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O. P. Rajora · B. P. Dancik

Chloroplast DNA variation in *Populus*. II. Interspecific restriction fragment polymorphisms and genetic relationships among *Populus deltoides*, *P. nigra*, *P. maximowiczii*, and *P.* × *canadensis*

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Abstract Restriction fragment analysis was conducted to determine interspecific chloroplast DNA (cpDNA) variation and genetic relationships among *Populus deltoides*, *P. nigra*, *P.* × *canadensis* (*P. deltoides* × *P. nigra*), and *P. maximowiczii*. Total cellular DNAs of these poplars were digested with 16 restriction endonucleases, and Southern blots of the restriction digests were probed with six different cloned cpDNA fragments from *Petunia*. *P. deltoides*, *P. nigra*, and *P. maximowiczii* each had a distinct chloroplast genome, separated by many restriction-site and restriction-fragment-length mutations, predominantly in the large single-copy region of the genome. *P.* × *canadensis* shared the same cpDNA restriction fragment patterns as *P. deltoides* var. *deltoides*. *P. nigra* was most diverged from *P. deltoides*, and *P. deltoides* showed close cpDNA relationships to *P. maximowiczii*. Nucleotide substitutions per site in cpDNA were 0.0036 between *P. deltoides* and *P. maximowiczii*, 0.0071 between *P. nigra* and *P. maximowiczii*, and 0.0077 between *P. deltoides* and *P. nigra*. We suggest that *P. nigra* should be classified in a new separate section, the *Nigrae*.

Key words Poplars · Chloroplast DNA
Phylogenetics · Interspecific variation
Restriction fragment polymorphisms

Introduction

Several features of the chloroplast (cp) genome, such as its small size, its conservative mode of evolution, its predominant uniparental mode of inheritance, together with abundance of chloroplast DNA (cpDNA) in plant tissues (Palmer 1987; Zurawski and Clegg 1987; Palmer et al. 1988), make cpDNA an extremely valuable molecule for interspecific genetic, evolutionary and phylogenetic studies in plants (Palmer 1987; Palmer et al. 1988; Crawford 1990; Clegg and Zurawski 1992). Variation in cpDNA has received great attention in forest trees and has been increasingly used for these and for other genetic and biosystematic investigations (reviews in Strauss et al. 1992; Wagner 1992).

The genus *Populus* L. (Salicaceae), consisting of about 30 species, is divided into six sections (Ekenwalder 1977; Dickmann and Stuart 1983). Placement within a section traditionally has been based on morphology and reproductive characters, and interspecific crossability (FAO 1958, 1979; Zsuffa 1975). Members of the same section have the ability to hybridize with each other, either naturally or artificially (Zsuffa 1975). *Populus* species of the *Aigeiros* Duby and *Tacamahaca* Spach. sections are related and sexually compatible (FAO 1958, 1979; Eckenwalder 1977). Natural hybridization occurs among several species of these sections (Zsuffa 1975). Therefore, knowledge of genetic relationships of the *Populus* species of these sections is of primary biological importance.

Populus deltoides Bartr ex. Marsh. (eastern and plains cottonwoods of North America), and *P. nigra* L. (European black poplar) of the section *Aigeiros*, and *P. maximowiczii* Henry (Japanese poplar) of the section *Tacamahaca*, are important both biologically and economically for the breeding of the hybrid poplar varieties used in intensive poplar culture programs (Dickmann and Stuart 1983). These three *Populus* species have geographically separate natural ranges on three different continents (*P. deltoides* in North America, *P. nigra* in Europe and western Asia, and *P. maximowiczii* in northeastern Asia) (FAO 1979), and are sexually compatible with each other (Zsuffa 1975). Natural hybridization between *P. deltoides* and *P. nigra* has given rise to *P.* × *candensis* Moench syn. *P.* × *euramericana* (Dode) Guinier (Zsuffa 1975). Although *P. deltoides* and *P. nigra* are classified within the same section, these species can hybridize in one direction only, namely *P. deltoides* (♀) × *P. nigra* (♂) (Zsuffa 1975). *Populus maximowiczii*, however, is sexually compatible with both *P. deltoides* and *P. nigra* in both directions (Zsuffa 1975).

Populus nigra was noted to be distinctive from the North American species of the section *Aigeiros*, showing resemblance to the balsam poplars of the section *Tacamahaca* (Eckenwalder 1977). Other recent studies have also highlighted the differences of *P. nigra* from other *Aigeiros* poplar species. In previous studies, one of us (Rajora) examined the genetic relationships among *P. deltoides*, *P. nigra*, and *P. maximowiczii* based on allozymes, leaf morphology, and interspecific pollen competition (Rajora 1986, 1989; Rajora and Zsuffa 1990). In controlled pollination studies, *P. deltoides* exhibited higher reproductive affinities to *P. maximowiczii* than to *P. nigra* (Rajora 1989). Genetic distances based on allozyme and leaf morphological

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O. P. Rajora (✉) · B. P. Dancik
Department of Forest Science, University of Alberta, Edmonton, Alberta T6G 2H1, Canada

data placed *P. nigra* closer to *P. maximowiczii* than to its consectional *P. deltoides* (Rajora 1986; Rajora and Zsuffa 1990). Additionally, the cp genome of *P. nigra* was found to be distinct from that of other species within its own section, but showing higher similarities to the cp genome of *P. alba* L. of the section *Leuce* Duby (Smith and Sytsma 1990). However, this study did not include *P. maximowiczii* and *P.* × *canadensis*.

We aimed to determine interspecific cpDNA and mitochondrial DNA (mtDNA) variation, and further resolve the genetic relationships among *P. deltoides, P. nigra, P. maximowiczii*, and *P.* × *canadensis* based on cpDNA and mtDNA polymorphisms. Both cpDNA and mtDNA were found to follow a maternal mode of inheritance in interspecific crosses of these *Populus* species (Rajora and Dancik 1992; Rajora et al. 1992). The results on mtDNA variation and the genetic relationships of these poplars were reported earlier (Barrett et al. 1993). *P. nigra* was found to be least diverged from *P. tremuloides* Michx. of the section *Leuce*, and most diverged from *P. deltoides* and *P.* × *canadensis*, based on mtDNA variation. In this paper, we present the results on interspecific cpDNA variation and on the genetic relationships among *P. deltoides, P. nigra, P. maximowiczii*, and *P.* × *canadensis*.

Materials and methods

Poplar material

Fourteen individuals of *P. deltoides* representing var. *deltoides* and var. *occidentalis*, 13 individuals of *P. nigra* representing var. *nigra*, var. *italica*, and var. *plantierensis*, and eight individuals of *P. maximowiczii*, were sampled and are described in an accompanying paper (Rajora and Dancik 1994a). *P.* × *canadensis* samples consisted of 17 cultivars: 'Baden 431','Blanc du Poitou', 'Canada Blanc', 'Dorskamp 925', 'Eugenei', 'Gelrica', 'Grandis', 'Heidemij', 'I-55/56', 'I-132/56', 'I-214', 'Jacometti', 'Ostia', 'Regenerata', 'Robusta', 'Steckby', and 'Zurich 03/3' (Rajora and Zsuffa 1989). These cultivars originated in France, Italy, The Netherlands, Germany and Spain (Rajora and Zsuffa 1989). Each cultivar was represented by one individual clone procured from the Ontario Forest Research Institute, Ontario Ministry of Natural Resources, Maple, Ontario. The cultivars were propagated in a greenhouse at the University of Alberta by rooting of their shoot cuttings.

DNA extraction and chloroplast DNA restriction fragment analysis

The methods used for DNA extraction, restriction, electrophoresis, Southern blotting, cpDNA probe preparation, molecular hybridization, and autoradiography are described in an accompanying (Rajora and Dancik 1994a) or an earlier (Rajora and Dancik 1992) paper. Sixteen restriction enzymes and six heterologous cpDNA probes from *Petunia hybrida*, with 83 enzyme-probe combinations, as described in Rajora and Dancik (1994a), were used to determine interspecific cpDNA restriction fragment variation in *Populus*.

Data analysis

The proportion of the cpDNA restriction fragments shared by any two species (F) was determined. F values were then used to estimate the number of nucleotide substitutions per site (Nei 1987) in a pairwise fashion among the *Populus* species. The unweighted pair-group method with arithmetic mean (UPGMA; Sneath and Sokal 1973) for nucleotide substitutions per site from fragment data (Nei 1987) was used to determine the clustering of the *Populus* species based on cpDNA nucleotide divergence.

Results

Eighty-three enzyme-probe combinations yielded 278 restriction sites in *P. deltoides*, 272 in *P. nigra*, and 282 in *P. maximowiczii*, with a total of 286 different restriction sites over all three species. Of these sites, 242 were shared by all three *Populus* species examined. Intraspecific cpDNA variation was observed among individuals within *P. deltoides, P. nigra*, and *P. maximowiczii*, and is described in an accompanying paper (Rajora and Dancik 1994a). Knowledge of this intraspecific cpDNA variation, particularly that between var. *deltoides* and var. *occidentalis* of *P. deltoides*, revealed by the *Cla*I-P6 and *Eco*RI-P6 enzyme-probe combinations (Rajora and Dancik 1994a), facilitated the correct interpretation of interspeci- fic cpDNA variation among the *Populus* species (Table 1).

Each of *P. deltoides*, *P. nigra*, and *P. maximowiczii* showed distinct autoradiographic restriction fragment patterns of cpDNA (Figs. 1 and 2). Interspecific cpDNA variation was observed among these three *Populus* species for 20 combinations of 11 restriction enzymes (*Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sma*I, and *Xba*I) and five probes (P3, P6, P8, P10 and P12) (Table 1). *Bcl* I, *Bgl* II, *Eco*RI, and *Xba*I restriction digests showed the majority of the observed interspecific cpDNA variation (Table 1). *Bgl*II restriction digests showed interspecific cpDNA variation among the *Populus* species for four probes, *Eco*RI for three probes, *Bam*HI, *Bcl* I, *Hin*dIII, and *Xba*I for two probes each, and *Ava*I, *Cla*I, *Eco*RV, *Pst*I, and *Sma*I for one probe each (Table 1). No interspecific cpDNA variation among the *Populus* species was observed for the restriction digests of *Kpn*I, *Pvu*II, *Sac*I, *Sal*I, and *Xho*I, and for the probe S8. The 20 restriction enzyme-probe combinations revealed a total of 45 restriction fragment polymorphisms (RFPs) among *P. deltoides*, *P. nigra*, and *P. maximowiczii* (Table 1; Figs. 1 and 2). Fifteen of these RFPs were inferred to be restriction-site polymorphisms (RSPs) and 30 to be restriction-fragment-length polymorphisms (RFLPs) (Table 1). The RSPs were characterized by gain or loss of a restriction site, whereas, with the exception of the *Bcl*I-P6 RFLP in *P. nigra*, RFLPs were characterized by fragment length differences of 50 bp to 800 bp in *P. nigra* (Table 1). The exceptional RFLP may be due to the gain of a *Bcl*I restriction site within the 15.6-kb fragment observed in *P. deltoides* and *P. maximowiczii*, resulting in two fragments in *P. nigra*, one of 10.2 kb with homology to the *Petunia* cpDNA fragment P6 (thus detected) and another of 5.4 kb with no homology with P6 (thus not detected). The RFLPs observed may have resulted from deletion and insertion phenomena. However, restriction-site mapping of cpDNA in *Populus* is required to verify the cause of the interspecific RFLPs.

Table 1 Interspecific chloroplast DNA (cpDNA) restriction-fragment polymorphisms and mutations observed among *P. deltoides* (PD), *P. nigra* (PN) and *P. maximowiczii* (PM).- = absent; RS = restriction-site mutation; RFL = restriction-fragment-length mutation; RSP = restriction-site polymorphism; RFLP = restriction-fragment-length polymorphism; LSC = large single copy; IR = inverted repeat

(A) Details of the restriction-fragment polymorphisms

Genome region	Restriction enzyme	Probe	cpDNA restriction fragments				Taxa	Mutations
			Total number	Variant/changed fragments (size in kb)				Type (number)
1. Large single-copy (LSC) region								
1.1 L SC region homologous to the 21-kb *Pst*I fragment of *Petunia* cpDNA								
	*Bcl*I	P3	4	18.3,	7.6,	7.2	PD, PM	RS (2); RFL (1)
			6	9.15, 9.15	7.7,	3.6, 3.6	PN	
	*Eco*RI	P3	4	5.0	–		PD	RFL (1); RS (1)
			5	4.8,	4.2		PN, PM	
	*Hin*dIII	P3	5	10.3,	1.4		PD, PM	RS (1); RFL (1)
			4	12.8	–		PN	
	*Xba*I	P3	6	4.6			PD	RS (1); RFL (1)
			5	–			PN	
			6	5.0			PM	
1.2 L SC region homologous to the 15.3-kb *Pst*I fragment of *Petunia* cpDNA								
	*Ava*I	P6	7	5.8			PD	RFL (2)
			7	5.3			PN	
			7	5.6			PM	
	*Bam*HI	P6	5	–	5.8,	4.8	PD	RS (1); RFL (4)
			5	–	5.0,	4.6	PN	
			6	6.1,	5.2,	4.7	PM	
	*Bcl*I	P6	4	15.6,	4.5, 2.75,	1.40	PD	RS (1); RFL (4)
			3	10.2,	7.25,	1.29	PN	
			4	15.6,	4.4, 2.75,	1.33	PM	
	*Bgl*II	P6	5	3.9			PD, PM	RFL (1)
			5	3.8			PN	
	*Cla*I	P6	7	5.6,	3.2/3.1		PD	RFL (2)
			6	5.3,	2.9		PN	
			7	5.6,	3.1		PM	
	*Eco*RI	P6	9	1.6,	1.0/0.9		PD	RFL (2)
			8	1.4,	0.8		PN	
			8	1.6,	1.0		PM	
	*Eco*RV	P6	8	–	1.9,	1.3	PD	RS (1); RFL (2)
			8	–	1.8,	1.2	PN	
			9	10.3,	1.9,	1.3	PM	
	*Hin*dIII	P6	3	6.4			PD, PM	RFL (1)
			3	6.0			PN	
	*Sma*I	P6	2/3	–			PD, PN	RS (1)
			3	8.0			PM	
	*Xba*I	P6	10	4.5, 2.0	3.7,	0.7	PD	RS (2); RFL (4)
			8	6.7,	3.5,	–	PN	
			10	5.0, 2.0,	3.6,	0.7	PM	
1.3 LSC region homologous to the 9.2-kb *Pst*I fragment of *Petunia* cpDNA								
	*Bgl*II	P8	5	5.5, 1.4	2.2, 0.7		PD	RS (2)
			3	6.9,	2.9		PN	
			4	5.5, 1.4,	2.9		PM	
1.4 LSC region homologous to the 9.0-kb *Pst*I fragment of *Petunia* cpDNA								
	*Bam*HI	P10	5	1.7/1.61			PD	RFL (1)
			4	1.58			PN, PM	
	Bg/II	P10	4	6.9,	5.6		PD, PM	RFL (1); RS(1)
			3	7.1	–		PN	
	*Eco*RI	P10	5	1.31			PD	RFL (1)
			5	1.26			PN, PM	
	*Pst*I	P10	1	–			PD, PN	RS (1)
			2	3.5			PM	
2. Inverted repeat (IR) region								
	*Bgl*II	P12	5	6.5			PD, PM	RFL (1)
			5	6.7			PN	

(B) Summary of the number of restriction-fragment polymorphisms between any two *Populus* species

Region	Probe	PD-PN			PD-PM			PN-PM		
		RSP	RFLP	Total	RSP	RFLP	Total	RSP	RFLP	Total
LSC	P3	5	3	8	1	2	3	4	2	6
LSC	P6	3	15	18	3	7	10	5	16	21
LSC	P8	2	0	2	1	0	1	1	0	1
LSC	P10	1	3	4	1	2	3	2	1	3
IR	P12	0	1	1	0	0	0	0	1	1
Total		11	22	33	6	11	17	12	20	32

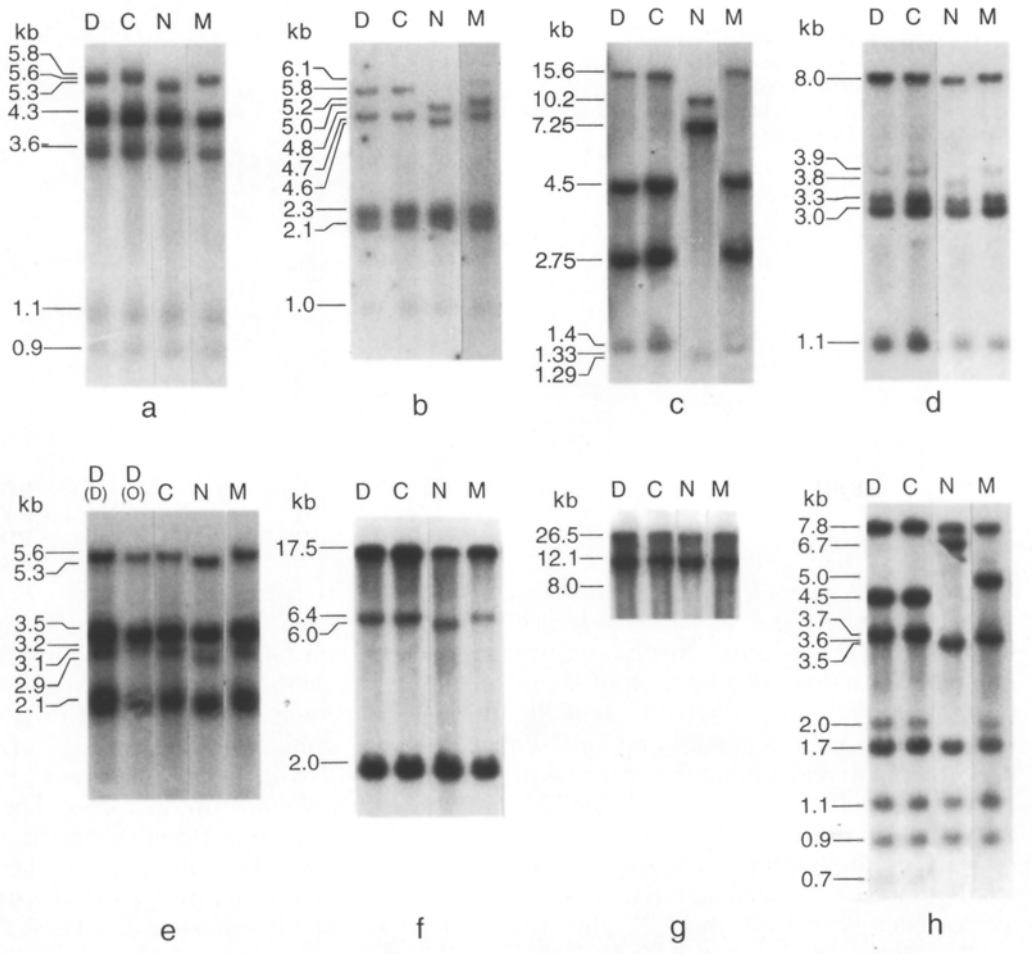

Fig. 1a–h Autoradiographic restriction-fragment patterns of chloroplast DNA (cpDNA) of *Populus* demonstrating interspecific variation in the large single-copy region homologous to the 15.3-kb *Petunia* cpDNA fragment P6, among *P. deltoides* (*D*), *P.* × *canadensis* (*C*), *P. nigra* (*N*), and *P. maximowiczii* (*M*). *D* (*D*) = *P. deltoides* var. *deltoides*, *D* (*O*) = *P. deltoides* var. *occidentalis*. *Populus* DNAs were restricted with (**a**) *Ava*I, (**b**) *Bam*HI, (**c**) *Bcl*I, (**d**) *Bgl*II, (**e**) *Cla*I, (**f**) *Hin*dIII, (**g**) *Sma*I and (**h**) *Xba*I, and Southern blots of their restriction digests were hybridized with the 15.3-kb *Petunia* cpDNA fragment P6

The observed restriction fragment polymorphisms in *Populus* were tentatively assigned to the same region as the cp genome location of the *Petunia* cpDNA probes because of the very high homologies suggested between the cpDNAs of *Populus* and *Petunia* by srong molecular hybridization, as is discussed in an accompanying paper (Rajora and Dancik 1994a). Comparative restriction-site mapping of the cp genomes in *P. deltoides*, *P. nigra*, and *P. maximowiczii* is required to determine the exact locations of the observed interspecific RFPs.

Of the total 45 interspecific RFPs, 44 were detected by the four *Petunia* cpDNA probes (P3, P6, P8 and P10) from the large singe-copy (LSC) region, and only one was detected by the probe P12 from the inverted-repeat (IR) region (Table 1). The large majority of the total interspecific RFPs among *P. deltoides*, *P. nigra*, and *P. maximowiczii* were observed in the LSC region homologous to the 15.3-kb *Petunia* cpDNA fragment P6 (Table 1; Fig. 1a–h). There were 28 interspecific RFPs (six RSPs and 22 RFLPs) in this region, nine (five RSPs and four RFLPs) in the region homologous to the *Petunia* cpDNA fragment P3 (Table 1; Fig. 2a–d), two (RSPs) in the region homologous to the *Petunia* cpDNA fragment P8 (Table 1; Fig. 2e), and five (two RSPs and three RFLPs) in the region homologus to the *Petunia* cpDNA fragment P10 (Table 1; Fig. 2f).

There were 33 diagnostic RFPs, including the only one in the IR region, between *P. deltoides* and *P. nigra*,

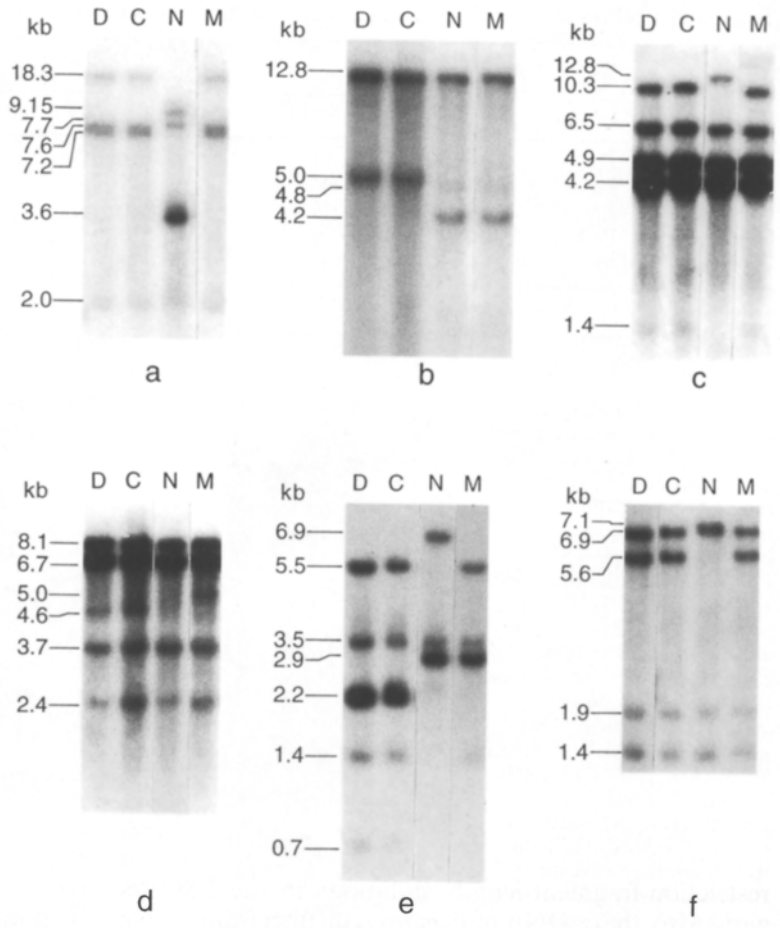

Fig. 2a–f Autoradiographic restriction-fragment patterns of chloroplast DNA (cpDNA) of *Populus* showing interspecific variation in the large single-copy region homologous to the *Petunia* cpDNA fragments P3 (**a, b, c** and **d**), P8 (**e**), and P10 (**f**) among *P. deltoides* (*D*), *P.* × *canadensis* (*C*), *P. nigra* (*N*), and *P. maximowiczii* (*M*). The restriction-fragment patterns were obtained by restricting *Populus* DNAs with (**a**) *Bcl*I, (**b**) *Eco*RI, (**c**) *Hin*dIII, (**d**) *Xba*I, and (**e** and **f**) *Bgl*II and hybridizing the Southern blots of the restriction digests with the *Petunia* cpDNA probe (**a, b, c** and **d**) P3–21-kb *Pst*I fragment, (**e**) P8–9.2-kb *Pst*I fragment, and (**f**) P10–9.0-knb *Pst*I fragment

17 between *P. deltoides* and *P. maximowiczii*, and 32 between *P. nigra* and *P. maximowiczii* (Table 1A, B). Restriction-site and/or restriction-fragment-length polymorphisms revealed by each of the seven enzyme-probe combinations (*Xba*I-P3, *Ava*I-P6, *Bam*HI-P6, *Bcl*I-P6, *Eco*RV-P6, *Xba*I-P6, and *Bgl*II-P8) unambiguously differentiated among the cpDNAs of *P. deltoides*, *P. nigra*, and *P. maximowiczii* (Table 1; Figs. 1a, b, c, h and 2d, e). *Populus* × *canadensis* cultivars exhibited the same autoradiographic restriction-fragment patterns of cpDNA as the suggested maternal contributor *P. deltoides* var. *deltoides* (Figs. 1 and 2). This was expected as cpDNA is maternally inherited in *P. deltoides* × *P. nigra* interspecific hybrids (Rajora and Dancik 1992). However, 12 cultivars had 1–5 novel cpDNA variants that were not observed in either *P. deltoides* or *P. nigra*. The data on these novel cpDNA fragments are presented in an accompanying paper (Rajora and Dancik 1994b). The natural *P.* × *canadensis* hybrids are considered to have originated as a result of hybridization between *P. deltoides* var. *deltoides*, whose maternal contribution is evident from both cpDNA (this study) and mtDNA (Barrett et al. 1993), and *P. nigra*, whose paternal contribution is supported by isozyme (Rajora and Zsuffa 1989) and DNA markers (unpublished data).

Estimates of the number of nucleotide substitutions per site ranged from 0.0006 to 0.0083 (Table 2). Among the three *Populus* species, *P. deltoides* was most diverged from *P. nigra* and most similar to *P. maximowiczii* based on estimates of restriction-fragment similarity and nucleotide substitutions per site (Table 2). Restriction-fragment patterns of cpDNA of five *P.* × *canadensis* cultivars ('Baden 431', 'Dorskamp 925', 'Eugenei', 'Heidemij', and 'Robusta') were identical to those of *P. deltoides* var. *deltoides*. The number of novel cpDNA fragments observed in the 12 *P.* × *canadensis* cultivars was taken into account when calculating the nucleotide divergence of *P.* × *canadensis* from *P. deltoides*, *P. nigra*, and *P. maximowiczii*. These *P.* × *canadensis* hybrids were most similar to *P. deltoides* and most diverged from *P. nigra* (Table 2).

The result from UPGMA cluster analysis (Fig. 3) were contrary to those expected on the basis of classical taxonomy for the consectional species *P. deltoides* and *P. nigra*. *P. deltoides* and *P.* × *canadensis* clustered closer to *P. maximowiczii* than to *P. nigra* (Fig. 3), suggesting *P. deltoides*' closer relationships to *P. maximowiczii* than to *P. nigra*.

Discussion

Our results demonstrate that each of the three species (*P. deltoides*, *P. nigra*, and *P. maximowizii*) has a distinct

Table 2 Chloroplast DNA restriction-fragment similarities (*F*) and nucleotide substitutions per site (*d*) among *P. deltoides*, *P. nigra*, *P. maximowiczii*, and *P.* × *canadensis*

Species		*P. deltoides*	*P. nigra*	*P. maximowiczii*
P. nigra	*F*	0.8721		
	d	0.0077		
P. maximowiczii	*F*	0.9378	0.8813	
	d	0.0036	0.0071	
P. × *canadensis*	*F*	0.9894	0.8627	0.9279
	d	0.0006	0.0083	0.0042

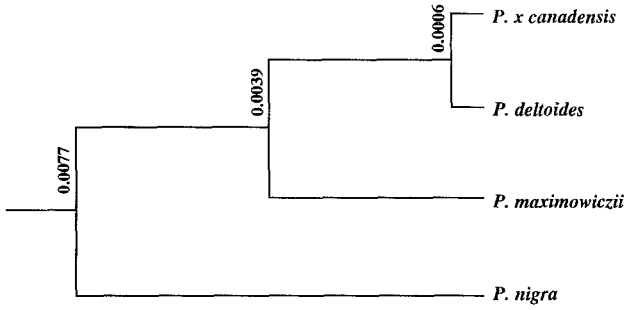

Fig. 3 A dendrogram of *Populus* species based on UPGMA analysis of chloroplast DNA nucleotide substitutions per site. The *numbers* at the nodes are estimates of nucleotide substitutions per site

chloroplast genome. Their cpDNA is differentiated by a number (17–33) of interspecific restriction-site and restriction-fragment-length mutations in the LSC region. Also, the cpDNA of *P. nigra* is distinct from that of *P. deltoides* and *P. maximowiczii* by a restriction-fragment-length mutation of 200 bp in the IR region. No differentiation of cpDNA between *P. deltoides* and *P. maximowiczii* was observed with the probe P12 from this IR region. *P. deltoides*, *P. nigra*, and *P. maximowiczii* can, therefore, be distinguished from each other by the restriction-fragment patterns of their cpDNA. These *Populus* species were also shown to have distinctive mitochondrial and nucler genomes (Rajora 1990; Rajora and Zsuffa 1990; Barrett et al. 1993).

Chloroplast DNA is maternally inherited in *P. deltoides* x *P. nigra* controlled crosses (Rajora and Dancik 1992). *Populus* × *canadensis*, a natural hybrid between *P. deltoides* and *P. nigra*, shared the same cpDNA restriction fragments as *P. deltoides* var. *deltoides*. None of the *P.* × *canadensis* cultivars/individuals was found to have cpDNA restriction fragments characteristic of either *P. deltoides* vàr. *occidentalis* or *P. nigra*. Therefore, results from this study confirm results from the mtDNA analysis (Barrett et al. 1993) that *P.* × *canadensis* received its maternal contribution from a *P. deltoides* var. *deltoides* progenitor. These results from both cpDNA and mtDNA are consistent with the historical information on the origin of *P. x canadensis* hybrids. These interpecific *Populus* hybrids are believed to have originated in France by free interbreeding of *P. deltoides* var. *deltoides*, imported from southeastern Canada, with the native *P. nigra* (Zsuffa 1975).

In our study, only 1 out of 45 interspecific cpDNA polymorphisms was observed with probe P12 from the IR region and the remaining 44 were revealed with the four probes from the LSC region (Table 1). In the IR region, one restriction-fragment-length mutation separated the cpDNA of *P. nigra* from that of *P. deltoides* and *P. maximowiczii*. No restriction-site mutation was observed in this region. These observations are consistent with the general observation in plants that the rate of nucleotide substitutions in the inverted repeat is greatly reduced in comparison to that of single-copy sequences (Wolfe et al. 1987; Palmer 1990).

The ratio of restriction-fragment-length mutations to nucleotide substitutions in our study was 2:1. This was due to the concentration of the restriction-fragment-length mutations in the region of the *Populus* chloroplast genome homologous to the *Petunia* cpDNA fragment P6 (Table 1; Fig. 1). The large majority of the interspecific cpDNA restriction-fragment polymorphisms were observed in this region, and the ratio of fragment-length mutations to nucleotide changes was 3.7:1. In other LSC regions probed, either the proportion of fragment-length mutations to restriction-site mutations was about equal or else no restriction-fragment-length mutations were observed. These results suggest that, in *Populus*, the region of the chloroplast genome homologous to the 15.3-kb *Petunia* cpDNA fragment P6 has evolved more rapidly than the other regions examined. It has been noted that non-coding regions of the chloroplast genome evolve more rapidly than do coding regions and that deletion/addition mutations accumulate in non-coding regions at a rate that is at least equal to that of nucleotide substitutions (Zurawski and Clegg 1987; Clegg and Zurawski 1992). The *Petunia* cpDNA fragment P6 comes the LSC region of the chloroplast genome which consists of a non-coding region (Palmer et al. 1983).

Estimates of nucleotide substitutions, as a measure of nucleotide divergence among the *Populus* species (Table 2), suggest that *P. deltoides* is least diverged from *P. maximowiczii* and most diverged from *P. nigra*; thus *P. deltoides* is more closley related to *P. maximowiczii* than to its consectional *P. nigra*. The higher cpDNA restriction-fragment similarities of *P. deltoides* to *P. maximowiczii* than to *P. nigra* are consistent with higher mtDNA restriction-fragment similarities and higher reproductive affinities of *P. deltoides* to *P. maximowiczii* than to *P. nigra* (Rajora 1989; Barrett et al. 1993). Therefore, the results suggest that *P. nigra* evolved along an evolutionary branch separate from that by which *P. deltoides* and *P. maximowiczii* have evolved.

A similar pattern of species relationships is evident among *P. deltoides*, *P. nigra* and *P. maximowiczii* when we compare our estimates of cpDNA divergence against the same values for mtDNA from the same individuals of these *Populus* species (Barrett et al. 1993). Nucleotide-substitution estimates (*d*) between *P. deltoides* (*PD*) and *P. nigra* (*PN*) and between *P. deltoides* and *P. maximowiczii* (*PM*) were similar for cpDNA (Table 2) and

mtDNA ($PD - PN = 0.0067$; $PD - PM = 0.0036$). Also, our estimate of nucleotide divergence between *P. deltoides* and *P. nigra* was similar to, but slightly lower than, that reported by Smith and Sytsma (1990) for cpDNA between these species ($d = 0.0095$). This may be due to the proportion of the respective genome examined. We used six probes covering 73.5 kb of the *Petunia* chloroplast genome, whereas Smith and Sytsma (1990) used probes covering the entire *Petunia* chloroplast genome (151.5 kb; Palmer et al. 1983) to examine poplar cpDNA. Our estimates of interspecific cpDNA nucleotide divergence in *Populus* are higher than those reported for *Salix* L. species (Brunsfeld et al. 1992) which belong to the same family, the Salicaceae.

P. nigra is more divergent from *P. deltoides* than it is from species in the section *Leuce* Duby for both cpDNA (Smith and Sytsma 1990) and mtDNA (Barrett et al. 1993). Smith and Sytsma (1990) suggest that for cpDNA this is the result of an ancestral hybridization event between a female member of the section *Leuce* (*P. alba*) and a "pre *P. nigra*" ancestor as the male contributor, resulting in transmission of organellar genomes from the maternal ancestor into the new hybrid, and continued transmission and maintenance of these organellar genomes through successive backcrossing of the hybrids to the paternal ancestor. Our results from the present study also suggest that *P. nigra* evolved along a different evolutionary path to that of *P. deltoides*. *P. deltoides* and *P. nigra* have lower reproductive affinities between themselves than each of these species has with members of the section *Tacamahaca*, including *P. maximowiczii* (Zsuffa 1975; Rajora 1989). Also *P. nigra* has higher allozyme and leaf morphological similarities to *P. maximowiczii* than to *P. deltoides* (Rajora 1986; Rajora and Zsuffa 1990). All these observations suggest that *P. nigra* warrants a reclassification separate from that of *P. deltoides*. We suggest that *P. nigra* should be classified in a separate new section, the *Nigrae*, within the genus *Populus*.

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