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Evolutionary Biology of *Trillium* and Related Genera (Trilliaceae) I. Restriction Site Mapping and Variation of Chloroplast DNA and its Systematic Implications

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Abstract **A physical map of chloroplast DNA (*cp*DNA) was constructed for *Trillium kamtschaticum* using three restriction enzymes, *Apa*I, *Pst*I and *Xho*I. The chloroplast genome is 154.5 kbp in size with each portion of the inverted repeat 26.5 kbp in length, separated by large (82.7 kbp) and small (18.8 kbp) single copy regions. The *cp*DNA of *T. kamtschaticum* is similar in structure to the *cp*DNAs of most angiosperms thus far studied. Chloroplast DNA variation in 25 taxa of *Trillium* was investigated using 14 restriction enzymes, with *Paris tetraphylla* as an outgroup. Phylogenetic analysis showed that sessile-flowered species (subgenus *Phyllantherum*) form a monophyletic group. Pedicellate-flowered species do not form a monophyletic group, and belong to at least three different groups. *T. kamtschaticum*, an East Asian diploid species, was found to be most closely related to a group of species from eastern North America.**

Key words: **Arcto-Tertiary element, *cp*DNA, phylogeny, RFLP-analysis, Trilliaceae, *Trillium*.**

The genus *Trillium* L. [Liliaceae *sensu* Krause (1930) or Trilliaceae *sensu* Dahlgren et al. (1985)] is distributed in temperate or boreal woodlands in the Northern Hemisphere. The genus comprises about 46 perennial herbaceous species and occupies three disjunct regions: eastern Asia (about ten species), eastern North America (29 species) and western North America (seven species). This distribution indicates a so-called Arcto-Tertiary origin (Gray, 1846, 1860; Li, 1952; Axelrod, 1966; Graham, 1972; Tiffney, 1985; Samejima and Samejima, 1987; Ohara, 1989). The genus has been divided into two subgroups, the pedicellate-flowered group (subgenus *Trillium*) and the sessile-flowered group (subgenus *Phyllantherum*) (Freeman, 1975). Evolutionary divergence within the genus *Trillium* has been investigated extensively in studies of gross morphology (Berg, 1958; Freeman, 1975; Ihara and Ihara, 1978; Samejima and Samejima, 1962, 1987), palynology (Takahashi, 1982, 1983, 1984), genome constitutions and chromosomal variations as revealed by hetero-chromatin banding patterns (Haga and Kurabayashi, 1954; Kurabayashi, 1958; Fukuda and Channel, 1975; Fukuda and Grant, 1980; Dyer, 1964a, b, c; Utech, 1980), artificial hybridization (Haga and Channell, 1982), and life history characteristics (Kawano et al., 1986, 1992; Ohara, 1989; Ohara and Kawano, 1986a, b; Ohara and Utech, 1986, 1988).

In order to elucidate the phylogeny of *Trillium* more rigorously, we chose to analyze chloroplast DNA (*cp*DNA) variation within the genus and its relatives. In recent years, *cp*DNA analysis has proven to be useful for resolving phylogenetic relationships among species and genera in many different angiosperm families (Palmer et al., 1988; Soltis et al., 1992).

To begin the analysis of *cp*DNA in *Trillium*, we estimated the size of the *cp*DNA molecule in *T. kamtschaticum* which is a sole diploid species in Asia, and then prepared a physical map by restriction enzyme analysis. This provided a foundation for an extensive survey of restriction fragment length polymorphisms (RFLPs) in 24 *Trillium* species from North America, one *Trillium* species from Japan, and one outgroup species, *Paris tetraphylla*, which proved to be one of the most closely related genera according to our recent molecular analyses of *rbc*L gene of the Trilliaceae (Kato et al., in press). By analyzing the presence or absence of restriction sites within species, we were able to construct a rooted phylogenetic tree for *Trillium*. This tree was then compared to the results of cladistic analyses based on the morphological characters for most of the presently known *Trillium* species (Kawano and Kato, in preparation). On the basis of these results, we could assess previous proposals regarding divergence within the genus and discuss its supposed Arcto-Tertiary origin.

Materials and Methods

1. *cpDNA Purification, Size Estimation and Physical Map Construction*

Fresh leaves of *T. kamtschaticum* were collected from a wild population near Chitose City, Hokkaido, in spring of 1992. Chloroplast DNA was isolated from fresh green leaf tissues following the method of Hirai et al. (1985). About 25 g of fresh leaf tissue were blended in liquid nitrogen. After the liquid nitrogen evaporated completely, the powder was suspended in 500 ml of Buffer A (50 mM Tris-HCl, pH 8.0; 0.35 M sucrose; 7 mM EDTA; 5 mM 2-mercaptoethanol) containing 0.1% bovine serum albumin. The liquid suspension was filtered through two layers of cheese cloth, and then two layers of Miracloth (Calbiochem). The filtrate was centrifuged at 1000×g for 10 min. The pellet was resuspended in 5 ml of Buffer A. The second resuspension was loaded on top of a stepwise 20–45–60% sucrose gradient made in 50 mM Tris-HCl, pH 8.0, 0.3 M sorbitol, 7 mM EDTA, and centrifuged at 7000×g for 30 min. The green band at the interphase between 20 and 45% sucrose was collected, diluted in a 1:3 ratio with Buffer B (50 mM Tris-HCl, pH 8.0; 20 mM EDTA), and centrifuged at 3000×g for 10 min. The chloroplast pellet was resuspended in 3 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), 1 ml of 10% sodium dodecylsarcosinate (in TE buffer) and 0.1 ml of 10 mg/ml Proteinase K. The mixture was incubated for more than 1 hr at 37°C. Chloroplast DNA was purified from the mixture by CsCl equilibrium density gradient centrifugation with ethidium bromide.

Purified *cp*DNAs were digested with three restriction enzymes *Apa*I, *Pst*I and *Xho*I, singly and in combination. The sizes of the resulting fragments were determined by electrophoresis in 0.5%, 0.7% and 1.5% agarose gels.

Table 1. Sources of DNA from 24 *Trillium* species and *Paris tetraphylla*.

DNA no.	Taxon	Chromosome no.*	Collection site	Collector
1.	*Trillium kamtschaticum* Pallas	10	Japan; Hokkaido; Samani-cho	H. Kato
2.	*T. erectum* L. var. *erectum*	10	Pennsylvania; Westmoreland Co.	S. Kawano et al.
3.	var. *album* (Michaux) Pursh	10	Tennessee; Sevier Co.	M. Ohara et al.
4.	*T. sulcatum* Patrick	10	North Carolina; Burke Co.	S. Kawano et al.
5.	*T. cernuum* L.	10	Wisconsin; Iron Co.	S. Kawano et al.
6.	*T. rugelii* Rendle	10	Cultivated in the Botanical Garden of Hokkaido Univ. (origin unknown)	Unknown
7.	*T. undulatum* Willdenow	10	Pennsylvania; Westmoreland Co.	S. Kawano et al.
8.	*T. grandiflorum* (Michaux) Salisbury	10	Pennsylvania; Westmoreland Co.	S. Kawano et al.
9.	*T. ovatum* Pursh	10	California; Del Norte Co.	S. Kawano et al.
10.	*T. rivale* S. Watson	10	California; Del Norte Co.	S. Kawano et al.
11.	*T. recurvatum* Beck	10	Arkansas; Newton Co.	M. Ohara et al.
12.	*T. lancifolium* Rafin	10	South Carolina; McCormick Co.	M. Ohara et al.
13.	*T. sessile* L.	10	Pennsylvania; Westmoreland Co.	S. Kawano et al.
14.	*T. decipiens* Freeman	10	Florida; Jackson Co.	M. Ohara et al.
15a.	*T. reliquum* Freeman	10	Georgia; Columbia Co.	M. Ohara et al.
15b.	*T. reliquum* Freeman	10	Georgia; Early Co.	M. Ohara et al.
16.	*T. stamineum* Harbison	10	Mississippi; Lauderdale Co.	M. Ohara et al.
17.	*T. maculatum* Rafin.	10	Georgia; Early Co.	M. Ohara et al.
18.	*T. foetidissimum* Freeman	10	Louisiana; West Feliciana Parish	M. Ohara et al.
19.	*T. luteum* (Muhlenberg) Harbison	10	North Carolina; Graham Co.	M. Ohara et al.
20.	*T. viride* Beck	10	Missouri; Washington Co.	M. Ohara et al.
21.	*T. viridescens* Nuttall	10	Arkansas; Washington Co.	S. Kawano et al.
22.	*T. petiolatum* Pursh	10	Washington; Chelan Co.	M. Ohara et al.
23.	*T. albidum* Freeman	10	California; Sonoma Co.	M. Ohara et al.
24.	*T. chloropetalum* (Torrey) Howell	10	California; Santa Cruz Co.	M. Ohara et al.
25.	*T. kurabayashii* Freeman	10	California; Del Norte Co.	M. Ohara et al.
26.	*Paris tetraphylla* A. Gray	10	Japan; Hokkaido; Mt. Hakodate-yama	H. Kato

* Samejima and Samejima(1987), Hara(1969).

The restriction fragments were transferred to a nylon membrane (Hybond-N, Amersham) from agarose gels. Cloned tobacco *cp*DNA fragments (kindly supplied by M. Sugiura, Nagoya University; Sugiura et al., 1986) were used as probes for physical mapping (The position of probes are shown in Fig. 5.). The probes were labeled and detected using DIG labeling kit and DIG luminescent detection kit (Boehringer Mannheim).

2. *Total DNA Extraction and RFLP Analysis*

Total DNAs were extracted from 25 species of *Trillium* and one species of *Paris* (Table 1). *Paris tetraphylla* A. Gray was used as the outgroup for the phylogeny reconstruction because this species was paraphyletic with several species of *Trillium, Kinugasa* and *Daiswa* based on our *rbc*L sequence analysis (Kato et al., in press). Fresh samples were obtained from field populations and from living plants in collections at Kyoto University and Hokkaido University. Voucher specimens for the plants within each population sampled are all deposited in the Herbarium at the Department of Botany, Faculty of Science, Kyoto University (KYO).

Total DNA extraction was performed using the method described by Shinwari et al. (1994). Extracted DNAs were digested with 14 restriction enzymes (*Apa*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sca*I, *Xba*I, *Xho*I). Agarose gel electrophoresis, bi-directional transfer of DNA fragments from the gels to nylon membranes, and southern hybridization with cloned *cp*DNA of tobacco were performed as described above.

Phylogenetic analysis was performed using PAUP version 3.1.1 (Swofford, 1993) to find the most parsimonious trees; both Wagner parsimony and Dollo parsimony were employed. A bootstrap analysis was also conducted with 100 replicates (Felsenstein, 1985).

Results

1. T. kamtschaticum cp*DNA: Genome Size and Structure*

Restriction fragments from the purified *cp*DNA of *T. kamtschaticum*, digested with three restriction enzymes *Apa*I, *Pst*I and *Xho*I, are shown in Fig. 1. Estimates for the size of each restriction fragment are shown in Table 2. Estimates for size of the total chloroplast genome ranged from 151.5 kbp to 153.3 kbp, with a mean of 152.3 kbp (Table 2).

The results of Southern hybridization of *T. kamtschaticum cp*DNA with tobacco probes are summarized in Table 3, and an example is given in Fig. 2. In this experiment, the tobacco probe B10 (9.4 kbp) was hybridized to *T. kamtschaticum cp*DNA digested with *Apa*I, *Pst*I and *Xho*I singly and in combination. The positions of restriction sites within and around the probed DNA region (Fig. 3) were inferred unambiguously from the hybridization results. Consecutive hybridizations with 19 tobacco *cp*DNA probes allowed us to construct a circular physical map for the *cp*DNA of *T. kamtschaticum* (Fig. 4). The arrangement of *T. kamtschaticum cp*DNA was found to be colinear with that of tobacco (Fig. 5).

Southern hybridization with tobacco probes from the inverted repeat (IR) revealed that *T. kamtschaticum* has IRs which are each approximately 26.5 kbp in length. The large single copy (LSC) and the small single copy (SSC) regions were estimated to be about 82.7 kbp and 18.8 kbp in size, respectively. Consequently, the total size of the chloroplast genome was about 154.5 kbp. This estimate is very close to the average estimate (152.3 kbp) based on the restriction fragments produced by single enzyme digestion (Table 2).

2. *Restriction Site Variation and Phylogenetic Analyses*

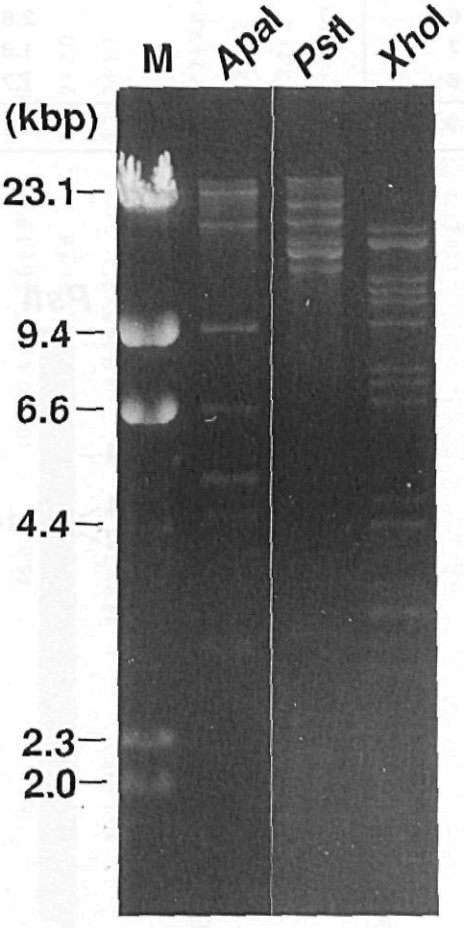

Fig. 1. Restriction patterns of *Trillium kamtschaticum cp*DNA generated by digestion with *Apa*I, *Pst*I and *Xho*I. M = lambda DNA digested with *Hin*dIII.

Table 2. Molecular size (in kbp) of restriction fragments of *T. kamtschaticum cp*DNA generated by digestion with *Apa*I, *Pst*I and *Xho*I.

No.	*Apa*I	*Pst*I	*Xho*I
1	50.0	45.0	18.0
2	35.0	23.0	16.0×2
3	20.0	20.0	11.5
4	9.5×2	16.0	11.0
5	7.0	14.5×2	10.5
6	5.0×2	13.0	9.5
7	4.5	3.2	8.0
8	3.0×2	2.1	7.6
9		2.0	7.0
10			4.7
11			4.5×2
12			3.7
13			3.5
14			3.4×2
15			3.1
16			2.8
17			1.8
18			1.7
Total (kbp)	151.5	153.3	152.2

The survey of Southern hybridization for 25 *Trillium* species and *Paris tetraphylla* revealed a total of 41 restriction site mutations (Figs. 6 and 7; Table 4). There were also several small fragment size differences which could not be attributed to specific restriction site mutations. These differences are probably the results of DNA deletions or insertions. Our phylogenetic analyses were based on 41 restriction site changes. The presence or absence of each mutation is given for all the taxa tested (Table 5). Twenty-one restriction site mutations were shared by two or more taxa and were therefore potentially informative for phylogenetic analysis. The remaining 20 mutations were found in only one species (autapomorphic mutations). DNAs from two different populations of *T. reliquum* (DNA nos. 15a and 15b) were the same.

Wagner parsimony analysis of the data in Table 5 revealed a single most parsimonious tree of 43 steps, including four homoplastic mutations (Fig. 8) (23 steps when autapomorphic characters are excluded). The consistency index for this tree is 0.95 (0.91 when autapomorphic mutations are excluded). All homoplastic characters had a consistency index of 0.5. Dollo parsimony analysis resulted in two equally parsimonious trees with 44 steps (data not shown). The positions of *T. undulatum* and *T. rivale* were different in the trees produced by Wagner and Dollo analyses. Bootstrap values for the Wagner analysis are shown in

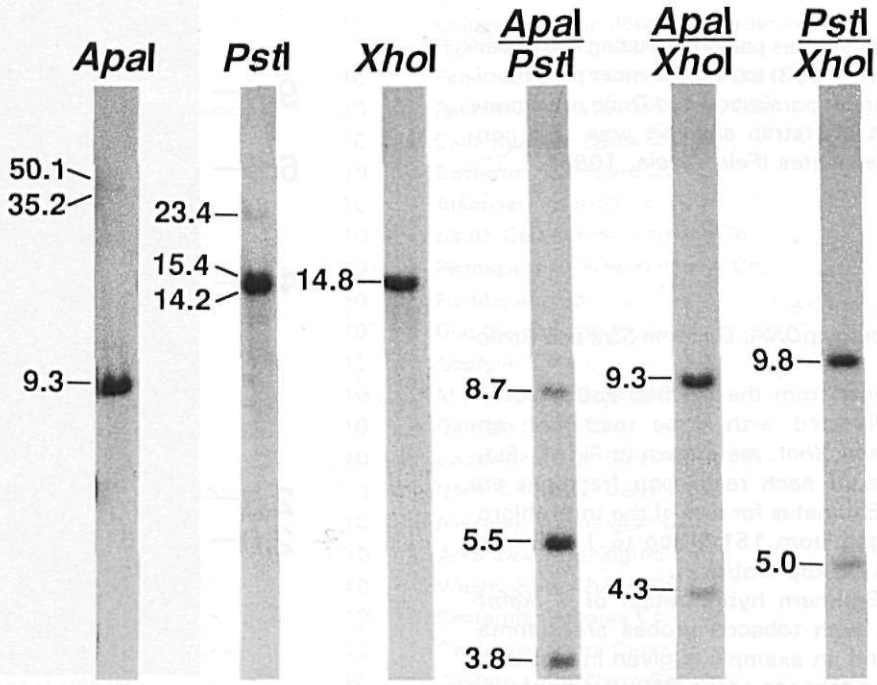

Fig. 2. Hybridization of the cloned tobacco B10 (Ba14–6-22, see Fig. 5) fragment as the probe to *T. kamtschaticum cp*DNA digested with *Apa*I, *Pst*I or *Xho*I singly, or in their combination. Size of each fragment is indicated on the left side of the lane.

Table 3. Restriction fragments of *T. kamtschaticum cp*DNA (size in kbp) detected by the tobacco *cp*DNA probes. The locations of the tobacco probes in chloroplast genome are shown in Fig. 5.

Tobacco *cp*DNA probe*	*Apa*I	*Pst*I	*Xho*I	*Apa*I/*Pst*I	*Apa*I/*Xho*I	*Pst*I/*Xho*I
Ba15	35.2	12.8	4.9	12.8	4.9	3.9
Ba10a	35.2	12.8	11.4	12.8	11.4	8.4
Ba4	35.2	12.8+19.3	11.4	12.8+12.6	11.4	8.4+3.0
Ba13-12a	35.2	19.3	3.1+1.8+3.4	12.6	3.1+1.8+3.4	3.4+3.1+1.8
Ba10b	35.2+6.7	19.3	6.8	12.6+6.7	5.5+1.3	6.8
Ba9a-9b	6.7+4.7+1.5+1.3+0.8	45.6+19.3+2.1	7.1+6.8	6.7+1.5+1.3+0.8	5.5+2.3+1.5+1.3+1.2+0.8	6.8+3.8+2.1+1.2
Ba16	4.7	45.6	10.3	4.7	2.4	10.3
Ba3	50.1	45.6	10.3+1.7	39.4	7.9+1.7	10.3+1.7
Ba17	50.1	45.6	16.5	39.4	16.5	16.5
Ba18-22c	50.1	45.6	16.5	39.4	16.5	16.5
Ba12b-20	50.1	45.6	16.5	39.4	16.5	16.5
Ba1	50.1	45.6	16.5+7.4+3.5+2.8+1.5	39.4	16.5+7.4+3.5+2.8+1.5	16.5+7.4+3.5+1.5+0.9
Ba7-23-24	50.1+35.2	14.2×2+2.0	14.8×2+4.5×2+2.8	8.7×2+2.0	4.5×2+4.3×2+2.8	9.8×2+4.4×2+1.9
Ba8	50.1+35.2	14.2×2+1.1	4.9+4.5×2	8.7×2+1.1	4.9+4.5×2	4.4×2+3.9+1.0
Ba14	50.1+35.2	14.2×2	14.8×2	8.7×2	4.3×2	9.8×2
Ba6-14-22	50.1+35.2+9.3×2	23.4+15.4+14.2×2	14.8×2	8.7×2+5.5×2+3.8×2	9.3×2+4.3×2	9.8×2+5.0×2
Ba11	9.3×2+5.3×2	23.4+15.4	14.8×2	5.3×2+3.8×2	9.3×2+1.2×2	5.0×2
Ba5	18.8+5.3×2+3.1×2	23.4+15.4	9.3+3.5×2+3.4+0.8×2	5.3×2+3.1×2	3.5×2+2.9×2+0.6×2	10.7+6.1+3.5×2+3.4+0.8×2
Ba2	18.8	23.4+15.4+3.1+1.3	11.9+9.3	11.2+3.1×2+1.3	12.4+6.4	10.7+6.1+3.1+1.2

* Sugiura et al. (1986).

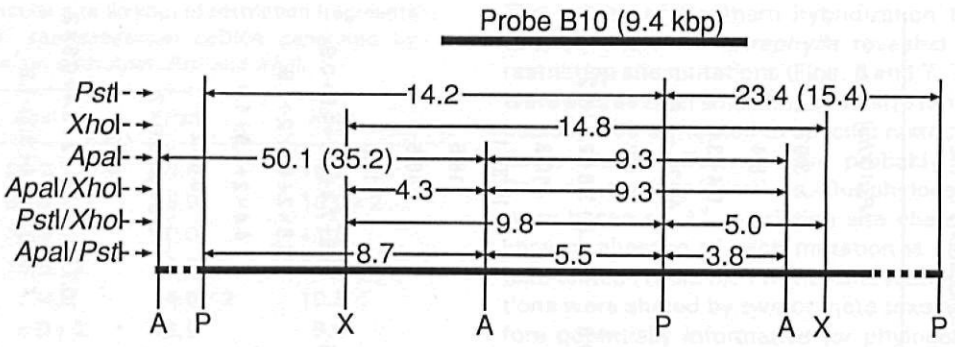

Fig. 3. Part of the physical map of *Trillium kamtschaticum cp*DNA, inferred from the result of Southern hybridization (see Fig. 2). The size of restriction fragment is shown. Restriction sites are shown for *Apa*I (A), *Pst*I (P) and *Xho*I (X). Fragment length is given in kbp. This region corresponds to the inverted repeats, so that double or two different sized (in parenthesis) fragments were observed.

Fig. 8.

Monophyly of sessile-flowered species was supported with a bootstrap value of 99%. Among the sessile group species, two less strongly supported clades are evident: (1) *T. decipiens* and *T. reliquum* (supported by a 65% bootstrap value) and (2) all other sessile-flowered species (61% bootstrap value).

Monophyly of *T. erectum, T. sulcatum, T. cernuum* and *T. rugelii* was supported (96% bootstrap value). Furthermore, these species formed a well supported clade with the eastern Asiatic species, *T. kamtschaticum* (98% bootstrap value). *T. grandiflorum* and *T. ovatum* were distinguished by six and three autapomorphic site changes, respectively, and appear separate from other pedicellate-flowered species. Two other pedicellate-flowered species, *T. undulatum* and *T. rivale,* were also distinguished by autapomorphic site changes, and one of the site changes was identical with that of *Paris tetraphylla*, another Trilliaceae genus (Fig. 6). These two *Trillium* species diverge at the root of the tree, close to the outgroup species, *P. tetraphylla.*

Discussion

1. *Comparison of Chloroplast Genome Structure*

The plant chloroplast genome is a circular DNA molecule typically between 120 kbp and 210 kbp in size. Its structure has been studied for many wild as well as cultivated plants (cf. Downie and Palmer, 1992). The

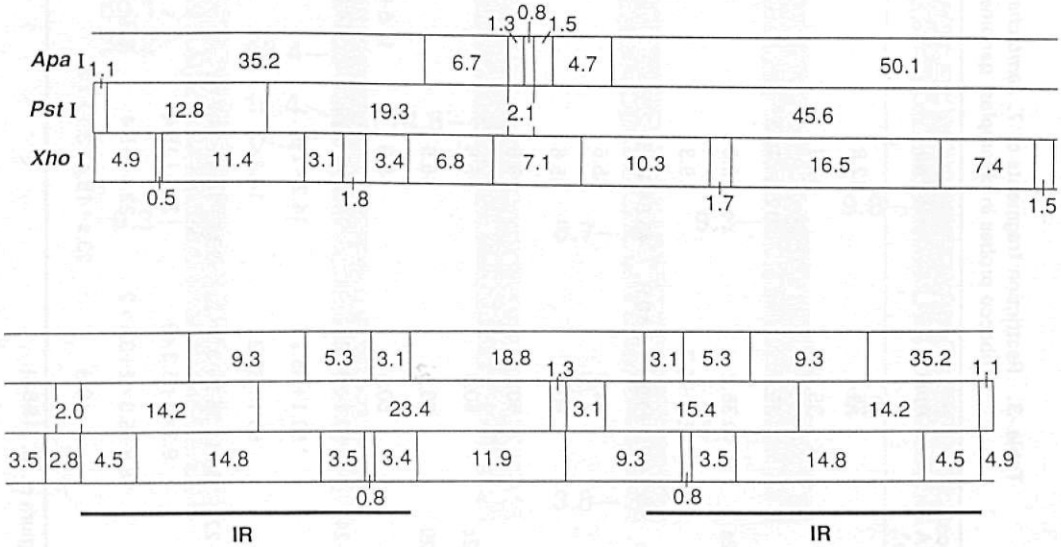

Fig. 4. Rectriction site map of the chloroplast genome of *Trillium kamtschaticum*. Numbers indicate fragment length in kbp. IR=inverted repeat.

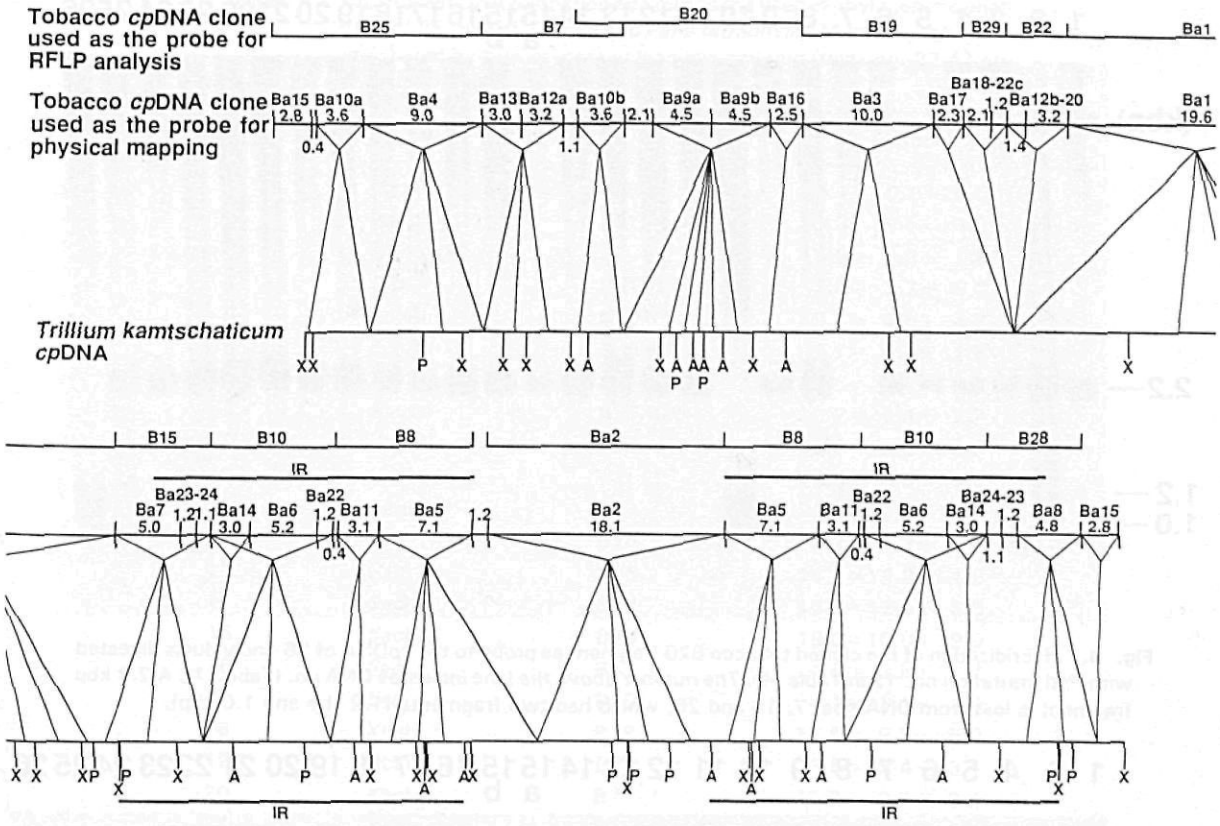

Fig. 5. Arrangement of homologous sequences between *Trillium kamtschaticum* and tobacco *cp*DNAs. *T. kamtschaticum cp*DNA fragments hybridized to each tobacco probe are indicated by vertical lines. IR indicate the inverted repeats. The symbols for restriction sites are as follows: A, *Apa*I, P, *Pst*I, X, *Xho*I. The top line indicates the position of tobacco probes, each of which consists of several small clones, used for RFLP analysis of 24 *Trillium* species.

results of these studies have shown that analysis of variation in the restriction enzyme cleavage sites of *cp*DNA is a powerful tool for the elucidation of phylogenetic relationships in flowering plants, particularly among congeneric species and closely related genera (Palmer et al., 1988).

The results obtained in this study indicate that the chloroplast genome of *T. kamtschaticum* is similar in size and structure to the chloroplast genomes of tobacco (Shinozaki et al., 1986), yam (Terauchi et al., 1989), daffodil (Hansmann, 1987), onion (Chase and Palmer, 1989; Katayama et al., 1991), asparagus (Chase and Palmer, 1989), *Narcissus* (Chase and Palmer, 1989) and orchid (Chase and Palmer, 1989). The arrangement of *T. kamtschaticum cp*DNA was colinear with that of tobacco (Fig. 5). The gene order in tobacco *cp*DNA is the most common order in angiosperms so far examined (Palmer et al., 1988). In contrast to this common order, three major inversions have been found in the large single copy region of *cp*DNAs of various graminaceous plants: *Oryza sativa* (Hirai et al., 1985; Kanno and Hirai, 1992; Kanno et al., 1993), *Triticum aestivum* (Quigley and Weil, 1985; Howe et al., 1988) and *Zea mays* (Palmer and Thompson, 1982). These inversions are lacking in the other monocotyledonous plants as mentioned above (Chase and Palmer, 1989; Katayama et al., 1991; Downie and Palmer, 1992).

Because the tobacco *cp*DNA is colinear with *Trillium cp*DNA, the heterologous tobacco probes were very effective for analyzing *Trillium cp*DNAs; individual probes did not hybridize to more than one location within the target *cp*DNAs (cf. Downie and Palmer, 1992).

2. *Evolutionary Divergence within the Genus* Trillium

The present molecular analysis provided a very interesting but quite unexpected picture of phylogenetic relationships among *Trillium* species (Fig. 8). Pedicellate-flowered species within the genus did not form a distinct monophyletic group (Fig. 8). Most *Trillium* species (all but *T. undulatum* and *T. rivale*) examined belonged to one weakly supported clade (68% boot-

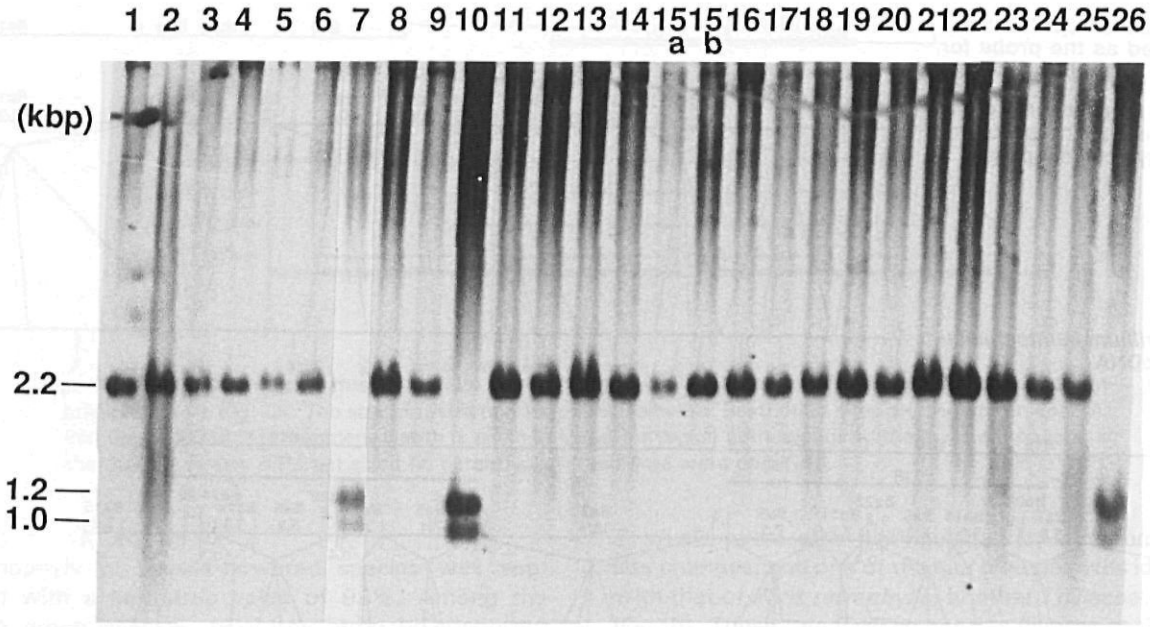

Fig. 6. Hybridization of the cloned tobacco B20 fragment as probe to the *cp*DNA of 26 individuals digested with *Pst*I (mutation no. 12 in Table 4). The number above the lane indicates DNA no. (Table 1). A 2.2 kbp fragment is lost from DNA nos. 7, 10 and 26, which had two fragments (1.2 kbp and 1.0 kbp).

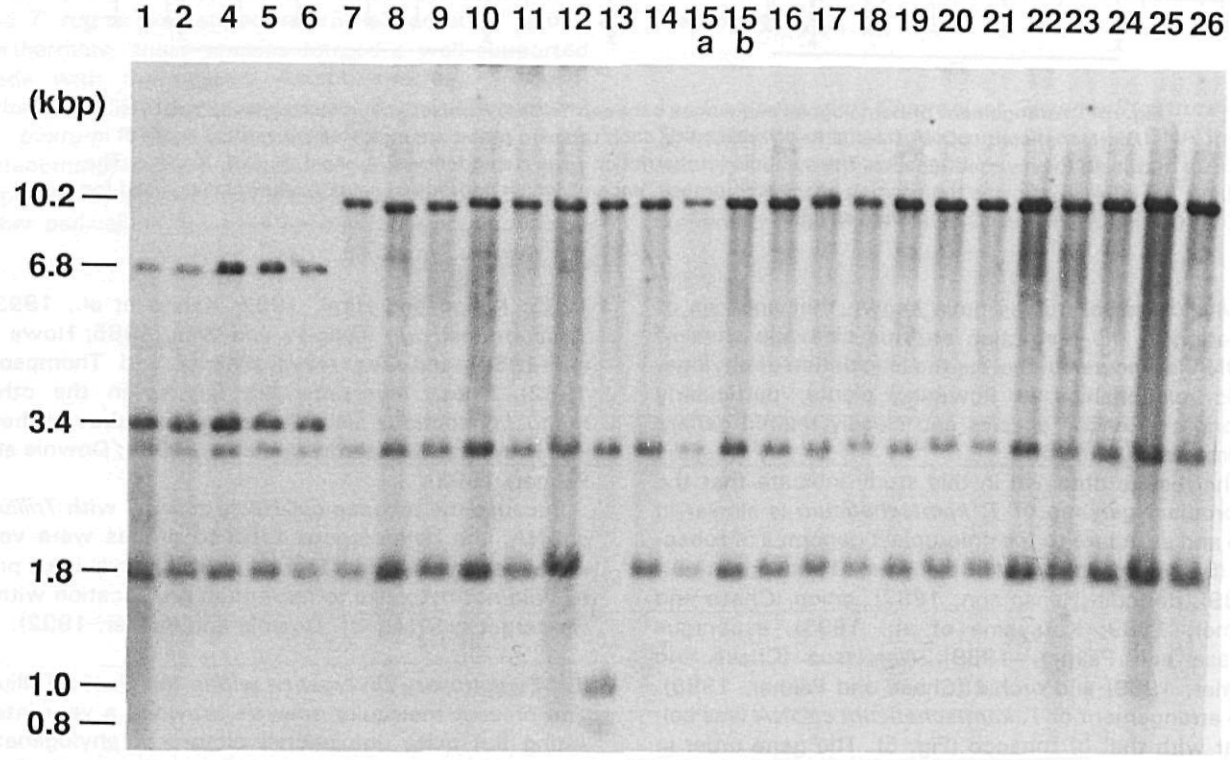

Fig. 7. Hybridization of the cloned tobacco B7 fragment as probe to the *cp*DNA of 25 individuals digested with *Xho*I (mutation no. 20 in Table 4). The number above the lane indicates DNA no. (Table 1). A 10.2 kbp fragment in DNA nos. 7–26 is lost for DNA nos. 1–6. For the latters two fragments (6.8 kbp and 3.4 kbp) were instead observed. A 1.8 kbp fragment is lost from DNA no. 13, which had two fragments (1.0 kbp and 0.8 kbp).

Table 4. Chloroplast DNA restriction site mutations used in phylogenetic analyses of 25 taxa of *Trillium* and *Paris tetraphylla*. Mutations no. 1-21 are shared by two or more individuals and mutations no. 22-41 are autapomorphic.

Mutation no.	Enzyme	Region (tobacco fragment)	Mutation (kbp)
1	*Apa*I	Ba5,B28	50.1=30.1+20.0
2	*Bam*HI	Ba1	13.3= 8.5+ 4.8
3	*Dra*I	B7	5.6= 3.8+ 1.8
4	*Dra*I	Ba1	7.0= 3.7+ 3.3
5	*Eco*RI	B19	8.8= 4.5+ 4.3
6	*Eco*RV	B25-B7	7.7= 4.9+ 2.8
7	*Eco*RV	Ba1	10.5= 8.7+ 1.8
8	*Hin*dIII	B28-B25	10.2= 7.4+ 2.8
9	*Kpn*I	B20	32.0=23.5+ 8.5
10	*Kpn*I	Ba1	14.5= 9.5+ 5.0
11	*Kpn*I	B8	30.0=29.3+ 0.7
12	*Pst*I	B20	2.2= 1.2+ 1.0
13	*Sac*I	B25	14.5=13.2+ 1.3
14	*Sac*I	B19	16.0=12.5+ 3.5
15	*Sac*I	B10	19.0=10.0+ 9.0
16	*Sac*I	B8	4.5= 2.8+ 1.7
17	*Sca*I	B20	4.1= 3.1+ 1.0
18	*Xba*I	B19	15.5= 9.5+ 6.0
19	*Xba*I	Ba1	6.5= 3.4+ 3.1
20	*Xho*I	B7	10.2= 6.8+ 3.4
21	*Xho*I	B15	19.3=14.8+ 4.5
22	*Bam*HI	Ba1	19.1=13.3+ 5.8
23	*Bam*HI	Ba1	8.5= 4.2+ 2.5+1.8
24	*Bam*HI	Ba1	4.8= 3.2+ 1.6
25	*Bgl*II	B25	7.4= 4.2+ 3.2
26	*Eco*RV	B25	5.9= 3.1+ 2.8
27	*Eco*RV	B28	4.5= 3.2+ 1.3
28	*Hin*dIII	B25	10.2= 8.7+ 1.5
29	*Hin*dIII	B7	20.6=11.0+ 9.6
30	*Kpn*I	B20	23.5=13.5+10.0
31	*Kpn*I	B19	5.0= 2.8+ 2.2
32	*Kpn*I	B8	7.0= 6.0+ 1.0
33	*Sac*I	B25	14.5=11.0+ 3.5
34	*Sac*I	Ba1	17.7=12.5+ 5.2
35	*Xba*I	B7-B20	6.7= 4.4+ 1.2
36	*Xba*I	B20	17.7=12.8+ 4.9
37	*Xba*I	B19	15.5=14.3+ 1.2
38	*Xba*I	B29-B22	6.0= 3.5+ 2.5
39	*Xho*I	B7	1.8= 1.0+ 0.8
40	*Xho*I	B15	4.5= 3.1+ 1.4
41	*Xho*I	B10	14.8= 9.9+ 4.9

strap value), consisting of members of the pedicellate-flowered species and all of the sessile-flowered species, with *Paris tetraphylla* as an outgroup. It was rather unexpected that *T. undulatum* and *T. rivale* would be paraphyletic to the above mentioned large clade. The pedicellate-flowered species, *T. gran-*

Table 5. Data matrix representing 41 *cp*DNA restriction site mutations in *Trillium* and *Paris tetraphylla*. Presence and absence of restriction sites are indicated by "1" and "0", respectively.

DNA no.	Taxon	Mutation no. 1 1234567890	1111111112 1234567890	2222222223 1234567890	3333333334 1234567890	4 1
1.	*Trillium kamtschaticum*	0111010000	1010000001	1100010010	1000100000	0
2.	*T. erectum* var. *erectum*	0101010100	1010001011	1101010010	1000100000	0
3.	*T. erectum* var. *album*	0101010100	1010001011	1100010010	1000100000	0
4.	*T. sulcatum*	0101010100	1010001011	1100010010	1000100000	0
5.	*T. cernuum*	0111010100	1010001011	1100010010	1000100000	0
6.	*T. rugelii*	0111010100	1010001011	1100010010	1000100000	0
7.	*T. undulatum*	1110010010	1100000000	1100010011	1000110000	0
8.	*T. grandiflorum*	1110010010	1000000100	1110001010	0100100000	1
9.	*T. ovatum*	1110010010	1000010100	1100110110	1001100000	0
10.	*T. rivale*	1110010010	1100000010	1100010010	1000000000	0
11.	*T. recurvatum*	1010100011	0001010100	1000010010	1000100000	0
12.	*T. lancifolium*	1010100011	0001010000	1100010010	1000100000	0
13.	*T. sessile*	1010100010	0001010100	1100010010	1010100010	0
14.	*T. decipiens*	1010110010	0001110100	1100010010	1000100001	0
15a,b	*T. reliquum*	1010110010	0001110100	1100010010	1000100100	0
16.	*T. stamineum*	1010100010	0001010100	1100010000	1000100000	0
17.	*T. maculatum*	1010100010	0001010100	0100010010	1000100000	0
18.	*T. foetidissimum*	1010100010	0001010100	0100010010	1000100000	0
19.	*T. luteum*	1010100010	0001010100	0100010010	1000100000	0
20.	*T. viride*	1010100010	0001010100	0100010010	1000100000	0
21.	*T. viridescens*	1010100010	0001010100	0100010010	1000100000	0
22.	*T. petiolatum*	1010100010	0001010100	1100010010	1000100000	0
23.	*T. albidum*	1010100010	0001010100	1100010010	1000100000	0
24.	*T. chloropetalum*	1010100010	0001010100	1100010010	1000100000	0
25.	*T. kurabayashii*	1010100010	0001010100	1100010010	1000100000	0
26.	*Paris tetraphylla*	1110011010	1100000010	1100010010	1000101000	0

diflorum and *T. ovatum*, are paraphyletic to the whole sessile-flowered species, and each of the two species has many autapomorphic site changes. It is clear that the sessile-flowered species form a monophyletic group within the genus. This grouping was supported by a very high bootstrap value (99%, Fig. 8). Thus, there is good molecular support for recognizing subgenus *Phyllantherum* (the sessile-flowered species) as a distinct subgrouping.

Berg (1958) suggested that the sessile-flowered species (subgenus *Phyllantherum*) are monophyletic because of their fruit and pollen structure, and also other morphological similarities. It is also noteworthy that there is little *cp*DNA variation among the sessile-flowered species, indicating their higher affinities. In contrast, there is much higher *cp*DNA variation among pedicellate-flowered species, suggesting their polyphyletic origins, just as seen in the cladistic analyses of gross morphological characters (Kawano and Kato, in preparation).

We have recently sequenced the chloroplast *rbc*L gene from a wide range of species and genera in the Trilliaceae (Kato et al., in press). The *rbc*L gene is highly conserved in plants generally, and we were unable to resolve the phylogeny of species within the subgenera *Trillium* and *Phyllantherum*. The genera surveyed were *Trillium, Paris, Daiswa,* and *Kinugasa*; which represent the Trilliaceae *sensu* Dahlgren et al. (1985). From the *rbc*L data, it was evident that these four genera are very closely related phylogenetically, although their exact phylogeny was unable to be determined.

From comparisons of pollen morphology, Takahashi (1982) suggested that the sessile-flowered species are closely related to the pedicellate-flowered species, *T. erectum* and *T. cernuum*. All of these species have granulate pollen grains (with the exception of *T. petiolatum* which has corrugate-type pollen). Our *cp*DNA data, however, indicated that the closest relatives of the sessile-flowered species are the pedicellate-flowered species, *T. ovatum* and *T. grandiflorum* (Fig. 8). These two species have spinulate pollen grains (Takahashi, 1982) and distinct life history traits among

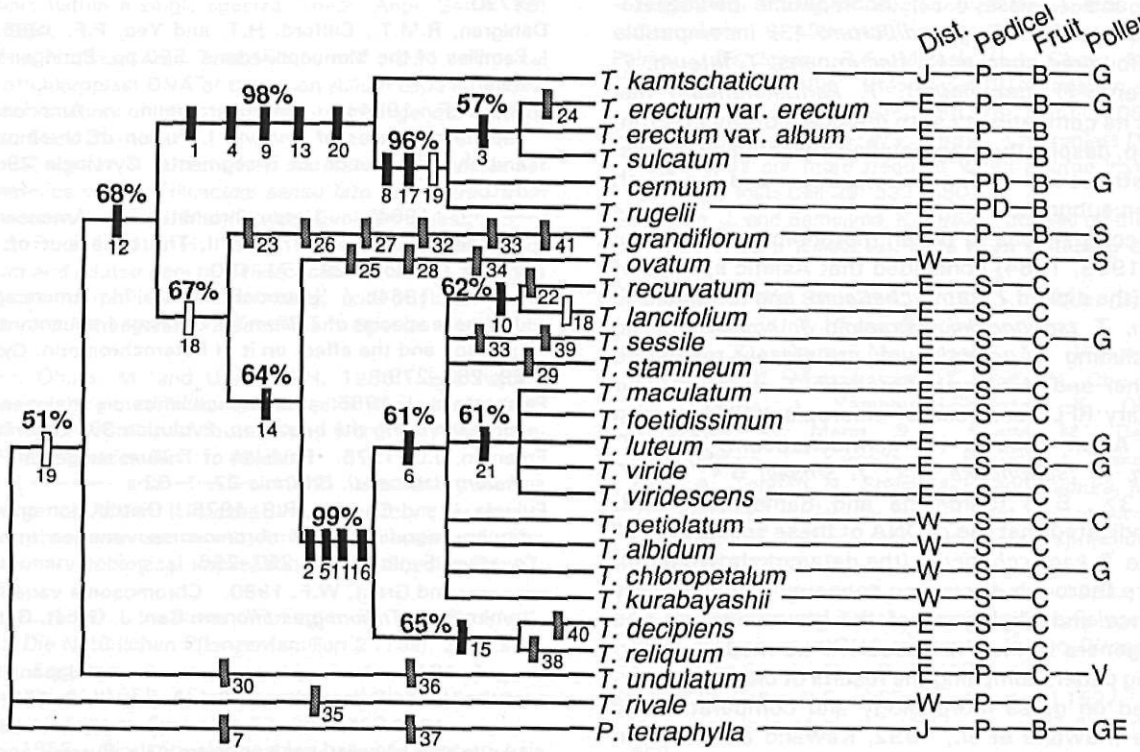

Fig. 8. Wagner parsimony tree constructed by the PAUP computer program. Consistency index (CI)= 0.913; retention index (RI)=0.983, both statistics exclude autapomorphies. Site changes and confidence values (%) for each nodes from bootstrap analysis (100 replications) are shown. Black bars represent the site changes shared by two or more DNAs. Open bars represent homoplastic site changes. Grey bars represent autapomorphic site changes. Geographical distributions and selected morphological characters are noted. Abbreviations: distribution (J, Japan; E,eastern North America; W, western North America), pedicel (P, pedicellate-erect; PD, pedicellate-declinate; S, sessile), fruit (B, berry; C, capsule), pollen (C, corrugate; G, granulate; V, verrucate; S, spinulate; GE, gemmate). Morphological data from Berg (1958), Smejima and Samejima (1987) and Takahashi (1982, 1983, 1984).

the pedicellate-flowered species (Kawano et al., 1992).

Among other pedicellate-flowered species, the species, *T. erectum, T. sulcatum, T. cernuum, T. rugelii* (all from eastern North America) and *T. kamtschaticum* (from East Asia), form a very well supported clade (Fig. 8). Utech (1980), and Samejima and Samejima (1987) have suggested that pedicellate-flowered *Trillium* species in North America can be divided into two subgroups within subgenus *Trillium*; the Grandiflorum subgroup with about eight species including *T. grandiflorum, T. ovatum, T. undulatum* and *T. rivale*, and the Erectum subgroup with about six species including *T. erectum, T. sulcatum, T. cernuum* and *T. rugelii*. The present *cp*DNA analysis supports the monophyly of the Erectum subgroup, but it shows that the Grandiflorum subgroup is heterogeneous and does not form a single clade.

In view of all available data, including the results of cladistic analyses based on morphological characters (Kawano and Kato, in preparation), the pedicellate-flowered group can be divided into at least two major subgroups, i.e., the Erectum subgroup (*T. erectum, T. sulcatum, T. cernuum, T. flexipes, T. rugelii, T. vaseyi, T. catesbaei, T. grandiflorum,* and *T. ovatum*) and the Undulatum subgroup (*T. undulatum, T. persistens, T. nivale, T. rivale,* and *T. pusillum*), which is more or less concordant with the results of the present molecular analysis.

3. *The Relationships between Eastern Asian and North American Pedicellate-flowered Species*

From the chloroplast DNA phylogeny in Fig. 8, the East Asian diploid species, *T. kamtschaticum,* appears to have an affinity to the Erectum subgroup, as was noted by Utech (1980) based on gross morphological similarities. Haga and Channell (1982) reported that North American species of *Trillium* can be classified into three compatibility groups according to their ability to crossbreed with *T. kamtschaticum*: (1) compatible pedicellate-flowered species (*T. erectum, T. gleasonii*=*T.*

flexipes and *T. vaseyi*) (2) incompatible pedicellate-flowered species (*T. grandiflorum*) (3) incompatible sessile-flowered species (*T. decumbens, T. luteum, T. sessile* and *T. stamineum*). *T. kamtschaticum* has retained its compatibility with members of the Erectum subgroup, despite the long isolation indicated by the disjunct distributions of *T. kamtschaticum* and the North American subgroup.

From comparisons of pollen morphology, Takahashi (1982, 1983, 1984) concluded that Asiatic species of *Trillium* (the diploid *T. kamtschaticum*, and tetraploid *T. apetalon, T. tschonoskii*, hexaploid *T. smallii*, and so on), excluding *T. govanianum*, are closely related to each other and the North American *T. erectum*. Our preliminary RFLP analyses of chloroplast DNA among several Asian species (*T. kamtschaticum*, 2×; *T. apetalon, T. tschonoskii*, 4×; *T. smallii*, 6×; and *T. hagae*, 3×, 6×) (Samejima and Samejima, 1962, 1987) indicated that the *cp*DNA of these species are all similar to *T. kamtschaticum* (the data not shown here).

A more thorough discussion concerning evolutionary divergence and phylogeny of the genus *Trillium* and related genera in the Trilliaceae will be made in a forthcoming paper, compiling the results of cladistic analysis based on gross morphology and comparative life histories (Kawano et al., 1992; Kawano and Kato, in preparation).

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