. The vegetational response to serpentine soils. Ecology **35**: 275-288.

Yoshitama, K., Shida, Y., Oyamada, T., Takasaki, N. and Yahara, S. 1997. Flavonol glycosides in the leaves of *Trillium apetalon* Makino and *T. kamtschaticum* Pallas. J. Plant Res. **110**: 443-448.

Zomlefer, W.B. 1996. The Trilliaceae in the southeastern United States. Harvard Papers Bot. **9**: 91-120.

(*Received September 11, 1998; accepted December 25, 1998*)