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Comparative analysis of chloroplast DNA in *Pyrus* species: physical map and gene localization

Abstract A physical map of chloroplast DNA (cpDNA) of pear [*Pyrus ussuriensis* var. *hondoensis* (Nakai et Kikuchi) Rehder] was constructed using five restriction enzymes, *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I. This information will make it possible to investigate the phylogenetic relationships between *Pyrus* species. Pear cpDNA was found to be a circular molecule with a total size of about 156 kb in which two inverted repeats of 24.8 kb divide the molecule into small (17 kb) and large (90 kb) single-copy regions. The endonuclease recognition sites in the physical map were determined by single and double digestion of 13 lambda phage clones which covered the entire sequence of the pear cpDNA. Twenty nine genes were localized on the physical map of the pear cpDNA. The structure of pear cpDNA was almost the same in terms of genome size and gene order as that of tobacco cpDNA. RFLP analysis was carried out on cpDNAs from five *Pyrus* species (*Pyrus pyrifolia*, *Pyrus ussuriensis*, *Pyrus calleryana*, *Pyrus elaeagrifolia* and *Pyrus communis*). Two mutations, a recognition-site mutation and a length mutation (deletion), were found only in the cpDNA of *P. pyrifolia* cultivars. These mutations were localized on the physical map of pear cpDNA. The number of mutations of cpDNA in *Pyrus* species are small in comparison with those of other angiosperms, suggesting a high degree of genome conservatism in *Pyrus* species.

Keywords Pear · *Pyrus ussuriensis* var. *hondoensis* · Chloroplast DNA · Physical map · Structural analysis

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Introduction

The genus *Pyrus* belongs to the subfamily Maloideae of the Rosaceae. Approximately 30 species are distributed in East and West Asia, Europe, and Africa (Rehder 1915; Nakai 1916; Hedrick 1921; Kikuchi 1946). Challice and Westwood (1973) recognized 22 species by numerical taxonomy using morphological characters and phenolic compounds. The classification of the *Pyrus* species is complicated by the lack of morphological characters distinguishing species, which results from continuous interspecific hybridization and introgression (Rubtsov 1944; Kikuchi 1946; Yu 1979). Nakai (1918, 1919) proposed the occurrence of four *Pyrus* species in Japan, i.e. *Pyrus pyrifolia* [Burm.] Nakai (= *Pyrus serotina*), *Pyrus dimorphophylla* Makino, *Pyrus hondoensis* [Nakai *et* Kikuchi] Rehder and *Pyrus aromatica* [Nakai *et* Kikuchi] Rehder. *P. hondoensis* (called 'Aonashi' in Japanese) and *P. aromatica* (called 'Iwateyamanashi' in Japanese) are endemic in Japan. These two species were included in *Pyrus ussuriensis* and treated as its varieties (*P. ussuriensis* var. *hondoensis* and *P. ussuriensis* var. *aromatica*) by Kikuchi (1924) and Ohwi and Kitagawa (1983).

Almost all modern cultivars cultivated in Japan are included in *P. pyrifolia*, although the origin of *P. pyrifolia* is unclear. Kajiura et al. (1983) surveyed a species-specific flavonoid in 31 *Pyrus* species including 171 East Asian pear cultivars and wild clones. A flavonol aglycone was detected in *P. ussuriensis, Pyrus bretschneideri*, and *Pyrus macrostipes* which originated in Korea and North China. Sixteen old Japanese cultivars including *P. aromatica* (= *P. ussuriensis* var. *aromatica*) and *P. serotina* (= *P. pyrifolia*) 'Nijisseiki' also contained this specific flavonol aglycone. Kajiura et al. (1983) suggested that some of the old Japanese cultivars and 'Nijisseiki' seemed to be related to *P. aromatica* through hybridization.

Recently, RFLP analyses of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) had been applied to investigate the relationships between some *Pyrus* genotypes and Asian pears (Iketani 1993; Iketani

et al. 1993, 1998). Four types (AAA, BAA, BAB and BBB) of chloroplast genomes in Asian pear were distinguished by the combination of three independent restriction-site mutations generated by three restriction enzymes. They indicated inconsistencies between the chloroplast genome types and the morphological classification, i.e. different chloroplast genome types were observed within one species (Iketani et al. 1998).

Chloroplasts of vascular plants and green algae contain multiple (10 to 200) copies of circular DNA molecules ranging in size from 120 kb to 217 kb (Palmer 1991) and are uniparentally transmitted to progeny in most plants. The characteristic feature in the structure of cpDNA is the long inverted repeats (IR) ranging from 10 kb to 85 kb which divide the rest of the molecule into small and large single-copy regions (SSC and LSC) respectively. Most cpDNAs in vascular plants show a typical genome structure having 20–26 kb of IR, 10–20 kb of SSC, and 80–100 kb of LSC (Palmer et al. 1985). It is well known that the genome structure and gene content of cpDNAs are conserved among divergent plants (Downie and Palmer 1992). The sequence information on the entire chloroplast DNA enables us to compare precisely the gene contents and arrangement of gene order among divergent plants such as liverwort (Bryophyte), pine (Pinaceae), tobacco (Solanaceae), *Arabidopsis thaliana* (Brassicaceae), spinach (Chenopodiaceae), *Oenothera elata* (Onagraceae), *Lotus japonicus* (Leguminosae), beechdrops (Orobanchaceae), rice (Gramineae), maize (Gramineae) and wheat (Gramineae) (Ohyama et al. 1986; Shinozaki et al. 1986; Hiratsuka et al. 1989; Wolfe et al. 1992; Wakasugi et al. 1994; Maier et al. 1995; Sato et al. 1999; Hupfer et al. 2000; Kato et al. 2000; Schmitz-Linneweber et al. 2001; Ogihara et al. 2002).

Conservatism of cpDNA allows us to compare phylogenetic relationships at various taxonomic levels (Palmer et al. 1985). In spite of the conservatism of the chloroplast genome, structural alterations such as insertions, deletions, inversions and translocations in cpDNA have been found in related plants by comparing the structure of cpDNAs (Downie and Palmer 1992). Tracing the mutational events in cpDNA provides useful tools to trace the course of evolution by reconstructing the relevant plant phylogeny (Downie and Palmer 1992; Doyle et al. 1992; Katayama and Ogihara 1996). Also polymorphisms in cpDNA make it possible to reveal plastid inheritance in *Pyrus* species in the future.

Although structural analysis of cpDNA has been carried out in a variety of plants, knowledge on the structure of cpDNA in the Rosaceae is very limited. In the present study, a physical map of the pear cpDNA was constructed in *P. ussuriensis* var. *hondoensis*. The molecular size, gene order and restriction fragment patterns of pear cpDNA (*P. ussuriensis* var. *hondoensis*) were compared with those of other *Pyrus* species and some angiosperms.

Materials and methods

Plant materials

P. ussuriensis var. *hondoensis* was used for the construction of a physical map of cpDNA. Nine accessions from three *Pyrus* species of East Asian pears (*P. pyrifolia, P. ussuriensis* and *Pyrus calleryana*), two *Pyrus* species of West Asian and European pears (*Pyrus elaeagrifolia* and *Pyrus communis*) and two accessions from *Prunus* species (*Prunus persica* and *Prunus avium*) were used for the RFLP analysis (Table 1).

All *Pyrus* accessions used for the present study were maintained at the Botanical Gardens, Osaka City University (OCU), and/or at the Experimental Farm, Kobe University (KU). All *Pyrus* species supplied from OCU were introduced from the Experimental Farm, Kyoto University, to OCU in 1957 by grafting. Classification of Japanese species followed Kikuchi (1946) and Ohwi and Kitagawa (1983).

Preparation of chloroplast DNA

Intact chloroplasts were isolated from *P. ussuriensis* var. *hondoensis* according to the method of Ogihara and Tsunewaki (1982) with modifications. Chloroplasts were isolated from about 150 g of fresh leaves into 500 ml of extraction buffer (0.35 M sucrose, 50 mM of Tris–HCl buffer pH 8.0, 3 mM of Na_2EDTA pH 8.0, 0.1% BSA, and 1 mM of 2-mercaptoethanol), and purified by the discontinuous sucrose gradient centrifugation. CpDNA was extracted from the purified chloroplasts according to the method of Kolodner and Tewari (1975). Pear cpDNA was digested with *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I solely or in combinations of two enzymes according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). The digested cpDNAs were fractionat-

Table 1 Distribution of mutations detected in five *Pyrus* and two *Prunus* species

[a] KU=Experimental Farm, Kobe University, OCU=Botanical Gardens, Osaka City University
[b] Site mutation (+) indicates new *Sal*I site generated compare to pear cpDNA (*P. ussuriensis* var. *hondoensis*)
[c] Length mutation (+) indicates that an approximately 300 bp deletion occurred
[d] Not determined

Code	Species	Source[a]	Accession	Name of cultivar or common name	Site mutation[b]	Length mutation[c]
1.	*Pyrus pyrifolia* [Burm.] Nakai	KU	1B0001K	Housui	+	+
2.	*P. pyrifolia* [Burm.] Nakai	KU	1B0002K	Choujuurou	+	+
3.	*P. pyrifolia* [Burm.] Nakai	OCU	4A0075	Nijisseiki	+	+
4.	*P. ussuriensis* var. *hondoensis* [Nakai *et* Kikuchi] Rehder	OCU	4A0135	Aonashi	–	–
5.	*P. ussuriensis* var. *aromatica* [Nakai *et* Kikuchi] Rehder	OCU	4A0128	Iwateyamanashi	–	–
6.	*P. calleryana* DEC.	OCU	4A0157	Callery pear	–	–
7.	*P. elaeagrifolia* PALL.	OCU	4A0126		–	–
8.	*P. communis* L.	KU	1A0001K	La France	–	–
9.	*P. communis* L.	KU	1A0002K	Bertlett	–	–
10.	*Prunus persica* BATSCH.	KU	2A0001K	Hakuhou	ND[d]	–
11.	*Prunus avium* L.	KU	2A0002K	Satounishiki	ND[d]	–

ed by electrophoresis on 0.7% agarose gels, ethidium bromide-stained bands were visualized with a UV transilluminator. Fragments of *Hin*dIII and *Pst*I-digested λphage DNA were used as molecular-weight markers.

Cloning of cpDNA and construction of a physical map

Cloning of cpDNA was carried out following the directions for the cloning system (Stratagene, USA). CpDNAs were partially digested with *Sau* 3AI (Takara Shuzo Co., Japan). After a partial fill-in reaction, cpDNA fragments were ligated with lambda FIX/*Xho*I-partial fill-in treated DNA (Stratagene, USA) and a packaging reaction was carried out using Gigapack Gold (Stratagene, USA). Recombinant phages were selected by plaque hybridization with plasmid clones of tobacco cpDNA as a probe. Hybridizations were carried out using ECL nonradioactive DNA labelling and detection kits (Amersham-Pharmacia Biotech, UK). In order to obtain overlapping clones, selected phages were screened by homologous hybridization. Recombinant phage DNAs were prepared using a Wizard Lambda Prep DNA Purification System (Promega, USA). The restriction sites of cloned fragments were determined by restriction fragment analysis and Southern hybridization. Each clone was connected to construct a physical map of the entire pear cpDNA. Twenty nine genes amplified by PCR from tobacco cpDNA clones were localized on the physical map of pear cpDNA (Katayama and Ogihara 1993).

Preparation of total DNA and Southern hybridization

Total DNAs of *Pyrus* species were extracted by the method of Doyle and Doyle (1987) with a slight modification. Total DNAs of *Prunus* species were extracted according to Reiter et al. (1992) with a slight modification to reduce contamination by polysaccharides. Total DNAs of *Pyrus* and *Prunus* species were digested with *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Digested total DNAs were fractionated by electrophoresis on 1.0% agarose gels; Southern hybridizations were carried out using ECL nonradioactive DNA labelling and detection kits (Amersham-Pharmacia Biotech, UK). Cloned fragments of pear cpDNA were used as probes.

Results and discussion

Restriction endonuclease analysis and genome size estimation

Restriction fragment patterns of pear cpDNA (*P. ussuriensis* var. *hondoensis*), after digestion with *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I, solely or in combinations of two enzymes, are shown in Fig. 1. The molecular size of the chloroplast genome was estimated to range from 155.8 kb for the *Bam*HI digest to 157 kb for the *Pst*I digest based on the molecular sizes of individual fragments (Table 2). According to these estimates, the molecular size of pear cpDNA was considered to be approximately 156 kb. This estimate indicates that the features of the genome of the pear cpDNA are equivalent in size to those of tobacco and *A. thaliana* (Shinozaki et al. 1986; Sato et al. 1999), in contrast to the cpDNAs of gramineous plants (Hiratsuka et al. 1989; Maier et al. 1995; Schmitz-Linneweber et al. 2001; Ogihara et al. 2002).
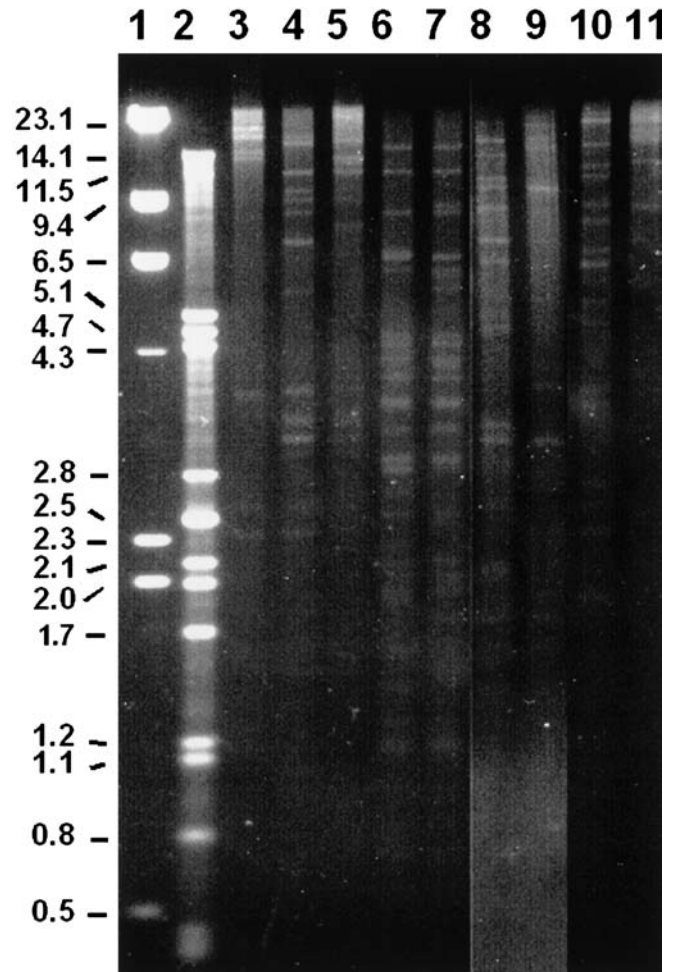

Fig. 1 Restriction fragment patterns of pear cpDNA (*P. ussuriensis* var. *hondoensis*) generated by single and double digestions with *Sal*I (*lane 3*), *Sal*I+*Xho*I (*lane 4*), *Xho*I (*lane 5*), *Sal*I+*Bam*HI (*lane 6*), *Bam*HI (*lane 7*), *Sal*I+*Sac*I (*lane 8*), *Sac*I (*lane 9*), *Sal*I+*Pst*I (*lane 10*), and *Pst*I (*lane 11*). *Lanes 1 and 2* show molecular weight markers of λDNA digested with *Hin*dIII and *Pst*I, respectively

Clone bank and the physical map of pear cpDNA

Pear cpDNA (*P. ussuriensis* var. *hondoensis*) was digested with *Sau*3AI, and the resulting fragments were cloned into a λ phage (λFIX II) vector (Zabarovsky and Allikmets 1986). Recombinant phages were selected by plaque hybridization with tobacco cpDNA clones as a probe (Shinozaki et al. 1986). To obtain overlapping clones covering the entire region of pear cpDNA, the 13 clones were isolated by homologous hybridization. These were as follows: pPhcp-1(12.5 kb), pPhcp-2(11.8 kb), pPhcp-3(15.5 kb), pPhcp-4(18.7 kb), pPhcp-5(19.8 kb), pPhcp-6(14.4 kb), pPhcp-7(18.0 kb), pPhcp-8(14.0 kb), pPhcp-9(13.5 kb), pPhcp-10(15.5 kb), pPhcp-11(13.0 kb), pPhcp-12(15.0 kb) and pPhcp-13(15.2 kb). These fragments covered the pear cpDNA completely (Fig. 2). Inserted fragments and restriction sites were confirmed by digestion with *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I solely

Table 2 Molecular size of restriction fragments of pear cpDNA generated with *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I. Fragment copy number is given in parentheses

| *Sal*I		*Xho*I		*Bam*HI		*Sac*I		*Pst*I	
Frag.	kb	Frag.	kb	Frag.	kb	Frag.	kb	Frag.	kb
Sl-1	39.0	X-1	30.6	B-1	16.5	Sc-1	41.1	P-1	27.0
Sl-2	22.0 (2)	X-2	16.5	B-2	12.0	Sc-2	21.0	P-2	21.6
Sl-3	17.0 (2)	X-3	13.6 (2)	B-3	9.5	Sc-3	19.2	P-3	21.1
Sl-4	15.0	X-4	12.5	B-4	8.9	Sc-4	18.8	P-4	20.5
Sl-5	13.5	X-5	11.5 (2)	B-5	6.5 (2)	Sc-5	14.8	P-5	16.0
Sl-6	3.7	X-6	10.8	B-6	5.8 (2)	Sc-6	10.6	P-6	15.4
Sl-7	2.3	X-7	9.0	B-7	4.4	Sc-7	10.4	P-7	15.0
Sl-8	2.2 (2)	X-8	6.6	B-8	4.3	Sc-8	4.3	P-8	11.0
Sl-9	0.5	X-9	3.7	B-9	4.2	Sc-9	3.4 (2)	P-9	9.4
		X-10	3.5 (2)	B-10	4.1	Sc-10	2.4		
		X-11	3.3	B-11	3.9 (3)	Sc-11	2.1		
		X-12	3.1	B-12	3.5	Sc-12	2.0 (2)		
		X-13	1.0 (2)	B-13	3.0 (2)	Sc-13	0.5 (2)		
		X-14	0.6	B-14	2.8 (2)				
				B-15	2.3				
				B-16	2.2				
				B-17	2.0 (5)				
				B-18	1.9				
				B-19	1.8 (2)				
				B-20	1.4				
				B-21	1.3 (2)				
				B-22	1.2 (5)				
				B-23	1.1 (3)				
				B-24	1.0 (2)				
				B-25	0.9 (2)				
				B-26	0.6 (2)				
				B-27	0.5 (2)				
				B-28	0.4 (2)				
				B-29	0.2 (2)				
Total	156.4		155.9		155.8		156.5		157.0
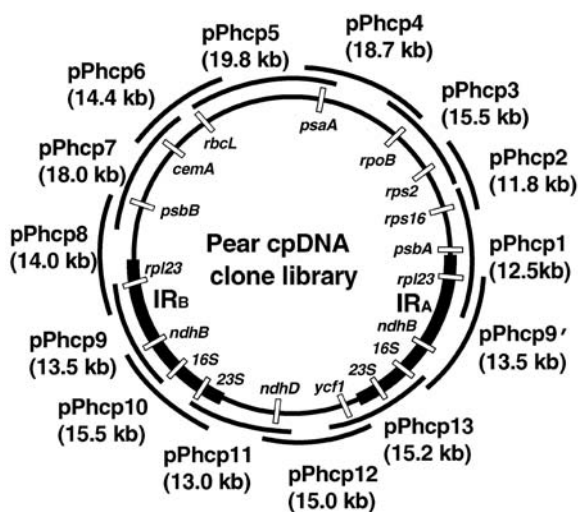
Fig. 2 Bank of overlapping clones of pear cpDNA (*P. ussuriensis* var. *hondoensis*). The extent of the inverted repeats (IRA and IRB) and the position of the genes on the pear cpDNA are indicated *inside the circle*. The clone names and insert sizes of the 13 phage clones are drawn *outside the circle*. pPhcp9′ in IRA represents duplication of pPhcp9 in IRB

or in combinations of two enzymes. Consequently, the physical map of cpDNA from Rosaceous plants was constructed for pear using the five restriction enzymes (Fig. 3).

Localization of the 29 genes on the physical map of pear cpDNA

Twenty nine genes were mapped onto the physical map of pear cpDNA (*P. ussuriensis* var. *hondoensis*) by Southern hybridization (Fig. 3). Pear cpDNA showed the same gene order as that of tobacco, *A. thaliana* and spinach cpDNAs, but differed from that of *L. japonicus* and *O. elata* cpDNAs which possess large inversions, and cpDNAs of gramineous plants which harbor large deletions and inversions (Fig. 4) (Palmer et al. 1988; Hiratsuka et al. 1989; Hachtel et al. 1991; Doyle et al. 1992; Maier et al. 1995; Sato et al. 1999; Hupfer et al. 2000; Kato et al. 2000; Schmitz-Linneweber et al. 2001; Ogihara et al. 2002).

It has been known that tobacco has a cpDNA of standard structure in angiosperms, but some legumes, *Oenothera* species and Gramineous plants have different ones. Three deletions (Del-A:59 bp, Del-B:48 bp and Del-C:113 bp) had been observed in pear cpDNA compared to tobacco cpDNA by sequence analysis
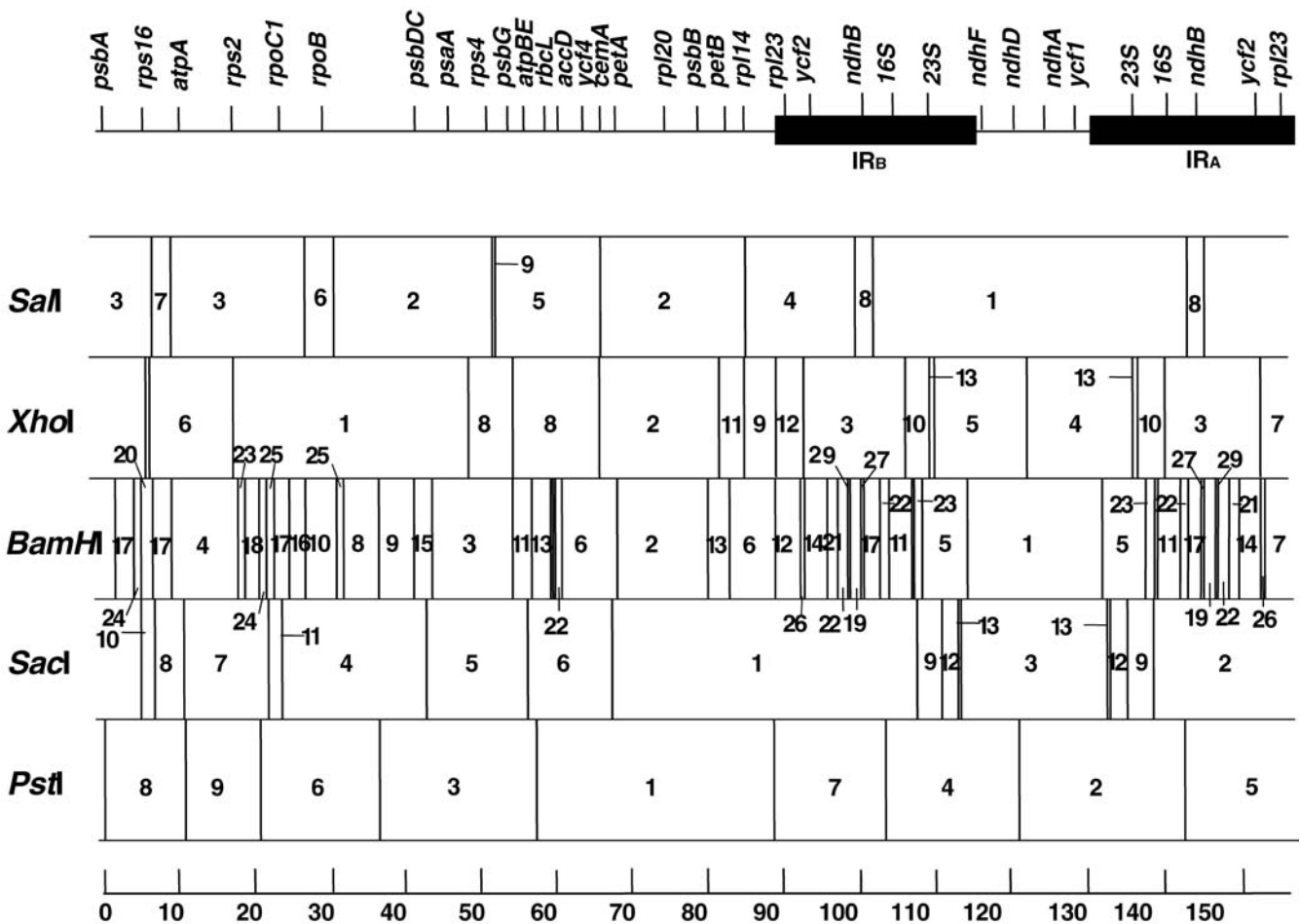
Fig. 3 Physical map of pear cpDNA (*P. ussuriensis* var. *hondoensis* in a linear form showing restriction sites of five restriction enzymes, *Sal*I, *Xho*I, *Bam*HI, *Sac*I and *Pst*I. IR$_A$ and IR$_B$ are the inverted repeat regions. The *numbers* given in the physical map correspond to those presented in Table 2. The location of 29 genes is indicated on the upper line

(Katayama and Uematsu, unpublished data). These deletions had been detected in the non-coding region of 570 bp between the *rbc*L gene (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase; RuBisCO) and the *acc*D gene (encoding one of the subunits of acetyl-CoA-carboxylase; ACCase) within the large single-copy region (LSC). In spite of these structural alterations, pear cpDNA showed an identical gene order to that of tobacco cpDNA. The size of each IR region of pear cpDNA was estimated to be 24.8 kb. The large single-copy region was calculated to be about 90 kb; and the small one, about 17 kb. These lengths are equivalent in size to those of tobacco, *A. thaliana* and spinach (Shinozaki et al. 1986; Sato et al. 1999; Schmitz-Linneweber et al. 2001). Therefore, it is apparent that the pear cpDNA belongs to the standard type of cpDNA in angiosperms.

Polymorphisms in cpDNAs among *Pyrus* species

To detect the structural alterations of cpDNA among *Pyrus* species, RFLP analysis was applied to DNA from 11 accessions of five *Pyrus* species and two *Prunus* species (Table 2). Total DNAs of 11 accessions were digested with the same five restriction enzymes as used for the physical map construction. Southern hybridization was performed with 13 phage clones obtained from pear cpDNA as probes. The hybridization patterns obtained from the five *Pyrus* species were monomorphic except for two mutations; one was a site mutation and the other was a fragment length mutation. These two mutations were detected only in cpDNAs from *P. pyrifolia* (Fig. 4).

An additional *Sal*I site (14.9 kb + 7.1 kb = 22 kb) was detected only in 'Kousui', 'Choujuurou' and 'Nijisseiki' belonging to *P. pyrifolia* in the region corresponding to the 22 kb S-2 fragment shown in the physical map when the clone pPhcp-8 was used as a probe (Figs. 4 and 5a). This additional site mutation occurred in a region between the *psb*B gene (encoding CP47 of photosystem II) and the *pet*B gene (encoding cytochrome b6). Iketani et al. (1998) reported that an additional *Sal*I site was generated in cultivars of *P. pyrifolia* (genome type: BBB). The same site mutation was mapped on the cpDNA in *P. ussuriensis* var. *hondoensis* in the present study.

Fig. 4 Comparison of the gene order of cpDNA in pear, tobacco, *A. thaliana* and spinach. The upper square (□) and the triangle (▲) in the enlargement indicate the deletion and the site mutation respectively observed in *P. pyrifolia*. B, X and S in the enlargement indicate restriction sites of *Bam*HI, *Xho*I and *Sal*I respectively. The *brackets* at the bottom indicate the large inversions in comparison with *a L. japonicus, b O. elata* and *c* Gramineous plants
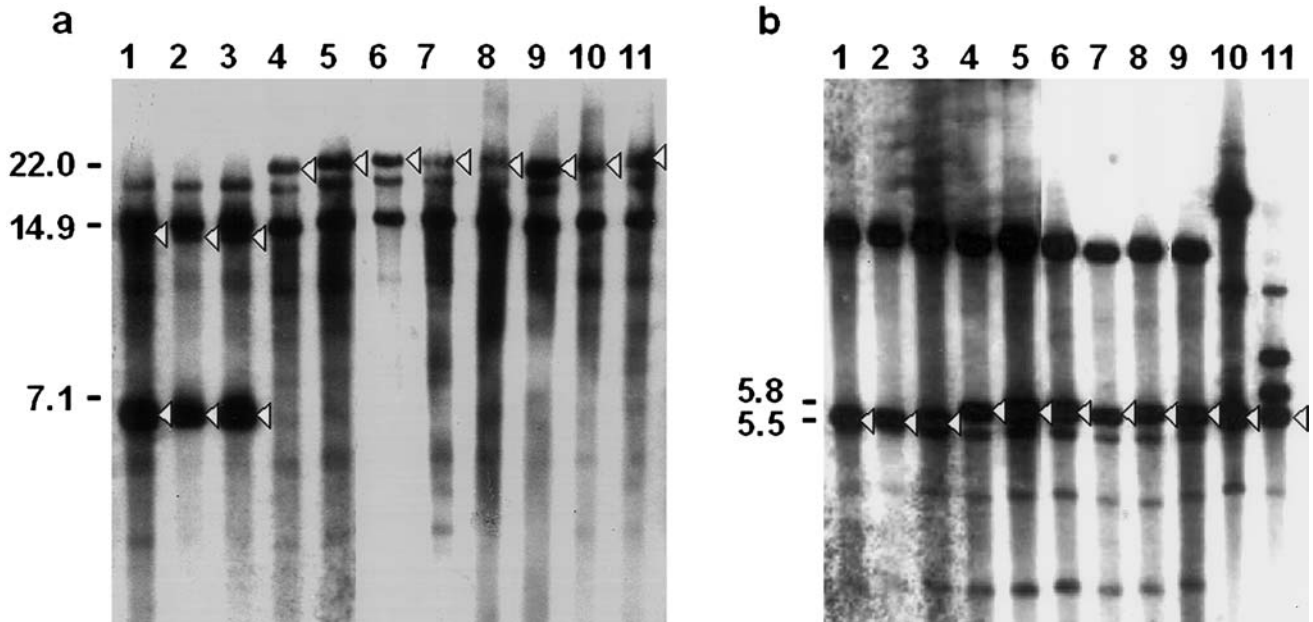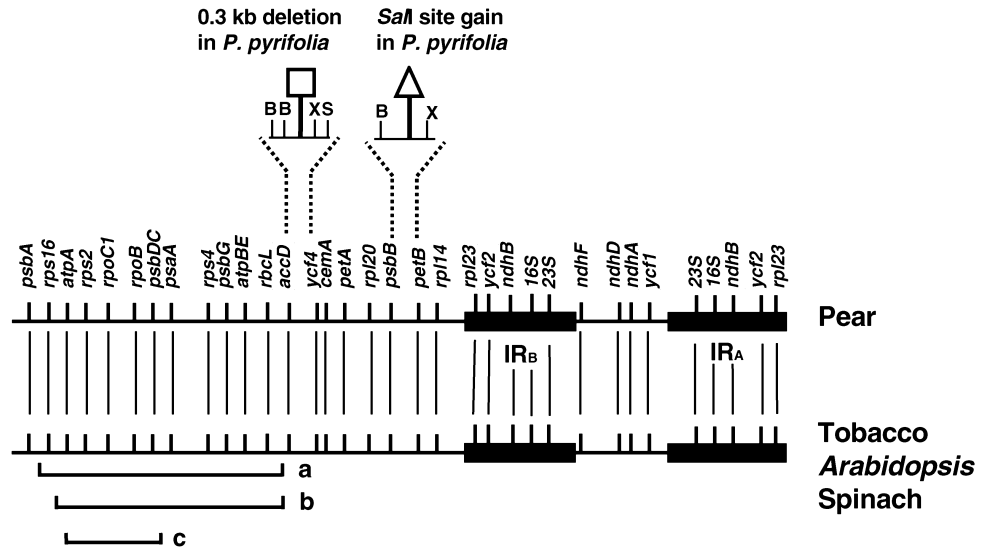

Fig. 5 Southern-blot analysis of total DNA from five *Pyrus* species and two *Prunus* species. **a** Digested with *Sal*I and probed with the pear cpDNA clone pPhcp-8. **b** Digested with *Bam*HI and probed with pPhcp-6. *Lane 1 P. pyrifolia* 'Kousui', *lane 2 P. pyrifolia* 'Choujuurou', *lane 3 P. pyrifolia* 'Nijisseiki', *lane 4 P. ussuriensis* var. *hondoensis, lane 5 P. ussuriensis* var. *aromatica, lane 6 P. calleryana, lane 7 P. elaeagrifolia, lane 8 P. communis* 'La France', *lane 9 P. communis* 'Bertlett', *lane 10 P. persica* 'Hakuhou', *lane 11 Prunus avium* 'Satounishiki'

A fragment length mutation was detected only in *P. pyrifolia* in the region corresponding to the Sl-5, X-5, B-6, Sc-6 and P-1 fragments shown in the physical map when the clone pPhcp-6 was used as a probe. This region of 'Kousui', 'Choujuurou' and 'Nijisseiki' was about 300 bp shorter (5.5 kb) compared to the corresponding region (5.8 kb) of the other *Pyrus* species and the two *Prunus* species (Figs. 4 and 5b). The Maloideae, including *Pyrus*, are thought to have arisen from an ancestral group which was supposed to be an allopolyploid of the Prunoideae and Spiraeoideae (Challice 1974). Our results indicate that the longer fragment was present in an ancestor common to *Pyrus* and *Prunus* species, and that the fragment length mutation occurred only in *P. pyrifolia* during the course of *Pyrus* differentiation. The alteration therefore appears to be a deletion. This deletion was mapped to a region between the *acc*D gene and *ycf*4 (hypothetical chloroplast reading frame) (Fig. 4). The region between the *rbc*L gene and the *cem*A gene (encoding an envelope membrane protein) including the *acc*D gene and *ycf*4 is known to be a recombinational hot spot in gramineous plants (Ogihara et al. 1988; Morton and Clegg 1993). This region of cpDNA may also be a hot spot region for recombination in *Pyrus*.

The two mutations, a site mutation and a deletion, are coupled and might have occurred simultaneously in the lineage giving rise to *P. pyrifolia*. However we need to

analyse cpDNA from other varieties in *P. pyrifolia* and other *Pyrus* species to determine whether these mutations occurred independently or not. Structural alterations such as deletions are useful tools to reveal the origin of *P. pyrifolia*.

The base substitution rate, p per base pair, was calculated concerning the 109 recognition sites investigated in this study after Brown et al. (1979). The value of $100 \times p$, which represents the substitution rate per 100 base pairs, was 0.15 in *Pyrus* cpDNA. It is lower than those of other taxa, i.e. 0.38 in *Pisum*, 0.87 in *Brassica* and 0.66 in *Triticum-Aegilops* (Palmer et al. 1983, 1985; Ogihara and Tsunewaki 1988). This result reveals the relatively low cpDNA diversity in *Pyrus* species. It is consistent with results indicating low genetic diversity in *Pyrus* species by Iketani et al. (1993, 1998). The low genetic diversity in *Pyrus* cpDNA might reflect its recent origin from a common ancestral population. Also further sequence analyses of nuclear genes are important to clarify the total genetic diversity in *Pyrus*.

References

Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76:1967–1971

Challice JS (1974) Rosaceae chemotaxonomy and the origin of the Maloideae. Bot J Linn Soc 69:239–259

Challice JS, Westwood MN (1973) Numerical taxonomic studies of the genus *Pyrus* using both chemical and botanical characters. Bot J Linn Soc 67:121–148

Downie SR, Palmer JD (1992) Use of chloroplast DNA rearrangements in reconstructing plant phylogeny. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 14–35

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Doyle JJ, Davis JI, Soreng RJ, Garvin D, Anderson MJ (1992) Chloroplast inversion and the origin of the grass family (Poaceae). Proc Natl Acad Sci USA 89:7722–7726

Hachtel W, Neuss A, Stein JV (1991) A chloroplast DNA inversion marks an evolutionary split in the genus *Oenothera*. Evolution 45:1050–1052

Hedrick UP (1921) The pear of New York. State New York, Department Agriculture, 29th Annual Report 2

Hiratsuka J, Shimada H, Whittier R, Ishibashi T, Sakamoto M, Mori M, Kondo C, Honji Y, Sun Cr, Meng BY, Li YQ, Kanno A, Nishizawa Y, Hirai A, Shinozaki K, Sugiura M (1989) The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol Gen Genet 217:185–194

Hupfer H, Swiatek M, Hornung S, Herrmann RG, Maier RM, Chiu W-L, Sears B (2000) Complete nucleotide sequence of the *Oenothera elata* plastid chromosome, representing plastome I of the five distinguishable Euoenothera plastomes. Mol Gen Genet 263:581–585

Iketani H (1993) Chloroplast DNA diversity in *Pyrus* and related genera. Gamma Field Symp 32:63–69

Iketani H, Manabe T, Matsuta N, Hayashi T (1993) Restriction fragment length polymorphism diversity of mitochondrial DNA in *Pyrus* and related genera. In: Hayashi T et al. (eds) Techniques on gene diagnosis and breeding in fruit trees. FTRS, Japan, pp 47–55

Iketani H, Manabe T, Matsuta N, Akihama T, Hayashi T (1998) Incongruence between RFLPs of chloroplast DNA and morphological classification in east asian pear (*Pyrus* spp.). Genet Res Crop Evol 45:533–539

Kajiura I, Nakajima M, Sakai Y, Oogaki C (1983) A species-specific flavonoid from *Pyrus ussuriensis* Max. and *Pyrus aromatica* Nakai et Kikuchi, and its geographical distribution in Japan. Japan J Breed 33:1–14

Kato T, Kaneko T, Sato S, Nakamura Y, Tabata S (2000) Complete structure of the chloroplast genome of a Legume, *Lotus japonicus*. DNA Res 7:323–330

Katayama H, Ogihara Y (1993) Structural alterations of the chloroplast genome found in grasses are not common in monocots. Curr Genet 23:160–165

Katayama H, Ogihara Y (1996) Phylogenetic affinities of the grasses to other monocots as revealed by molecular analysis of chloroplast DNA. Curr Genet 20:527–581

Kikuchi A (1924) On the origin of Japanese pear and the inheritance of the skin colors of their fruits. Japan J Genet 3:1–21

Kikuchi A (1946) Classification of the strains and varieties of the Chinese pear. Studies from the Inst Hort, Kyoto Imper Univ 3:1–11 (in Japanese)

Kolodner R, Tewari KK (1975) The molecular size and conformation of the chloroplast DNA from higher plants. Biochim Biophys Acta 402:372–390

Maier RM, Neckermann K, Igloi GL, Kossel H (1995) Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. J Mol Biol 251:614–628

Morton BR, Clegg MT (1993) A chloroplast DNA mutational hotspot and gene conversion in a noncoding region near *rbc*L in the grass family (Poaceae). Curr Genet 24:357–365

Nakai T (1916) Flora sylvatica Koreana. Pars VI. Pomaceae. The Government of Chosen

Nakai T (1918) Notulae ad plantas Japoniae et Koreae XVI. Bot Mag Tokyo 32:28–37

Nakai T (1919) Notulae ad plantas Japoniae et Koreae XXI. Bot Mag Tokyo 33:193–216

Ogihara Y, Tsunewaki K (1982) Molecular basis of the genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. I. Diversity of the chloroplast genome and its lineage revealed by the restriction pattern of ctDNAs. Jpn J Genet 57:371–396

Ogihara Y, Tsunewaki K (1988) Diversity and evolution of chloroplast DNA in *Tritucum* and *Aegilops* as revealed by restriction fragment analysis. Theor Appl Genet 76:321–332

Ogihara Y, Terachi T, Sasakuma T (1988) Intramolecular recombination of the chloroplast genome mediated by short direct-repeat sequences in wheat species. Proc Natl Acad Sci USA 85:8573–8577

Ogihara Y, Isono K, Kojima T, Endo A, Hanaoka M, Shiina T, Terachi T, Utsugi S, Murata M, Mori N, Takumi S, Ikeo K, Gojobori T, Murai R, Murai K, Matsuoka Y, Ohnishi Y, Tajiri H, Tsunewaki K (2002) Structural features of a wheat plastome as revealed by complete sequencing of chloroplast DNA. Mol Genet Genomics 266:740–746

Ohwi J, Kitagawa M (1983) New flora of Japan. Shibundo Publishers, Tokyo, pp 878–879 (in Japanese)

Ohyama K, Fukuzawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono K, Shiki Y, Takeuchi M, Chang Z, Aota S, Inokuchi H, Ozeki H (1986) Chloroplast gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322:572–574

Palmer JD (1991) Plastid chromosome: structure and evolution. In: Bogorad L, Vasil IK (eds) The molecular biology of plastids. Academic Press, San Diego, pp 5–53

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181–189

Palmer JD, Jorgensen RA, Thompson WF (1985) Chloroplast DNA variation and evolution in *Pisum*; patterns of change and phylogenetic analysis. Genetics 109:195–213

Palmer JD, Jansen RK, Micaels HJ, Chase MW, Manhart JR (1988) Chloroplast DNA variation and plant phylogeny. Ann Missouri Bot Gard 75:1180–1206

Rehder A (1915) Synopsis of the Chinese species of *Pyrus*. Proc Am Acad Art and Sci 50:225–241

Reiter RS, Young RM, Scolnik PA (1992) Genetic linkage of the *Arabidopsis* genome: methods for mapping with recombinant inbreds and random amplified polymorphic DNAs. In: Koncz C, Chua NH, Schell J (eds) Methods in *Arabidopsis* research. World Scientific, Singapore, pp 170–190

Rubtsov GA (1944) Geographical distribution of the genus *Pyrus* and trends and factors in its evolution. Am Nat 78:358–366

Sato S, Nakamura Y, Kaneko T, Asamizu E, Tabata S (1999) Complete structure of the chloroplast genome of *Arabidopsis thaliana* DNA Res 6:283–290

Schmitz-Linneweber C, Maier RM, Alcaraz JP, Cottet A, Herrmann RG, Mache R (2001) The plastid chromosome of spinach (*Spinacia oleracea*): complete nucleotide sequence and gene organization. Plant Mol Biol 45:307–315

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsubayashi T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kato A, Tohdoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence of the tobacco chloroplast genome; its gene organization and expression. EMBO J 5:2043–2049

Wakasugi T, Tsudzuki J, Ito S, Nakashima K, Tsudzuki T, Sugiura M (1994) Loss of all *ndh* genes as determined by sequencing the entire chloroplast genome of the black pine *Pirus thunbergii*. Proc Natl Acad Sci USA 91:9794–9798

Wolfe KH, Morden CW, Palmer JD (1992) Function and evolution of a minimal plastid genome from a nonphotosynthetic parasitic plant. Proc Natl Acad Sci USA 89:10,648–10,652

Yu TT (1979) Taxonomy of the fruit tree in China. Agriculture Press, Beijing, (in Chinese)

Zabarovsky ER, Allikmets RM (1986) An improved technique for the efficient construction of gene libraries by partial filling-in of cohesive ends. Gene 42:119–123