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Physical map of chloroplast DNA in sugi, *Cryptomeria japonica*

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Summary. To investigate the evolution of conifer species, we constructed a physical map of the chloroplast DNA of sugi, *Cryptomeria japonica*, with four restriction endonucleases, *Pst*I, *Sal*I, *Sac*I and *Xho*I. The chloroplast genome of *C. japonica* was found to be a circular molecule with a total size of approximately 133 kb. This molecule lacked an inverted repeat. Twenty genes were localized on the physical map of *C. japonica* cpDNA by Southern hybridization. The chloroplast genome structure of *C. japonica* showed considerable rearrangements of the standard genome type found in vascular plants and differed markedly from that of tobacco. The difference was explicable by one deletion and five inversions. The chloroplast genome of *C. japonica* differed too from that of the genus *Pinus* which also lacks one of the inverted repeats. The results indicate that the conifer group originated monophyletically from an ancient lineage, and diverged independently after loss of an inverted repeat structure.

Key words: *Cryptomeria japonica* – Chloroplast DNA – Physical map – Evolution of conifers

Introduction

A large number of the chloroplast genomes of angiosperms, ferns, algae, bryophytes and gymnosperms have been characterized by physical gene mapping. The chloroplast (cp) DNAs of tobacco, liverwort and rice have been completely sequenced (Ohyama et al. 1986; Shinozaki et al. 1986; Hiratsuka et al. 1989). The chloroplast genomes of all land plants have been shown to exist as a circular chromosome ranging in size from 120 to 217 kb, although dimeric forms are found (Palmer 1987). The cpDNAs of most land plants contain large inverted repeats. However, these inverted repeats are lacking in one group of legumes (Palmer and Thompson 1982; Palmer et al. 1987) and some conifer tree species (Lidholm et al. 1988; Strauss et al. 1988; White 1990a; Lidholm and Gustafsson 1991).

In gymnosperms, physical maps of cpDNA have been reported for *Ginkgo biloba* (Palmer and Stein 1986) and five conifer species, namely, *Pseudotsuga menziesii*, *Pinus radiata*, *P. monticola*, *P. contorta* and *P. thunbergii* (Strauss et al. 1988; White 1990a; Lidholm and Gustafsson 1991; Tsudzuki et al. 1992). Conifer cpDNAs show some unique features compared to the those of other plants including: (1) paternal or biparental inheritance (Neale et al. 1986; Szmidt et al. 1987; Neale and Sederoff 1989; Neale et al. 1989; Stine et al. 1989), (2) the lack of a large inverted repeat, except for *P. thunbergii*, and (3) intraspecific variation in some pine species (Wagner et al. 1987; White 1990b; Ali et al. 1991).

Among gymnosperms, the genus *Ginkgo* is considered to be one of the most ancient having been distributed throughout the world some 195–225 million years ago. Even so, it should be emphasized that the cpDNA structure of *G. biloba* is similar to that of most land plants e.g., petunia or tobacco). The genus *Pinus* is believed to be more recent than *Ginkgo* and its cpDNA structure differs greatly from that of the standard type found in land plants (Strauss et al. 1988). *Cryptomeria japonica* belongs to the family Taxodiaceae and is endemic in Japan. The phylogenetic relationships of *Cryptomeria* are unclear, because genetical markers are greatly limited (Yasue et al. 1987; Tsumura et al. 1989).

Chloroplast evolution in gymnosperms raises the following questions: (1) is the loss of the inverted repeat

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Table 1. Size in kbp and number of restriction fragments of *C. japonica* chloroplast DNA

No.	*Pst*I	*Pst*I + *Sal*I	*Sal*I	*Pst*I + *Sac*I	*Sac*I	*Pst*I + *Xho*I	*Xho*I
1	36.4	19.6	20.4	17.5	17.8	14.9	28.2
2	23.7	11.0	14.0	13.1	13.1	14.1	19.8
3	18.2	9.4	12.7	9.8	12.3	12.6	13.7
4	14.9	8.7	10.8 (2)	9.2	10.8	9.4	12.6
5	8.6	8.2	10.1	7.6 (2)	10.0	8.9	9.3 (2)
6	7.9	7.7	9.5	6.2	9.1	7.9	8.9
7	6.7	6.8	9.4	6.0	8.8	7.6	6.5
8	4.2	6.3	8.7	4.7	8.5	7.3	6.2
9	2.4 (2)	5.1	8.2 (2)	4.3 (2)	7.7	6.5	5.4
10	2.2	4.9	6.8	3.8	5.9	4.7	3.5
11	1.6	4.5	3.2	3.6 (3)	5.8	4.2 (3)	3.4
12	1.2	4.2	1.1	3.4 (2)	5.5	3.6	2.9
13	1.1	3.8		2.5	3.6	3.6	2.2
14	0.9	3.5		2.4	3.6	3.6	1.3
15	0.8	3.2 (2)		1.6 (2)	3.2	2.9	
16	0.7	2.9 (2)		1.4	2.5	2.2 (2)	
17		2.4 (2)		1.3	1.4	1.6	
18		1.6		1.2	1.3	1.5	
19		1.5		1.1 (2)	0.9	1.4	
20		1.2		0.9	0.8	1.3	
21		1.1		0.9	0.5	1.2	
22		1.1		0.7 (2)		1.1 (2)	
23		0.9		0.5		0.9	
24		0.8				0.8	
25		0.8				0.5	
26		0.7					
27		0.5					
28		0.5					
Total	133.9	131.4	133.9	132.1	133.1	133.7	133.2
Mean value	133.0 kbp						

(IR) commonly found in conifers? and (2) what is the arrangement of the gene order following loss of the IR in conifers? In order to address these questions, we have constructed a physical map of the cpDNA of *C. japonica*, and compared its genome structure to that of other conifers and other land plants. From an evolutionary point of view, *Cryptomeria* is interesting. It is considered to be one of the most ancient conifer species, having diverged from its ancestral group some 24–65 million years ago (Uemura 1981).

Materials and methods

Plant material

Open-pollinated seeds of *C. japonica* D. Don, collected from an individual clone (G-5), were germinated and grown for 3–6 months in the greenhouse. The needle tissue of these seedlings was used for cpDNA extraction.

Chloroplast DNA isolation and DNA manipulation

Chloroplast DNA was isolated from needles of *C. japonica* by using a sucrose discontinuous – gradient method (Ogihara and Tsumewaki 1982). The cpDNA was digested with four restriction endonucleases, namely *Pst*I, *Sal*I, *Sac*I and *Xho*I, either solely or in a combination of *Pst*I with *Sal*I, *Sac*I or *Xho*I, according to the manufacturer's instruction. The digested cpDNA was fractionated by 0.7% agarose-gel electrophoresis in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate and 2 mM EDTA, pH 8.0) for estimation of fragment sizes and/or Southern hybridization. Fractionated DNAs were transferred to nylon membranes (Hybond-N, Amersham Co. Ltd.).

*Sal*I digests of sugi cpDNA were ligated into pUC18. Competent *Escherichia coli* cells (strain JM109) were transformed and grown overnight on an LB agar plate containing IPTG, X-gal and 100 μg/ml of ampicillin. Recombinant colonies were grown in LB broth and glycerized for storage at −85 °C. Plasmid DNAs were extracted from 2-ml cultures in LB broth (Birnboim and Dolly 1979), and digested with *Sal*I to check the inserts.

Southern hybridization of sugi cpDNA was carried out with homologous and heterologous probes to assign the order of restriction fragments of cpDNA. For homologous hybridization, nine sugi *Sal*I-digested clones were used. For heterologous hybridization, clones of wheat and tobacco cpDNAs were employed (Ogihara and Tsunewaki 1982; Sugiura et al. 1986). These clones were digested with the appropriate restriction enzymes and inserted fragments were recovered from agarose gels after electrophoresis by the glass powder method (Vogelstein and Gillespie 1979). DNA fragments corresponding to the coding region of the gene were prepared after a computer search of restriction sites (GENETYX program, SDC Software Develop-

ment Co. Ltd.). The probe DNAs (0.1–0.2 µg) were labeled by the digoxygenin non-radioactive labeling method (Boehringer Mannheim Co. Ltd.). The nylon membranes were hybridized with probes for 18 h in hybridization buffer [5 × SSC, 0.5% (w/v) dry skim milk, 0.1% (w/v) N-lauroylsarcosine sodium salt, 0.02% (w/v) SDS, 50 mg/ml salmon sperm DNA] at 68 °C. Membranes were washed twice in 2 × SSC, 0.1% SDS for 15 min at room temperature and then twice in 0.1 × SSC, 0.1% SDS for 15 min at 68 °C. Immunological detection of the hybridized fragment was carried out following the protocol of the manufacturer (Boehringer Mannheim Co. Ltd.).

Results

Restriction endonuclease analysis and genome size estimation

Although conifer cpDNAs sometimes revealed intraspecific variation (Wagner et al. 1987; White 1990b), cpDNA patterns isolated from different clones of sugi were identical with each other (data not shown).

The restriction fragment patterns of *C. japonica* cpDNA digested with *Pst*I, *Sal*I, *Sac*I and *Xho*I, either solely or in combinations of two enzymes, are shown in Fig. 1. Chloroplast genome size was estimated to range from 131.1 (*Sac*I) to 133.9 (*Pst*I) kbp (mean of 133 kbp), based on the molecular sizes of individual restriction fragments and their copy number (Table 1). This genome size is in the range of gymnosperm cpDNAs. That of *G. biloba* is 141 kbp when one of inverted repeats (17 kbp) is omitted from the genome of 158 kbp (Palmer and Stein 1986). Other coniferous species have a cpDNA size of 120 and 121 kbp (Strauss et al. 1988; White 1990a; Lidholm and Gustafsson 1991; Tsudzuki et al. 1992).

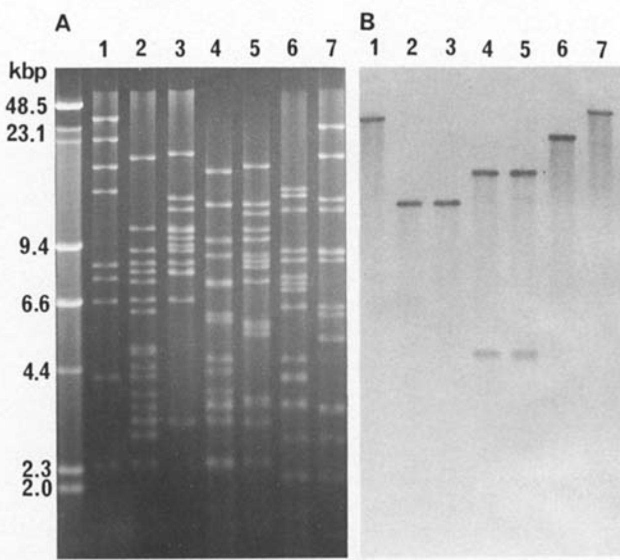

Fig. 1. Restriction fragment patterns of *C. japonica* cpDNA generated by single digestion with *Pst*I, *Sal*I, *Sac*I and *Xho*I or in combinations with *Pst*I and the other three enzymes (**A**), and their Southern hybridization pattern with the S9b of sugi cpDNA as a probe (**B**). *Lanes 1*, *Pst*I; *2*, *Pst*I and *Sal*I; *3*, *Sal*I; *4*, *Pst*I and *Sac*I; *5*, *Sac*I; *6*, *Pst*I and *Xho*I; *7*, *Xho*I

Construction of a physical map and mapping of photosynthesis-related genes

The recognition sites of four restriction endonucleases were estimated by the fragment patterns produced by single and double digestions of cpDNA, as presented in Table 1. The restriction fragment orders were confirmed by homologous hybridization with cloned sugi cpDNA fragments used as probes (Table 2). The fragment orders of sugi cpDNA were compared to those of tobacco (Sugiura et al. 1986) and wheat (Ogihara and Tsunewaki 1988). Mapping data (Fig. 2) show that the cpDNA of *C. japonica* lacks an inverted repeat and that the fragment order is highly rearranged in comparison to that of the standard type of angiosperm such as petunia and mung bean (Palmer and Stein 1986). Southern hybridization patterns with cloned DNAs of wheat cpDNA, which harbors three major inversions compared to tobacco cpDNA (Howe et al. 1988), were so complicated that some of them were not completely traced (data not shown). Twenty photosynthesis-related genes (Table 3) have been localized on the physical map to allow a precise comparison of the chloroplast genome structure between angiosperms and conifers. Gene-mapping data confirm that, like other conifers, the chloroplast genome of *C. japonica* lacks one of the inverted repeats (Strauss

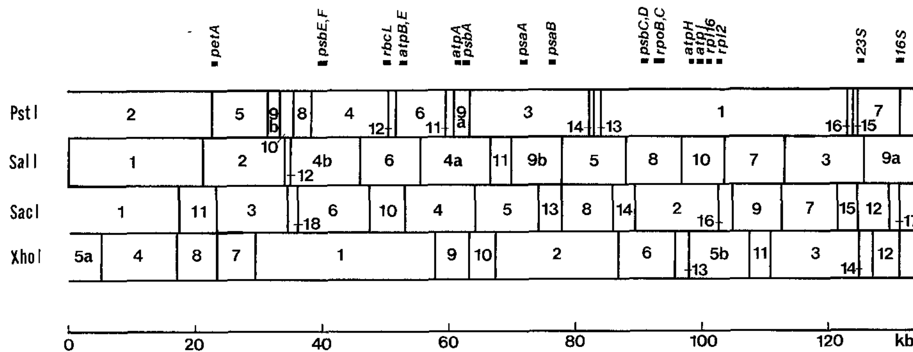

Fig. 2. Physical map of *C. japonica* in a linear form showing restriction sites of the four endonucleases *Pst*I, *Sal*I, *Sac*I and *Xho*I, and the location of 20 photosynthesis-related genes. The circular DNA has been opened at the *Sal*I site between S1 (20.4 kbp) and S9a (8.2 kbp)

Table 2. The cpDNA fragments of sugi hybridized with homologous (S5 to S12) and heterologous (pTBa1 and pTB10) probes

Probe (kb) DNA fragment	Restriction enzyme used for digestion						
	*Pst*I	*Pst*I/*Sal*I	*Sal*I	*Pst*I/*Sac*I	*Sac*I	*Pst*I/*Xho*I	*Xho*I
S5(10.1)	P1 P3 P13 P14	PS8 PS18 PS21 PS22	S5	PSc6 PSc21 PSc22	Sc8 Sc14	PX1 PX5 PX12 PX21 PX22	X2 X6
S7(9.4)	P1	PS3 PS15	S7	PSc5 PSc8 PSc13	Sc7 Sc9 Sc16	PX4 PX8 PX13	X3 X5b X11
S8(8.7)	P1	PS4	S8	PSc2	Sc2 Sc14	PX5 PX15	X6 X13
S9a(8.2)	P2 P7	PS9 PS16	S9a	PSc1	Sc1	PX7 PX14	X5a X12
S9b(8.2)	P3	PS5	S9b	PSc3 PSc11	Sc5 Sc13	PX2	X2
S10(6.8)	P1	PS7	S10	PSc2 PSc13	Sc2 Sc16	PX4	X5b
S11(3.2)	P3	PS3 PS15	S11	PSc3 PSc5	Sc5	PX2 PX13	X2 X10
S3(12.7)	P1 P7 P15 P16	NT[a]	S3	NT	SC7 Sc12 Sc15	NT	X2
S12(1.1)	P10	NT	S12	NT	Sc18	NT	X1
pTBa1(19.6)	P4 P5 P9b	NT	S2 S4b	NT	Sc3 Sc6 Sc11	NT	X1 X8 X10
pTB10(approximately 9.6)	P2	NT	S1 S9	NT	Sc1	NT	X5a

[a] NT, not tested

Table 3. Southern hybridizations of the cpDNA of *C. japonica* with gene probes

Gene probe	Plant source	Probes for gene mapping	Hybridized fragments			
		Clone, R.E., Frag.size	*Pst*I	*Sal*I	*Sac*I	*Xho*I
psaA	Tobacco	pTX9, *Bam*HI, 2.5 kb	P3	S9b	Sc5	X2
psaB	Tobacco	pTB30, *Eco*RI + *Bam*HI, 2.04 kb	P3	S9b	Sc13	X2
psbA	Tobacco	pTB28, *Xba*I + *Pst*I, 0.7 kb	P9a	S4a	Sc4	X9
psbC,D	Tobacco	pTB30, *Nco*I, 1.7 kb	P1	S8	Sc2	X6
psbE,F	Tobacco	pTS9, *Sac*I + *Eco*RV, 1.1 kb	P4	S4b	Sc6	X1
atpA	Tobacco	pTb25, *Sal*I + *Sma*I, 1.55 kb	P9a	S4a	Sc4	X9
atpB,E	Tobacco	pTS6, *Sma*I + *Pvu*II, 1.4 kb	P6	S6	Sc4 Sc10	X1
atpH	Tobacco	pTB25, *Nru*I, 0.7 kb	P1	S10	Sc2	X5b
atpI	Tobacco	pTB25, *Nco*I + *Sca*I, 0.7 kb	P1	S10	Sc2	X5b
petA	Tobacco	pTS6, *Nru*I + *Sal*I, 1.0 bp	P5	S2	Sc11	X8
rpl2	Tobacco	pTB28, *Pst*I + *Hin*dIII, 0.6 kb	P1	S10	Sc16	X5b
rpl16	Tobacco	pTS10, *Bam*HI + *Xba*I, 1.35 kb	P1	S10	Sc2	X5b
rpoB	Tobacco	pTB7, *Nco*I + *Sal*I, 1.7 kb	P1	S8	Sc2	X6
*rpo*C	Tobacco	pTB7, *Bam*HI + *Hin*dIII, 3.0 kb	P1	S8	Sc2	X6
*rbc*L	Wheat	B2, *Nru*I + *Pst*I, 1.2 kb	P4 P12	S6	Sc10	X1
16*s* rDNA	Tobacco	pTB8, *Sph*I + *Nru*I, 1.2 kb	P2 P7	S9a	Sc1 Sc17	X5a X12
23*s* rDNA	Tobacco	pTB8, *Xho*I, 0.6 kb	P7	S3	Sc12	X14

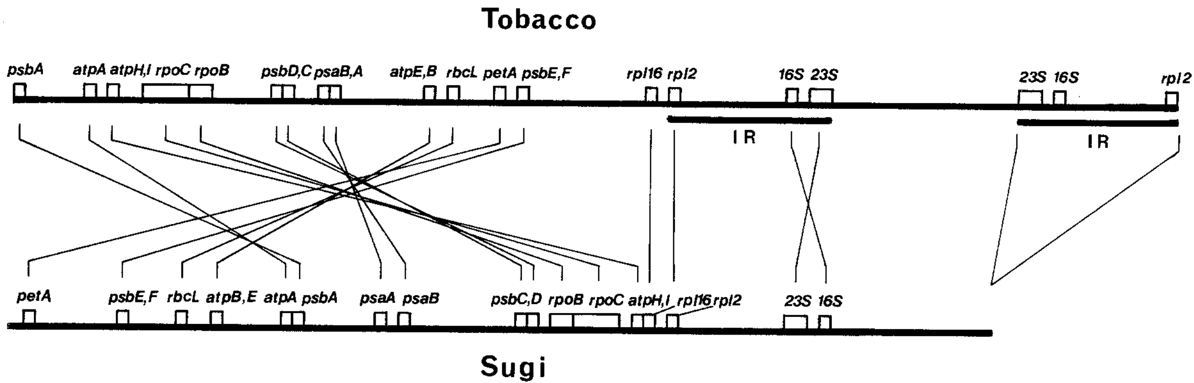

Fig. 3. A comparison of the gene order between tobacco and sugi cpDNAs and the hypothesized deletion and inversions involved in the evolution of the *C. japonica* chloroplast genome from a tobacco-like ancestral genome

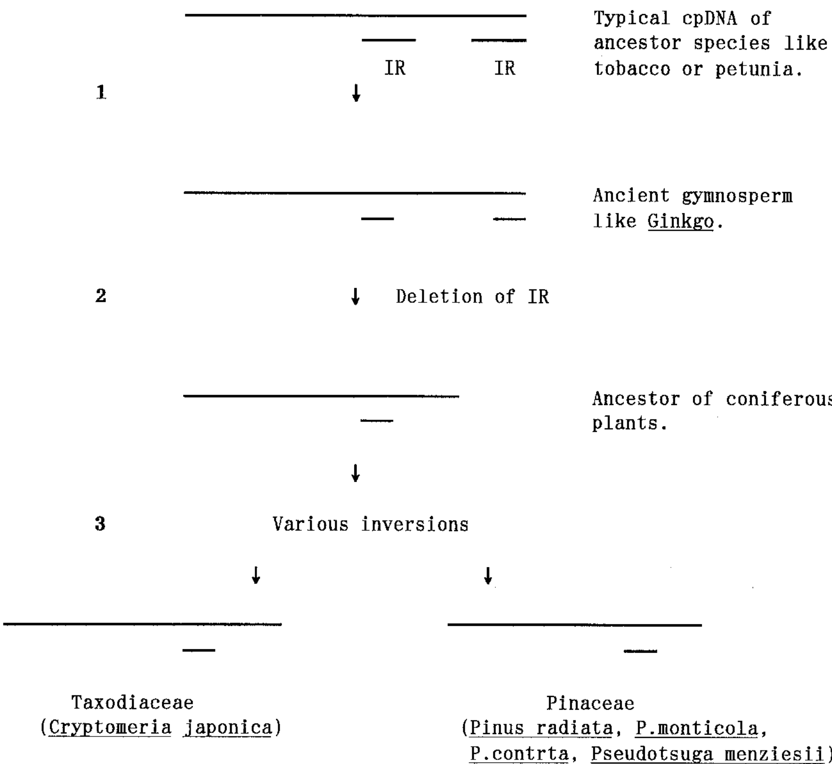

Fig. 4. Putative hypothesis of the cpDNA evolution of conifer species. Step 1, deletion of part of a repeat sequence; step 2, deletion of one of the inverted repeats; step 3, various inversions occurring during the differentiation of each family

et al. 1988; White 1990a; Lidholm and Gustafsson 1991) and is highly rearranged in comparison to other conifers. Allowing for only deletion and inversion, six events (one deletion and five inversions) are required to trace the cpDNA of *C. japonica* back to the ancestral type (Fig. 3). The sequence of these events as inferred from the gene order, is as follows: (1) loss of the large inverted repeat occurred first; (2) a large inversion took place between the *atpH,I* and *psbF,E* genes; (3) then, successively, four small inversions occurred; namely, between *psbA* and *atpE,B*, *atpE,B* and *psbE,F*, *psbE,F* and *petA*, and the 16 and 23 rDNAs.

Discussion

The present results show that the cpDNA of *C. japonica* lacks a large inverted repeat and has been considerably rearranged in comparison to that of the standard type cpDNA present in petunia and mung bean (Palmer and Stein 1986). The cpDNA of sugi also differs from that of pine in terms of genome size and gene order, although pine too lacks an inverted repeat (Strauss et al. 1988). It is well known that the genome structure of chloroplast DNA is highly conserved among different plant species (Palmer 1987). In fact, a basic structure of the cpDNA

genome is maintained in most plants, including green algae, mosses, and vascular plants (Palmer and Stein 1986; Palmer 1987). This conservatism of cpDNA has been confirmed by DNA sequencing of the entire genome in liverwort, tobacco and rice (Ohyama et al. 1986; Shinozaki et al. 1986; Hiratsuka et al. 1989). Accordingly, drastic changes in the chloroplast genome, such as the loss of large inverted repeats, deletions, inversions and translocations, are considered to be exceptional events in the lineage of plant differentiation (Palmer et al. 1988). These genome alternations are found at various taxonomic levels, e.g., species, genus, family and higher orders. As for the deletion of a large inverted repeat in cpDNA, two examples have been reported involving one group of legumes (Palmer and Thompson 1982) and some conifer trees belonging to the Pinaceae (Lidholm et al. 1988; Strauss et al. 1988; White 1990a; Lidholm and Gustafsson 1991). On the other hand, primitive and ancient *Ginkgo*, whose relatives are considered to be the ancestors of conifers (Doyle and Donoghue 1986), maintains two large inverted repeats (17 kbp) and the same gene order as that of the standard type cpDNA (Palmer and Stein 1986). Structural alternations of the chloroplast genomes of conifers can be traced from the standard type of vascular plant cpDNA. In the Pinaceae, the cpDNA genome type of *P. radiata* can be derived from the standard type by six mutations: two deletions and four inversions (Strauss et al. 1988). The cpDNAs of *P. monticola* (White 1990a) and *P. contorta* (Lidholm and Gustafsson 1991) have a similar structure to that of radiata pine while that of *P. menziesii* harbors one additional inversion to those of radiata pine (Strauss et al. 1988). Six mutational events, one deletion and five inversions, from the standard cpDNA type are required to produce that of *C. japonica*. But, when the gene order of *C. japonica* cpDNA is compared with that of *Pinus radiata*, the genome structures suggest independent changes so that no simple evolutionary path can be determined. These lines of evidence strongly suggest that loss of a large inverted repeat occurred at the time of the differentiation of coniferous plant(s) from other gymnosperms some 300 million years ago (Doyle and Donoghue 1986). Subsequent to this event the cpDNA genome of coniferous plants has diverged independently.

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