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Physical mapping of plastid DNA variation among eleven *Nicotiana* species

Y. Salts, R. G. Herrmann [1], N. Peleg, U. Lavi, S. Izhar, R. Frankel and J. S. Beckmann
Division of Plant Genetics and Breeding, ARO, The Volcani Center, Bet Dagan 50-250, Israel
[1] Botanisches Institut der Universität Düsseldorf, D-4000 Düsseldorf 1, Federal Republic of Germany

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Summary. Plastid DNA of seven American and four Australian species of the genus *Nicotiana* was examined by restriction endonuclease analysis using the enzymes *Sal* I, *Bgl* I, *Pst* I, *Kpn* I, *Xho* I, *Pvu* II and *Eco* RI. These endonucleases collectively distinguish more than 120 sites on *N. tabacum* plastid DNA. The DNAs of all ten species exhibited restriction patterns distinguishable from those of *N. tabacum* for at least one of the enzymes used. All distinctive sites were physically mapped taking advantage of the restriction cleavage site map available for plastid DNA from *Nicotiana tabacum* (Seyer et al. 1981). This map was extended for the restriction endonucleases *Pst* I and *Kpn* I. In spite of variation in detail, the overall fragment order was found to be the same for plastid DNA from the eleven *Nicotiana* species. Most of the DNA changes resulted from small insertions/deletions and, possibly, inversions. They are located within seven regions scattered along the plastid chromosome. The divergence pattern of the *Nicotiana* plastid chromosomes was strikingly similar to that found in the genus *Oenothera* subsection *Euoenothera* (Gordon et al. 1982). The possible role of replication as a factor in the evolution of divergence patterns is discussed. The restriction patterns of plastid DNA from species within a continent resembled each other with one exception in each instance. The American species *N. repanda* showed patterns similar to those of most Australian species, and those of the Australian species *N. debneyi* resembled those of most American species.

Key words: *Nicotiana* – Evolution of plastid DNA – Comparative restriction site mapping – Insertions and deletions

Abbreviations: ims = isonuclear male sterile; ptDNA = plastid, chloroplast DNA; Rubisco = ribulosebisphosphate carboxylase/oxygenase; kbp = kilobase pairs; LSU = large subunit of Rubisco

1 Introduction

Plastid and nuclear genomes interact in the production of important components including ribulose bisphosphate carboxylase/oxygenase (reviewed in Bottomley 1980), thylakoid membrane proteins (Herrmann et al. 1983) and plastid ribosomes (reviewed in Bedbrook and Kolodner 1979; Herrmann and Possingham 1980). This intracellular cooperation is highly specific. Genetic studies on interspecific hybrids from the genera *Nicotiana* (Frankel et al. 1979) or *Oenothera* (Stubbe 1959) have suggested that both organelles cannot be arbitrarily exchanged and that speciation in higher plants involves a coevolution of nuclear genome and plastome. A considerable intra- and interspecific variability in restriction patterns of ptDNA which can include changes in structural genes that may be directly involved in genome/plastome interactions possibly reflects this divergence. The large subunit of Rubisco encoded by the plastome, as well as the small subunit originating from nucleus/cytosol, have both been shown to differ among *Nicotiana* (Chen et al. 1976) and among *Oenothera* (v. Wettstein et al. 1978) species. Similarly, several variant polypeptides of thylakoid membranes in *Oenothera,* including components of the ATP synthase and the photosystem II reaction center, follow plastid or nuclear modes of inheritance (Herrmann et al. 1980b).

Plastid DNAs vary between different species belonging to the genus *Nicotiana* (Atchison et al. 1976; Vedel et al. 1976; Frankel et al. 1979; Rhodes et al. 1981). Recent reports of restriction maps for *N. tabacum* ptDNA (Jurgenson and Bourque 1980; Fluhr and Edelman 1981a; Seyer et al. 1981) establish a basis for an investigation into the nature of this variation and the location of these changes on the circular DNA molecule. This approach can provide information about plastome evolution in general, about *Nicotiana* ptDNA in particular, and aid in clarifying the relationships of plastomes within this genus. Moreover, evolutionary changes are not expected to occur at

equal rates all along a genome. Those parts of a genome that accumulate changes at faster rates may not fulfil essential functions. Therefore, defining expendable areas in plastid chromosomes could be of interest in genetic modification of these DNA molecules.

We report here the maps for ptDNA of eleven *Nicotiana* species, of both American and Australian origin. These maps include the location of variable and conserved regions in the *Nicotiana* plastid chromosomes.

2 Materials and methods

Plant material

The wild species, *ims* lines, and abbreviations for species used in this study are listed in Table 1. The *ims* lines *deb., meg., plu.,* and *sua.* were produced by repeated back crosses (17–22 generations) employing *N. tabacum* as the pollen parent for the four *Nicotiana* species denoted *ims* (Frankel et al. 1979). The line *ims debneyi* was derived from cultivar 'Red Russian', the remaining three were obtained from cultivar 'Hicks'. The ptDNAs of the *ims* lines were used as source for wild plastomes, as Frankel et al. (1979) have shown that *ims-deb., ims-meg., ims- plu.* and *ims-sua.* ptDNA gave restriction patterns indistinguishable from those of their maternal wild type. Hence their organelle DNA was regarded as being wild type.

Isolation of ptDNA

DNA was isolated from chloroplasts essentially as described in Salts and Beckmann (1981). Plant material was kept dark for 3–4 days to decrease starch content. Purified ptDNA was dissolved in H_2O (ca. 1 mg/ml), adjusted to 10 mM Tris/HCl, 0.5 mM EDTA (pH 8.0) and stored refrigerated.

Restriction endonuclease analysis

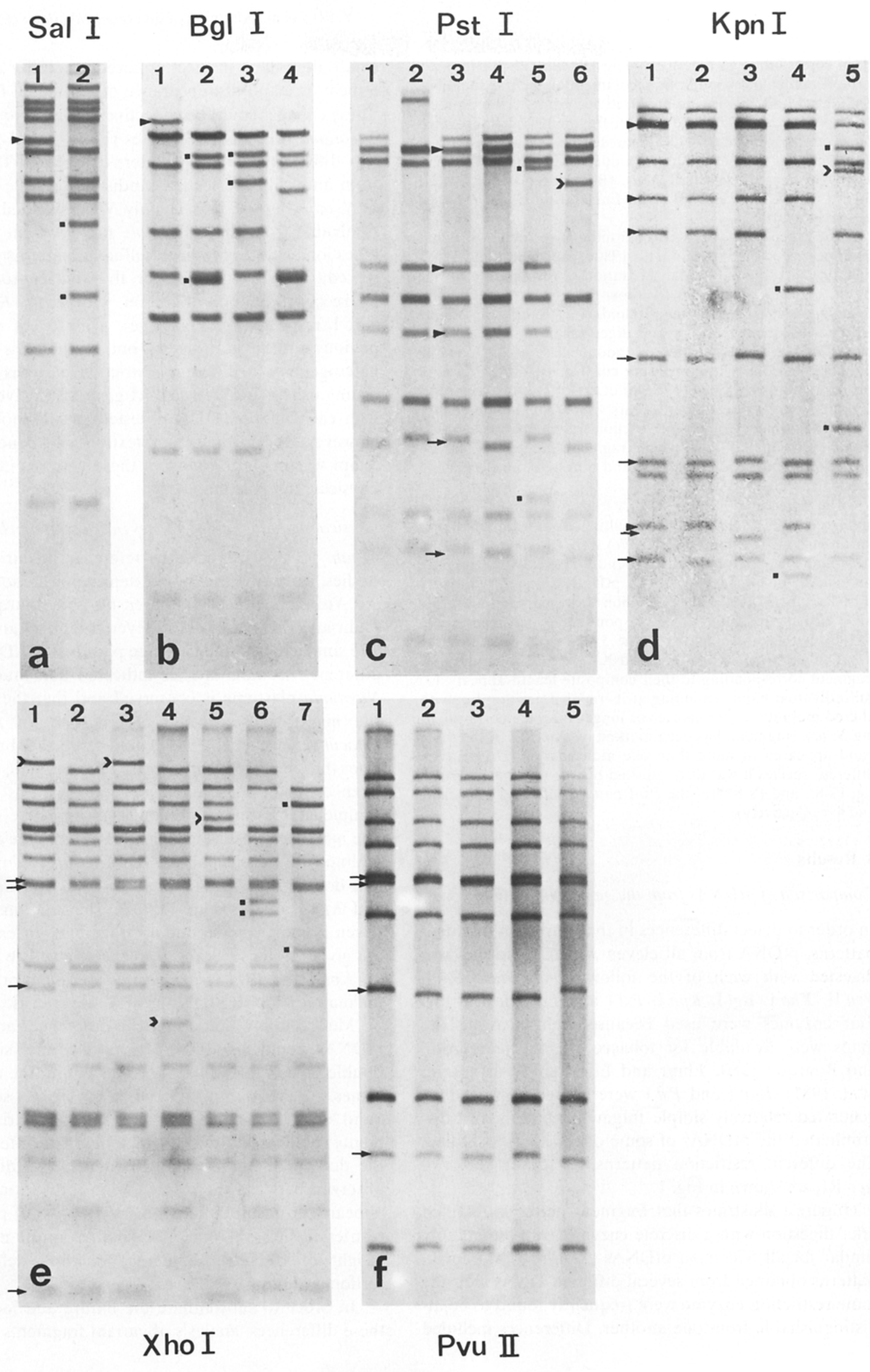
Restriction endonucleases *Bgl* I, *Xho* I, *Pvu* II, *Kpn* I and *Eco* RI were purchased from New England Biolabs (Bad Schwalbach) or Boehringer (Mannheim) and used individually

Table 1. Characteristics of *Nicotiana* species studied

Subgenus section	Species (abbreviations used)	Origin[a]	Chromosome no. (*n*)	Rubisco[b]	
				Large subunit	Small subunit
Rusticae					
Paniculatae	*N. glauca* (*gla.*)	Argentina – California	12	I	9′
	N. raimondii (*rai.*)	Peru	12	III	6, 10
Tabacum					
Tomentosae	*N. glutinosa* (*glu.*)	Peru – Chile	12	III	5, 5′
Genuinae	*N. tabacum* (*tab.*)	Peru	24	II	7, 9
Petunoides					
Alatae	ims *plumbaginifolia* (*plu.*)[c]	N.W. Argentina	10	II	3
Bigelovianae	*N. bigelovii* (*big.*)	California	24	III	4, 6
Repandae	*N. repanda* (*rep.*)	Golf of Mexico	24	I	9
Suaveolentes	ims *suaveolens* (*sua.*)	S.E. Austrlia	16	A	2, 5, 6, 8
	N. gossei (*gos.*)	Mid Australia	18	A	2, 6, 8
	ims *megalosiphon* (*meg.*)[c]	E. Australia	20	A	3, 5, 6
	ims *debneyi* (*deb.*)[c]	Coastal E. Australia	24	A	1, 3, 5

[a] According to Goodspeed (1954) and Burbidge (1960)
[b] According to Chen et al. (1976 and personal communication)
[c] In the *ims* lines the characteristics of the maternal species are given

Fig. 1. Restriction endonuclease fragment patterns of ptDNA from eleven *Nicotiana* species. The digests were sized by electrophoresis in horizontal agarose slab gels. The individual tracks illustrate groups of species with indistinguishable fragment patterns. Fragments arising by one or two additional restriction sites when compared to the corresponding *N. tab.* fragment "▸" are indicated by "■", those lacking restriction sites relative to those of *N. tab.* by ">". Fragments with changed mobility but unaltered relative position in the patterns are distinguished by *arrow.* The restriction patterns of *ims big.* and *ims glu.* substitution lines (Frankel et al. 1979) in Panel *b* , lane 4, *c* lane 2, and *e*, lane 2 will be discussed in a separate paper. The diffuse zone in the high molecular weight range of some tracks represents contaminating nuclear DNA (Seyer et al. 1981; Gordon et al. 1982). **Panel a:** *Sal* I patterns with ptDNA from *(1) N. tab., N. plu., N. gla., N. rai., N. glu., N. rep., N. sua., N. gos., N. meg.,* and *N. deb., (2) N. big.* (0.45% agarose gel). **Panel b:** *Bgl* I patterns with ptDNA from *(1) N. tab., N. plu., N. gla., N. big., N. rep., N. sua., N. gos., N. meg.,* and *N. deb., (2) N. glu.,* (3) *N. rai.* Note that the variant fragment B-1_{II} in lane 2 is two-molar (0.5% agarose gel). **Panel c:** *Pst* I patterns with ptDNA from *(3) N. tab., N. gla., N. rai., N. glu., N. big.,* and *N. deb., (1* and *4) N. sua., N. gos.,* and *N. meg., (5) N. plu., (6) N. rep.* (0.45% agarose gel). **Panel d:** *Kpn* I patterns with ptDNA from *(1) N. tab., N. plu., N. gla., N. big.,* and *N. deb., (2) N. glu., (3) N. rep.,* and *N. gos., (4) N. rai., (5) N. sua.,* and *N. meg.* (0.55% agarose gel). **Panel e:** *Xho* I patterns with ptDNA from *(1) N. sua., N. gos.,* and *N. meg., (3) N. deb., (4) N. big., (5) N. glu., (6) N. plu., (7) N. rep.* The patterns of *N. tab., N. gla.,* and *N. rai.* ptDNA which are indistinguishable from each other are not shown (0.6% agarose gel). **Panel f:** *Pvu* II patterns with ptDNA from *(1) N. tab., N. plu., N. gla., N. glu.,* and *N. deb., (2) N. sua., (3) N. meg., (4) N. rep.,* and *N. gos., (5) N. rai.,* and *N. big.* (0.6% agarose gel)



Sal I
Bgl I
Pst I
Kpn I
1
2
1
2
3
4
1
2
3
4
5
6
1
2
3
4
5
a
b
c
d
1
2
3
4
5
6
7
1
2
3
4
5
e
f
Xho I
Pvu II

or in combination to digest 0.5–4 μg DNA in 30–100 μl assay volume under the conditions recommended by the suppliers. *Sal* I, *Pst* I and *Kpn* I were prepared according to the protocol of Arrand et al. (1978). Restriction fragments were separated by electrophoresis in 0.4–1.8% agarose slab gels and visualized after staining with ethidium bromide. Undigested *Eco* RI or *Hind* III digested lambda DNA (Boehringer) and *Hae* III digests of ΦX 174 RF-DNA (New England Biolabs) were used as size standards.

The procedure for physical mapping of restriction sites has been described (Herrmann et al. 1980c; Herrmann and Whitfeld 1982). It involves initial separation of restriction cleavage products of a first enzyme on a low-gelling agarose tube gels (Seaplaque agarose, Marine Colloids Inc. Rockland, Maine, USA), subsequent recovery and digestion of individual DNA bands with a second restriction endonuclease at 37 °C in the liquefied agarose. The maps were constructed on the basis of the restriction sites for *Sal* I (Seyer et al. 1981) using reciprocal digestion with the enzyme pairs *Sal* I/*Kpn* I and Sal I/*Pst* I. The nomenclature for restriction fragments was adapted from Seyer et al. (1981), in which fragments are denoted by the first letter(s) of the enzyme used and numbered in decreasing molecular weight.

The differences among ptDNA from different species that appeared as (1) subfragments resulting from additional sites for an enzyme (compared to *N. tab.*) were assigned to the corresponding *N. tab.* fragment, e.g. band $S\text{-}5_{I}$ (the large) and $S\text{-}5_{II}$ (the small) in the case of *N. big.* ptDNA digested with *Sal* I (Fig. 1a, lane 2). (2) Fewer restriction sites compared to *N. tab.* were also named after the corresponding *N. tab.* bands, e.g. X-1-15 in the case of pattern lane 3 in Fig. 1e in which the *Xho* I site between *N. tab.* X-1 and X-15 is missing and a new fragment corresponding to their composite length appears. (3) An individual band remaining at its relative position but with altered mobility, either shorter or longer than the corresponding *N. tab.* fragment, has been marked by asterisks. If the same band appeared in more than one molecular weight between different species it was distinguished by one or more asterisks, e.g. Ps-8* and Ps-8** in the *Pst* I patterns of Fig. 1c (tracks 2 and 4, respectively).

3 Results

Comparison of ptDNAs from the genus Nicotiana

In order to detect differences in the restriction fragment patterns, ptDNA from all eleven *Nicotiana* species was digested with each of the following enzymes, *Sal* I, *Pvu* II, *Xho* I, *Bgl* I, *Kpn* I, *Pst* I and *Eco* RI. The first four enzymes were used because their cleavage site maps were available for tobacco ptDNA (Jurgenson and Bourque 1980; Fluhr and Edelman 1981a; Seyer et al. 1981). *Kpn* I and *Pst* I were chosen because they generated relatively simple fragment patterns and discriminated the ptDNAs of some of the species studied. The different restriction patterns, excluding those of *Eco* RI, are shown in Fig. 1.

Figure 1 illustrates that fragment patterns obtained after digestion with a discrete enzyme were remarkably similar for all *Nicotiana* ptDNAs examined. Moreover, patterns obtained from several different DNAs with the same restriction enzyme were frequently found to be indistinguishable from one another. Differences included small alterations in electrophoretic mobility as well as appearance and disappearance of individual fragments. These changes could be collectively utilized to group the *Nicotiana* ptDNAs into classes (Fig. 1). *Sal* I gave only two different restriction patterns (Fig. 1a). The DNAs from nine wild species were indistinguishable from that of *N. tab.* (Fig. 1a, lane 1), only *N. big.* yielded a pattern resolvable from that of *N. tab.* ptDNA (lane 2). Upon digestion with *Bgl I* three different patterns were observed (Fig. 1b), whereas four, five and six groups could be recognized upon digestion with *Pst* I, *Kpn I* and Xho I respectively (Fig. 1c–e). Differences were less obvious in the *Pvu* II digests, but, on the basis of microheterogeneity, 5 different restriction patterns could be distinguished (Fig. 1f). *Eco* RI gave distinctive patterns with each of the 11 DNAs tested (results not shown). However, due to their complexity (>40 bands) no attempt was made to interpret these patterns in terms of physical map position.

Physical mapping of differences in Nicotiana ptDNA

N. tab. ptDNA was used as reference in our mapping studies, since it is the most extensively characterized of the *Nicotiana* ptDNAs (Jurgenson and Bourque 1980; Fluhr and Edelman 1981a; Seyer et al. 1981 and Fig. 2). The similarities in the cleavage patterns of ptDNA from different *Nicotiana* species indicated that much of the *Nicotiana* plastome is conserved and that the cleavage site map for the plastid chromosome of *Nicotiana tabacum* (Seyer et al. 1981) could serve as a basis to locate the observed differences. To evaluate the differences in the *Pst* I and *Kpn* I patterns, this map was completed for the restriction sites for these enzymes. The map was constructed using essentially the approach outlined by Seyer et al. (1981). The sizes of the single and double digest fragments obtained with *Sal* I, *Pst* I and *Kpn* I are listed in Table 2. The relationships between primary and secondary fragments for each series are given in Tables 3 and 4, respectively. The positions for *Kpn* I and *Pst* I cleavage sites are incorporated into the map for *N. tab.* ptDNA (Fig. 2; Seyer et al. 1981).

Much information about the differences among the ptDNAs could be derived from the analysis of the double digests including *Sal* I plus one of the other enzymes. As the map position for each *N. tab.* secondary band was defined, changes apparent in variant fragments could be located just by looking at differences in the double digest restriction patterns. The differences observed between the ptDNA from the various species appeared to result either from gain or loss of particular restriction sites or from small changes in the molecular weight of discrete fragment indicating deletion/insertion mutations.

In order to substantiate the nature and location of these differences, analysis of variant fragments was per-

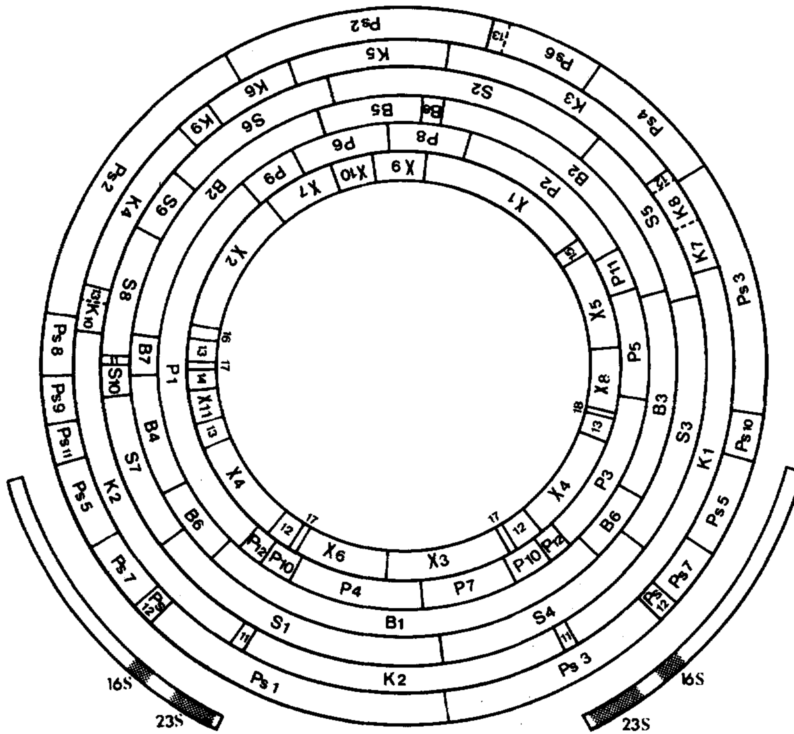


Fig. 2. Extended restriction endonuclease cleavage site map of *N. tabacum* ptDNA. The *Xho* I (*X*), *Pvu* II (*P*), *Bgl* I (*B*), and *Sal* I (*S*) maps are adapted from Seyer et al. (1981). The *Pst* I (*Ps*) and *Kpn* I (*K*) sites are drawn to scale relative to the *Sal* I restriction sites. The relative order of fragments separated by dashed lines remains to be established. the location of K-13 within S-8 and the stoichiometry and location of Ps-12 are tentative as well as the relative positions of a few *Pst* I and *Kpn* I cleavage sites that are very close to *Xho* I, *Pvu* II or *Bgl* I sites and have been determined solely from molecular weight calculations. The inverted repeat and the locations of the rDNA operons are indicated by the extended part

Table 2. Sizes in kbp and stoichiometries (in brackets) of single and double digestion fragments of *N. tabacum* ptDNA obtained by digestion with the restriction endonucleases *Sal* I, *Pst* I and *Kpn* I

Primary band no.	*Sal* I[a] kbp	*Pst* I kbp	*Kpn* I kbp	Secondary band no.	*Sal* I/*Pst* I kbp	*Sal* I/*Kpn* I kbp
1	27	25	34	a	25	22.0
2	23.8	22.2 (2×)	27.5 (2×)	b	17.5	16.9
3	22	18.85 (2×)	19.5	c	13.5	14.2
4	19.7	9.6	14.6	d	10.0	13.2
5	16.7	8.25 (2×)	12.1	e	9.0 (3×)	9.8 (4×)
6	15.2	7.0	6.0	f	8.05 (2×)	6.0
7	13.2	5.1 (2×)	4.6	g	7.4	5.75
8	11.4	4.3	4.25	h	7 (2×)	5.6
9	5.6	3.1	3.65	i	5.55	5.5
10	2.9	2.4	3.15	j	3.07 (3×)	4.6
11	0.65	1.83	0.9 (2×)	k	2.75	4.25
12		1.5 (2×)	0.65	l	2.65	3.65
13		1.25	0.5	m	2.60	3.4
				n	2.05 (2×)	3.15
				o	1.83	2.9
				p	1.5 (2×)	2.4
				q	1.25	2.1
				r	0.65	2
				s	0.5 (2×)	0.9 (2×)
				t		0.65 (2×)
				u		0.5
Total (kbp)	158.15	166.13	159.80		165.14	160.4

[a] Sizes taken from Seyer et al. (1981)

Table 3. Relationships of primary and secondary fragments produced by digestion of *N. tab.* ptDNA with *Sal* I and *Pst* I

Secondary fragments	Primary fragment from which secondary fragments are derived	
Sal I/*Pst* I	*Sal* I	*Pst* I
a	1	1[a]
b	4	3b
c	2	2a
d	5	3a
e (3×)	3, 6, 8	2b (2×), 3a
f (2×)	3, 7	5 (2×)[a]
g	6	2a
h (2×)	2, 5	4, 6[a]
i	9[a]	2b
j (3×)	3, 7, 8	7 (2×), 8
k	2	4
l	3	10[a]
m	10	9
n (2×)	1, 4	7 (2×)
o	7	11[a]
p	1, 4	12 (2×)[a]
q	2	13[a]
r	11[a]	8
s (2×)	7, 10	8, 9

Fragment stoichiometry in brackets
Primary fragments that contain no cleavage site for the other enzyme

Table 4. Relationships of primary and secondary fragments produced by digestion of *N. tab.* ptDNA with *Sal* I and *Kpn* I

Secondary fragments	Primary fragment from which secondary fragments are derived	
Sal I/*Kpn* I	*Sal* I	*Kpn* I
a	3[a]	1
b	1	2a
c	2	3
d	7[a]	2b
e (4×)	1, 2, 4 (2×)	1, 2a, 2b, 5
f	6	6[a]
g	8	4
h	9[a]	4
i	5	3
j	5	7[a]
k	5	8[a]
l	6	9[a]
m	6	4
n	8	10[a]
o	10[a]	2b
p	6	5
q	8	2b
r	5	1
s (2×)	1, 4	11 (2×)[a]
t (2×)	5, 11[a]	2b, 12[a]
u	8	13[a]

Fragment stoichiometry in brackets
[a] Primary fragments that contain no cleavage site for the other enzyme

formed in two additional ways. First, total ptDNA was digested with the enzyme that gave the distinctive pattern, the fragments were separated on low melting agarose gels, the exceptional band was excised, digested with a second enzyme and the resulting secondary fragments were run on a slab gel along with primary and double digests of *N. tab.* and the wild species ptDNA. In the second approach an enzyme was used with which the alteration remained cryptic (generally *Sal* I) and then the suspected bands were digested by the distinguishing enzyme. In this way, the variant band was identified and the position in the ptDNA map in which the change occurred was established.

Figures 3a and b illustrates these approaches. First, the 14.1 kbp exceptional *Xho* I primary fragment of *N. glu.* ptDNA (Fig. 1e, lane 5) that did not appear in *N. tab.* ptDNA was digested with *Sal* I (Fig. 3a, lane 6). Two bands appeared in the secondary digest corresponding to the two new bands in the double digest of *N. glu.* ptDNA. These exceptional bands can only be interpreted by assuming that the *Xho* I site existing between the adjacent fragments X-5 and X-15 in *N. tab.* ptDNA (cf. Fig. 5 in Seyer et al. 1981) is missing in *N. glu.* ptDNA thereby creating a fragment composed of both. The same reasoning can be applied to the appearance of a 24.2 kbp *Xho* I band X-1-15 in *N. gos.* ptDNA (Fig. 1e, lane 1). When this band was digested by *Sal* I (Fig. 3a, lane 9) the secondary fragments can only be interpreted to mean that the *Xho* I junction between X-1 and X-15 of *N. tab.* ptDNA (cf. Fig. 5 in Seyer et al. 1981) is missing in *N. gos.* ptDNA.

The second approach is illustrated in Fig. 3b. In *N. glu.* ptDNA, fragment K-10* (Fig. 1d, lane 2) was shorter than K-10 in *N. tab.* ptDNA (3.1 and 3.15 kbp, respectively). In order to establish that both fragments originate from the same ptDNA segment, fragment S-8 of both ptDNAs which should contain K-10 and K-10*, respectively (Fig. 2) were isolated and digested by *Kpn* I. As expected, fragments identical to K-10 (Fig. 3b, lane 7) and K-10* (Fig. 3b, lane 1) were found together with the two flanking fragments (Fig. 3b, lanes 4 and 12, cf. also lanes 2 and 6). (The difference is too small to be detectable in S-8.) Similarly, in *N. gos.* ptDNA *Kpn* I bands differed from their *N. tab.* counterparts, namely K-6* and K-7*, that appeared to be larger than K-6 and K-7, and K-9* which was smaller than K-9 (Fig. 1d, lanes 1 and 3). In fact, digestion of *Sal* I primary fragments S-5 and S-6 (Fig. 2) with *Kpn* I showed that these bands originated from the same DNA segments (Fig. 3b, lanes 3, 10 and 11, cf. also lanes 6 and 9).

The differences between the eleven *Nicotiana* ptDNAs along with the nature of alteration are listed in Table 5. Figure 4 summarizes these data relative to the restriction cleavage site map of *N. tab.* ptDNA.

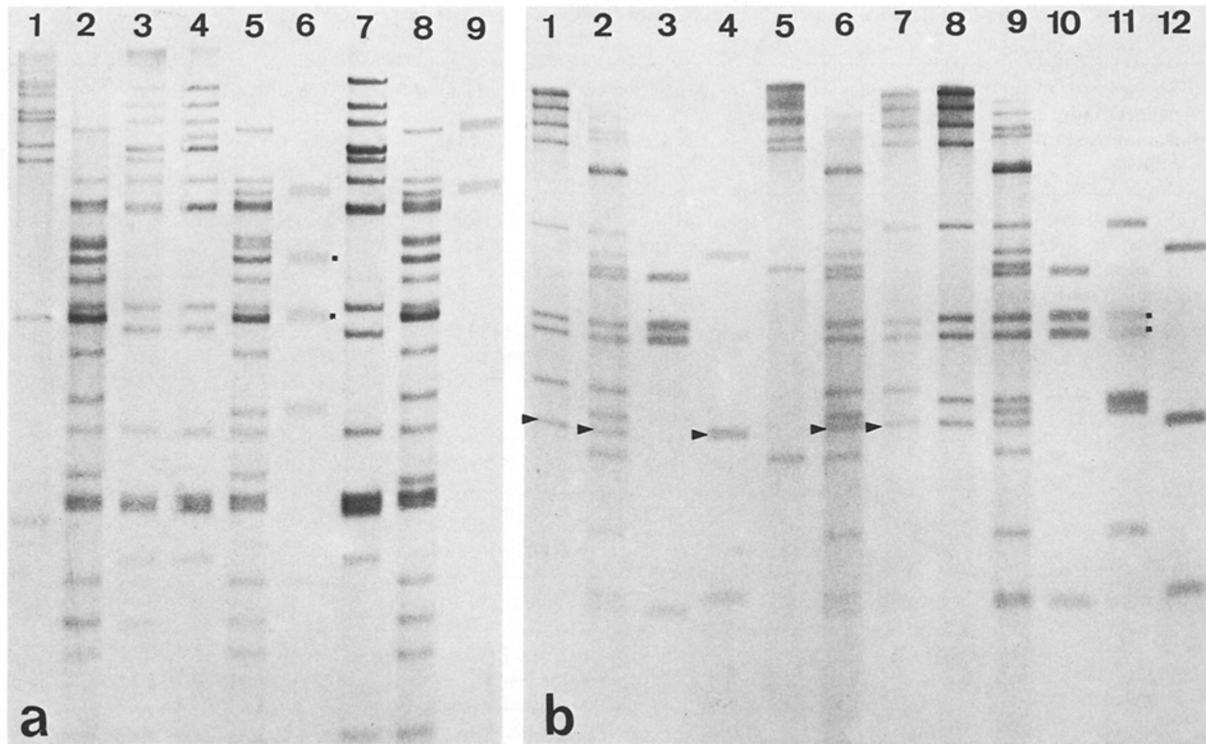


Fig. 3. Analysis of variant restriction fragments of *Nicotiana* plastomes. **Panel a:** Fragments X-5-15 (*N. glu.*) and X-1-15 (*N. gos*) were digested with *Sal* I and run on a 0.75% agarose slab gel which included ptDNA from *N. tab.*, *N. glu.*, and *N. gos.* digested either by *Sal* I, *Xho* I or both as reference. (Lanes *1–3*) *N. tab.* ptDNA digested by *Sal* I, *Sal* I plus *Xho* I, and *Xho* I, respectively, (*4* and *5*) *N. glu.* ptDNA digested by *Xho* I, and *Sal* I plus *Xho* I, respectively, (*6*) *N. glu.* X-5-15 digested by *Sal* I, (*7* and *8*) *N. gos.* ptDNA digested by *Xho* I, and *Sal* I plus *Xho* I, respectively, (*9*) *N. gos.* X-1-15 digested by *Sal* I. **Panel b:** Fragments S-2, S-8 (*N. glu.*) and S-5, S-6 and S-8 (*N. gos.*) were digested with *Kpn* I and run on a 1% agarose slab gel along with ptDNA from *N. tab.*, *N. glu.*, and *N. gos.* digested by *Sal* I, *Kpn* I or both. (Lanes *1* and *2*) *N. glu.* ptDNA digested by *Kpn* I and *Sal* I plus *Kpn* I, respectively, (*3*) *N. glu.* S-5 digested by *Kpn I,* (*4*) *N. glu.* S-8 digested by *Kpn* I, (*5–7*) *N. tab.* ptDNA digested by *Sal* I, *Sal* I plus *Kpn* I, and *Kpn* I, respectively, (*8* and *9*) *N. gos.* ptDNA digested by *Kpn* I, and *Kpn* I plus *Sal* I, respectively, (*10–12*) *N. gos.* S-5, S-6 and S-8, respectively, digested by *Kpn* I. Corresponding variant secondary fragments are marked in the parental DNAs and secondary digests. Cross contamination by other fragments from the primary gels are indicated by points. For further explanations see text

4 Discussion

The restriction endonuclease work presented in this study shows that the ten *Nicotiana* plastomes examined, six of American and four of Australian origin, contain discrete ptDNAs that are distinguishable from the *N. tab.* plastome. We have employed seven enzymes that differ in their recognition sequence to increase the probability of estimating plastome divergence. These enzymes have been useful in disclosing even intraspecific ptDNA variation (Herrmann et al. 1980), and the cleavage sites of four of them have been located on the 160 kbp plastid chromosome of tobacco (Jurgenson and Bourque 1980; Fluhr and Edelman 1981a; Seyer et al. 1981). We have refined this map by locating the 33 cleavage sites for the endonucleases *Kpn* I and *Pst* I (Fig. 2) and, using the additional repetitive *Bgl* I site in *N. glu.* ptDNA (Fig. 4) approximately 1.4 kbp away from the conserved symmetrical P-10/P-7 and P-10/P-4 junctions (Fig. 2), have determined the sizes for the repeated and small single-copy regions more accurately. This *Bgl* I cleavage site, which seems also to occur in the related *Petunia hybrida* ptDNA (Bovenberg et al. 1981), suggests that the sizes of the repetitive and small single-copy segments are closer to 22 and 21 kbp, respectively (cf. Seyer et al. 1981). Its appearance is not attended by substantial size changes.

The capacity of the chosen restriction enzymes to discriminate ptDNA from related species differs considerably, a property that is remarkably constant even for ptDNAs from different genera for a given enzyme (Schiller et al. 1982). *Sal* I produced invariant patterns except for *N. big.* (Figs. 1 and 4). In other instances, the general fragmentation characteristics for each enzyme were maintained; many fragments were common to all species. The majority of variable fragments remained at their relative positions (e.g., *Pvu* II or *Pst* I pattern). Others that disappeared are replaced by new fragments that total approximately the disappearing ones and vice versa, indicating that even these variable fragments are related and large-scale rearrangements are absent in the *Nicotiana* plastomes. Collectively, therefore, the restriction patterns obtained thus illustrate both the distinctness *and* the relatedness of the *Nicotiana* plastomes. The expected strict positional conservation

Table 5. Analysis of the observed differences between the plastomes of eleven *Nicotiana* species

Enzyme	Species with restriction patterns indistinguishable from those of *N. tab.* ptDNA	Species with observed changes	Alteration compared to *N. tab.* ptDNA (molecular weights in kbp)	Way of confirmation: result
Sal I	*N. plu., N. gla., N. glu., N. rai., N. rep., N. sua., N. gos., N. meg., N. deb.* (Fig. 1a, lane 1)	*N. big.* (Fig. 1a, lane 2)	extra *Sal* I cut in S-5 (16.7) proximal to S-3	S-5_{I} (10.2) and S-5_{II} (7.5) digested by *Bgl* I
Bgl I	*N. plu., N. gla., N. glu., N. rep., N. sua., N. gos., N. meg., N. deb.* (Fig. 1b, lane 1)	*N. rai.* (Fig. 1b, lane 3)	extra *Bgl* I cut in B-1 (39.7) and S-1 (27.0) close to the S-1/S-4 junction	S-1 and S-4 digested with *Bgl* I. *Bgl* I/*Xho* I double digestion of ptDNA. B-1_{I} (22.2) and B-1_{II} (16.9)
		N. glu. (Fig. 1b, lane 2)	2 extra *Bgl* I cuts in B-1 (39.7), one in each repeat copy within S-1 (27.0) and S-4 (19.7)	S-1 and S-4 digested with *Bgl* I. B-1_{II} (two molar 8.9) digested by *Xho* I; B-1_{I} (22.2)
Pst I	*N. gla., N. rai., N. glu., N. big., N. deb.* (Fig. 1c, lane 2)	*N. sua., N. gos., N. meg.* (Fig. 1c, lane 4)	Ps-8* (4.1) instead of Ps-8 (4.3)	
			Ps-10* (2.3) instead of Ps-10 (2.4)	
		N. plu. (Fig. 1c, lane 5)	extra *Pst* I site in Ps-2a (22.2) proximal to Ps-2b	S-6 digested by *Pst* I; Ps-$2a_{I}$ (18.0) and Ps-$2a_{II}$ (3.25)
		N. rep. (Fig. 1c, lane 6)	*Pst* I site between Ps-4 (9.6) and Ps-6 (7.0) is missing	Ps-4-6 (16.1); Ps-4-6 digested with *Sal* I
			Ps-8** (4.0) instead of Ps-8 (4.3)	*Sal* I/*Pst* I double digestion of ptDNA
			Ps-10** (2.1) instead of Ps-10 (2.4)	*Sal* I/*Pst* I double digestion of ptDNA
Kpn I	*N. plu., N. gla., N. big., N. deb.* (Fig. 1d, lane 1)	*N. glu.* (Fig. 1d, lane 2, Fig. 3b, lane 4)	K-10* (3.1) instead of K-10 (3.15)	S-8 digested by *Kpn* I
		N. rep., N. gos. (Fig. 1d, lane 3, Fig. 3b, lanes 10–12)	K-6* (7.0) instead of K-6 (6.9)	S-5 digested by *Kpn* I
			K-7* (4.7) instead of K-7 (4.6)	S-6 digested by *Kpn* I
			K-9* (3.55) instead of K-9 (3.65)	S-6 digested by *Kpn* I
		N. rai. (Fig. 1d, lane 4)	extra *Kpn* I cut in K-5 (12.1)	S-2 digested by *Kpn* I K-5_{I} (8.5) and K-5_{II} (3.03)
		N. sua., N. meg. (Fig. 1d, lane 5)	K-6* (7.0) instead of K-6 (6.9)	*Kpn* I/*Sal* I double digestion of ptDNAs
			extra *Kpn* I site in K-2a (27.5) proximal to S-1	S-1 and S-4 digested by *Kpn* I; K-$2a_{I}$ (21.25) and K-$2a_{II}$ (5.3)
			Kpn I site between K-4 (14.6) and K-9 (3.65) is missing	K-4-9 (17.65); *Kpn* I/*Sal* I double digestion of ptDNAs
Xho I	*N. gla., N. rai.*	*N. big.* (Fig. 1e, lane 4)	*Xho* I site between X-13 (3.0) and X-16 (1.55) is missing	X-13-16 (4.7)
		N. sua., N. gos., N. meg., N. deb. (Fig. 1e, lanes 1 and 3; Fig. 3, lane 9)	*Xho* I site between X-1 (21.9) and X-15 (2.1) is missing	X-1-15 (24.2); X-1-15 digested by *Sal* I

Table 5 (continued)

Enzyme	Species with restriction patterns indistinguishable from those of *N. tab.* ptDNA	Species with observed changes	Alteration compared to *N. tab.* ptDNA (molecular weights in kbp)	Way of confirmation: result
		N. sua., N. gos., N. meg. (Fig. 1e, lanes 1 and 7)	X-7* (8.9) instead of X-7 (9.2)	*Sal* I/*Xho* I double digestion of ptDNA
			X-10* (5.35) instead of X-10 (5.4)	*Sal* I/*Xho* I double digestion of ptDNA
		N. glu. (Fig. 1e, lane 5)	X-16* (1.50) instead of X-16 (1.55)	
		(Fig. 3a, lane 6)	*Xho* I site between X-5 (11.75) and X-15 (2.1) is missing	X-5-15 (14.1)
			X-8* (9.1) instead of X-8 (8.9)	*Sal* I/*Xho* I double digestion of ptDNA
			X-10** (5.45) instead of X-10 (5.4)	*Sal* I/*Xho* I double digestion of ptDNA
		N. plu. (Fig. 1e, lane 6)	extra *Xho* I site in X-3 (15.55) proximal to X-17	X-3_{I} (8.2) and X-3_{II} (7.6) digested by *Sal* I
		N. rep. (Fig. 1e, lane 7)	extra *Xho* I site in X-1 (21.9) proximal to X-15	X-1_{I} (15.55) digested by *Sal* I; X-1_{II} (6.3)
			X-7* (8.9) instead of X-7 (9.2)	*Sal* I/*Xho* I double digestion of ptDNA
			X-8** (8.6) instead of X-8 (8.9)	
			X-10* (5.35) instead of X-10 (5.4)	
Pvu II	*N. plu., N. gla., N. glu., N. deb.* (Fig. 1f, lane 1)	*N. sua.* (Fig. 1f, lane 2)	Pv-7* (10.15) instead of Pv-7 (10.0) Pv-7 (10.0)	
			Pv-9* (6.2) instead of Pv-9 (6.35)	
		N. meg. (Fig. 1f, lane 3)	Pv-6* (10.1) instead of Pv-6 (10.3)	
			Pv-9** (6.1) instead of Pv-9 (6.35)	
		N. rep., N. gos. (Fig. 1f, lane 4)	Pv-9*** (6.0) instead of Pv-9 (6.35)	
		N. rai., N. big. (Fig. 1f, lane 5)	Pv-11* (3.3) instead of Pv-11 (3.5)	

of restriction fragments in the *Nicotiana* plastid chromosomes (Herrmann et al. 1980; Fluhr and Edelman 1981b; Seyer et al. 1981; Shen et al. 1982) justifies the use of the restriction map from the *N. tab.* plastid chromosome to pinpoint the differences observed between species.

Restriction fragment polymorphism can arise by several processes, including (1) point mutations; (2) base modification; (3) insertions or deletions; or (4) inversions and transpositions, all of which have been demonstrated to occur in ptDNA. The principal mechanism that accounts for most, if not all, observed mutations in *Nicotiana* plastomes seems to be small deletions/insertions (up to 300 bp), as in other related ptDNAs. This is readily demonstrated if polymorphic fragments are flanked by (small) invariant secondary fragments. Deletion/insertion can occur without changes in cut frequency (Herrmann et al. 1980b; Gordon et al. 1982). For example, fragment S-8 of *N. glu.* and *N. tab.* digested with *Kpn* I releases K-13 (0.5 kbp) and terminal fragments of 5.75 and 2.10 kbp in both instances plus K-10* (3.10 kbp in *N. glu.*) which is 50 bp

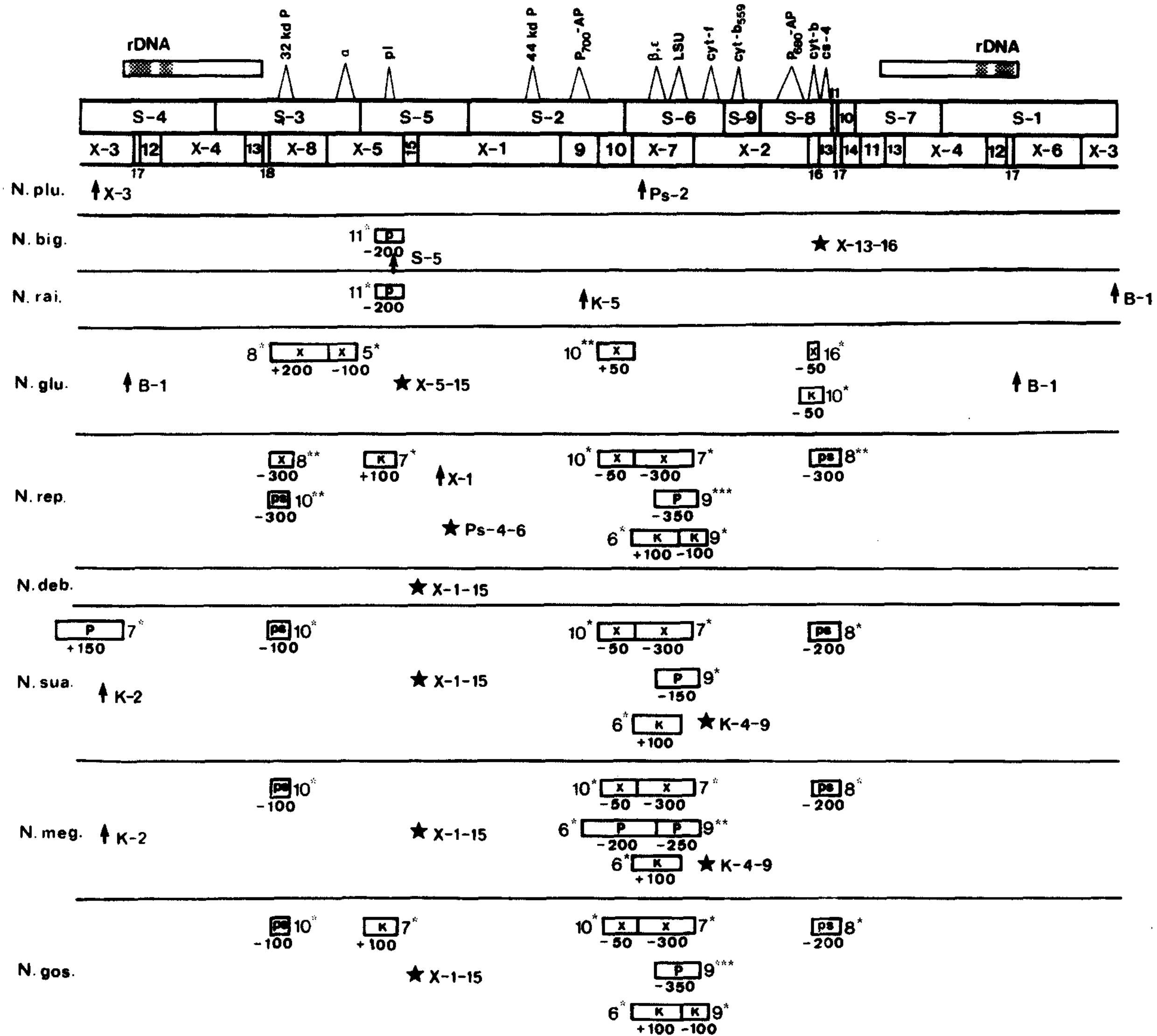


Fig. 4. Comparative restriction endonuclease cleavage site maps for ptDNA from eleven *Nicotiana* species. The complete *Sal* I and *Xho* I maps of *N. tab.* ptDNA (Fig. 2, Seyer et al. 1981) is used as reference and presented in linearized form by cutting the small single-copy region between fragments S-1 and S-4. The differences for each species are given relative to *N. tab.* ptDNA. Insertions (+) and deletions (–) are boxed for each enzyme and their size is indicated in bp. Mutations affecting restriction sites are marked by *arrow* (additional sites) or *asterisks* (missing sites). The position of the genes for thylakoid membrane proteins – the alpha, beta, epsilon and proteolipid (pl) subunits of the ATP synthase, the cytochromes f, b_6 and b_{559}, subunit 4 (cs-4) of the cytochrome complex, the P_{700} and P_{680} chlorophyll *a* apoproteins as well as the 44 kd chlorophyll *a*-conjugated and the herbicide-binding 32 kd proteins associated with photosystem II – and for the large subunit of Rubisco are taken from Bisanz et al., manuscript in preparation

shorter than K-10 (3.15 kbp in *N. tab.;* Fig. 3b, lanes 4 and 7). This difference which is obviously too small to be seen in S-8 is also evident in the *Xho* I patterns (X-16 vs. X-16**; 1.55/1.50; Fig. 4). Similarly, the 0.3 kbp deletion in *N. rep.* X-8** (8.6 kbp) compared to *N. tab.* X-8 (8.9 kbp) was corroborated by an equivalent size change of the internal fragment Ps-10** (2.1 vs. 2.4 kbp). Deletion/insertion may also be accompanied by changes in cut frequency if two or more enzymes possess cleavage sites at or in the vicinity of the mutation. For example, reciprocal and double digestions of *N. tab.* and *N. big.* ptDNA with *Sal* I show that the 16.7 kbp primary fragment S-5 of *N-tab.* disappears in *N. big.* and is replaced by two smaller fragments of 10.2 and 7.5 kbp (Fig. 1a, lanes 1 and 2). Only the S-5 internal, small fragment P-11* (3.5 kbp; Fig. 2) from *N. big.* which is approximately 200 bp smaller than the corresponding fragment in *N. tab.* is cut by *Sal* I. Other instances where gain or loss of closely spaced restriction sites may coincide with size alterations are found in the

Kpn I (K-2; K-4-9) and *Pvu* II patterns in *N. sua.* and *N. meg.* ptDNA (Fig. 4). The observed deletions/insertions do not markedly alter the contour lengths of the chromosomes (ca. 0.5–1%) as is evident from the invariant *Sal* I patterns.

As cleavage site changes frequently result from large nucleotide changes, it is unlikely that methylation known to occur in *Chlamydomonas* ptDNA (Bolen et al. 1982) or single base substitutions that have been demonstrated in various plastid genes (in *N. tab.*, e.g. Zurawski et al., 1982; Shinozaki and Sugiura 1982) account for fragment polymorphism in *Nicotiana.* Base modification would have to occur in some but not other *Nicotiana* species; owing to the inherent difficulty in discriminating point mutations and small deletions/insertions, restriction analysis alone cannot prove single base substitutions as the source for changes in cut frequency (but see Kung et al. 1982; Scowcroft 1979). Colinearity of the nucleotide sequences and conservation of restriction sites, finally, preclude large-scale rearrangements between *Nicotiana* plastomes. Inversions in segmentally organized ptDNAs are known, but rare and restricted to disparate organisms (Palmer and Thompson 1982; Herrmann et al. 1983). If they occur in *Nicotiana,* they must be small and within the average distance of two to three cut positions, i.e. at most 5 kbp (Schiller et al. 1982). Such rearrangements may have occurred in two places. The finding that the smaller subfragment obtained upon digestion of fragment X-5-15 in *N. glu.* with *Sal* I is smaller than the corresponding fragment in *N. tab.*, the adjacent fragment X-8*, in turn, larger than the *N. tab.* fragment X-8 (Fig. 3a), could have resulted by an asymmetric inversion including the *Xho* I site with its larger portion in X-5. It is worth mentioning in this context that an equivalent finding at approximately the same position has been noted by Gordon et al. (1982) in the chromosome of *Euoenothera* plastome V. Similar logic can be applied to the adjacent fragments K-6* and K-9* in *N. gos.* and *N. rep.* ptDNA that have changed their position relative to K-6 and K-9 in the *N. tab.* chromosome. The former are larger than K-6, the latter shorter than K-9 by about 100 bp each, which suggests an asymmetric inversion around the *Kpn* I site. However, these results are equally consistent with two independent opposing mutations, insertion *plus* deletion.

Distribution and biological significance of fragment variation

Genetic data show that an exchange of plastids and nuclei between species can greatly impair harmoniously balanced growth and development of the resulting genome/plastome hybrids (Stubbe 1959; Frankel et al. 1979). This incompatibility illustrates that acquisition of biological specificity involves the co-evolution of both cellular genetic compartments and, consequently, mutations in any one of these compartments have to fulfil the requirement that the entire system remains balanced and functional.

The level of divergence in *Nicotiana* plastid chromosomes is comparable to that found for other taxa (Palmer and Zamir 1982) and reflects both relatively conservative plastome evolution as well as a recent origin for the genus; the largest number of changes observed between a pair of ptDNAs is 13, for *N. rai.* and *N. rep.* (Fig. 4). Fragment variation has been observed in seven regions: four in the large, one in the small single-copy region, and two in the inverted repeat (one in each copy). Most changes are clustered in three regions within the large unique-sequence segment, centrally at fragment S-2/S-6 and at its two borders with the repeat (Fig. 4).

Genome/plastome disharmony frequently includes disturbances of thylakoid membrane development ("hybrid bleaching", Stubbe 1959). The recent identification of genes for the majority of thylakoid membrane proteins including those from *Nicotiana* (Fig. 4), spinach and *Oenothera* (Herrmann et al. 1983; Morris et al. 1983) has disclosed that allelic components with nuclear or plastid modes of inheritance could be potentially responsible for compartment coevolution since the topogenesis of multisubunit membrane complexes in genome/plastome hybrids may be impaired. However, attempts to relate discrete pattern differences to genetically defined loci have been difficult (Gordon et al. 1982; Bowman et al. 1983), although 25% of the actual coding capacity of plastid chromosomes is now known. Many deletions and insertions are found in the vicinity of genes for thylakoid membrane proteins or cause *intra*specific plastome variation without notable phenotypic significance, e.g. in the large intercistronic rDNA spacer (Herrmann et al. 1980; Bowman et al. 1983). Moreover, the heterogeneity pattern of *Nicotiana* plastomes resembles the patterns found for *Oenothera* (Gordon et al. 1982), *Brassica* (Palmer et al. 1983) and cereals (Bowman et al. 1983; Poulsen 1983). Both this striking conservation of "hot spots" and the biased divergence pattern cannot be mere coincidence. They suggest that progressing mutations in these regions of the plastid chromosome are neutral, in spite of the fact that ptDNA fragment patterns frequently diverge with decreasing taxonomic relationships (but see below), and follow classification of nucleo-cytosolic incompatibility (Herrmann and Possingham 1980; Gordon et al. 1982).

The mechanism(s) underlying deletion/insertion and inversion in ptDNA are unknown. They may include slipped mispairing during replication (Efstratiadis et al. 1980; Albertini et al. 1982), recombination or related processes and transposition (Calos and Miller 1980). Since insertion elements have not been identified in ptDNA and attempts to demonstrate intermolecular and interorganellar plastome recombination in higher plants have not been successful, e.g. in *Nicotiana* and even in *Oenothera* or *Pelargonium* where biparental transmission predominates, plastome divergence patterns probably arise during replication. The peculiar structural feature of plastid chromosomes, namely the large 22–25 kbp inverted duplication may be an additional element. Replication of higher plant plastid chromosomes must include copy correction to ensure homogeneity of the duplicated segment which necessarily requires (intramolecular) strand pairing. This mechanism has

been inferred from the symmetrical appearance of changes that occur *within* the two copies of the repetition (Adelberg and Bergquist 1972; Bedbrook and Bogorad 1976; Herrmann et al. 1980b; Grant et al. 1980; Gordon et al. 1982; Myers et al. 1982; cf. also *N. glu.* in Fig. 4). It is conceivable that both processes are coupled with the exchange of chromosome segments that leads to isomeric ptDNA molecules within a species (Palmer 1983). The repeated segment also appears to confer structural stability to the chromosome (Palmer and Thompson 1982). Plastid DNAs containing this palindromic structure are essentially colinear in their gross sequence arrangement (disregarding the numerous small deletions/insertions; Herrmann et al. 1980b; Fluhr and Edelmann 1981b; Seyer et al. 1981; Palmer and Thompson 1982; Palmer and Zamir 1982; Kung et al. 1982; Shen et al. 1982; Palmer et al. 1983) while the loss of one repetitive segment found in Fabacean plastomes (Kolodner and Tewari 1979; Koller and Delius 1980; Chu et al. 1981; Palmer and Thompson 1981) coincides with significant increases in rearrangement and transposition frequencies (Palmer and Thompson 1981, 1982). Only two kinds of inversions have been observed in DNAs that retain their repeats, a 45–50 kbp inversion that distinguishes the plastomes of Solanaceae, Chenopodiaceae, Brassicaceae, Cucurbitaceae and corn from those of the Rosoidean members mung bean (Palmer and Thompson 1981, 1982) and *Oenothera* (Herrmann et al. 1983), and a smaller, 20 kbp inversion within this segment in cereal plastomes (Palmer and Thompson 1982; Bowman et al. 1983; Poulsen 1983).

In the plastid chromosome of *Euglena gracilis,* which differs fundamentally in its anatomy, a single origin of replication has been located within the unique sequence segment, approximately 5 kbp away from the tandem triplication in the vicinity of a region that is particularly prone to intraspecific variation (Koller and Delius 1982). This position is intriguing in terms of both its location and the heterogeneity of the segment. Information about position(s) for replication origins in plastid chromosomes of higher plants has not been published but several observations provide evidence that replication and divergence patterns are correlated. Restriction analysis strongly suggests that the inversion of single-copy regions relative to each other that creates isomeric molecules in higher plants occurs at the edges of or within the repetition. Myers et al. (1982) have postulated facilitated breakage of DNA during copy correction to explain the generation of induced mutant *Chlamydomonas* plastomes harbouring deletions that extend from single-copy sequences into the inverted repeat. These plastomes replicate but apparently have lost their capability for copy correction as they maintain *asymmetric* repetitive segments. This implies that essential control elements for the latter process reside in single-copy border region(s) and perhaps extend into the repeated segment(s). It is probably not pure coincidence that the limitations of repeat borders, even between closely related species, are not strict, an extreme example for this has recently been described from *Nicotiana accuminata* (Shen et al. 1982), and that the break points that have led to the large 45–50 or 20 kpb inversions within the 90 kbp single-copy segment (see above) are located just at variant regions. Variance centrally in the large single-copy segment may be caused by forming unicircular head-to-tail dimers that may fuse in this part of the chromosome, at or within the duplication (cf. Fig. 3 in Kolodner and Tewari 1979). Head-to-head dimers (Kolodner and Tewari 1979) could result from ligation of sister molecules terminating replication suggesting that copy editing proceeds rather than follows (Palmer and Thompson 1982) this "intermolecular recombination". The different changes in the region X-7/X-10, X-13/X-16 or X-1/X-15/X-5, multiple variation of discrete fragments (e.g., P-9 or K-9) in *Nicotiana* ptDNA (Figs. 1 and 4) as well as alterations in *Euoenothera* plastome DNAs that resulted from several smaller events (Gordon et al. 1982) leave little doubt that the same or similar positions may be altered several times independently precluding that changes in this regions are spread by recombination. If our hypothesis is correct, divergence patterns could well reflect approximate phylogenetic scales without necessarily being correlated with genome/plastome incompatibility. However, as the positions of more than a dozen plastome-coded genes for the thylakoid membrane are conserved (Herrmann et al. 1983) the possibility that heterogeneous sites are involved in genome/plastome compatibility in a regulatory rather than structural way cannot be ruled out (cf. Bowman et al. 1983).

Information concerning relationships in *Nicotiana* comes from morphological, genetic and more recently from molecular data. Taxonomic data indicates that the genus originated in South America and dispersed to North America and Australia during Pliocene/Eocene, i.e. 60 million years ago (Goodspeed 1954). Nascent species were probably 6-paired while members of advanced sections possessed 12–24 pairs of intermediate figures of chromosomes. These generally arose by interspecific crosses, allopolyploidization and introgressive hybridization. Several recent studies have analyzed plastome variation within *Nicotiana* by isoelectric focussing studies on the plastid-encoded, maternally inherited large subunit of Rubisco (Chen et al. 1976) and by restriction analysis of ptDNA (Frankel et al. 1979; Kung et al. 1982; Shen et al. 1982). We have not attempted to estimate plastome divergence within *Nicotiana* in greater detail. There are inherent limitations in computing critically and independently divergence and directionality of plastome divergence from restriction enzyme analyses alone. Among these are the possibility that single changes may be detected by several restriction enzymes or that many may escape detection; the difficulty of discriminating primary, multiple and convergent mutations at the *same site;* and the small number of species surveyed. Our analysis rests on only eleven of approximately sixty recognized *Nicotiana* species that belong to 7 sections in 3 subgenera and possess varying chromosome sets and ploidy levels (Table 1; Fig. 4). In addition, natural interspecific crosses and offspring may suffer from genetic imbalance and in certain species biparental transmission occurs occasionally. If complementary genetic information for organelle biosynthesis is lost from hybrid nuclei, or intracellular genetic compartments compete or disharmonize with increasing evolutionary disjunction of species (Stubbe 1959; Sears 1980), plastomes may not merely sort out by chance and may be more prone to changes. All this complicates comparisons, can lead to contradictory or erroneous inference and thus preclude unambiguous designations of pedigrees. For example, *N. tab.* and *N. gla.* are almost indistinguishable by our DNA analyses, yet they possess LSU of types I and II, respectively (Chen et al. 1976). At the same time, the

Australian species *N. deb.* displays only one visible restriction polymorphism from these two American species, in a region unrelated to LSU (Fig. 4), yet it still harbours another LSU, of type A (Chen et al. 1976). Type I is also seen in *N. rep.* which differs considerably from *N. gla.* in DNA analysis (Fig. 4; Table 1). Furthermore, restriction analysis would suggest that the American species *N. rep.* resembles three Australian species more than any of the other American species studied and, conversely, the Australian *N. deb.* resembles most American species (Fig. 4). All species indigenous to Australia belong to the section Suaveolentes, subgenus Petunoides (Table 1) which exhibits affinities to South American Alatae, Accuminatae and Noctiflorae (Goodspeed 1954; Burbidge 1960). Only if DNA changes and their extent are put at equivalent map positions, all Australian species behave as a group. They lack the restriction site X-1/X-15 when compared to ptDNA of American species and three of them share a 100 bp deletion in Ps-10* which differs in magnitude (300 bp) from that found in *N. rep.* at the same position (Fig. 4). Among the Australian species studied, *N. deb.* and *N. sua.* are considered to be most advanced; the taxonomic positions of *N. meg.* and *N. gos.* are unclear. Our data suggest that *N. sua* and *N. meg.* are related. Their plastomes share an extra cut in the equivalent *N. tab.* fragment K-2, the fragments K-4-9 and a 100 bp insertion in K-6. It is highly unlikely that all three changes accumulated independently in the plastomes of both species.

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