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Nicotiana Chloroplast Genome III. Chloroplast DNA Evolution

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Summary. *Nicotiana* chloroplast genomes exhibit a high degree of diversity and a general similarity as revealed by restriction enzyme analysis. This property can be measured accurately by restriction enzymes which generate over 20 fragments. However, the restriction enzymes which generate a small number (about 10) of fragments are extremely useful not only in constructing the restriction maps but also in establishing the sequence of ct-DNA evolution. By using a single enzyme, Sma I, a elimination and sequential gain of its recognition sites during the course of ct-DNA evolution is clearly demonstrated. Thus, a sequence of ct-DNA evolution for many *Nicotiana* species is formulated. The observed changes are all clustered in one region to form a "hot spot" in the circular molecule of ct-DNA. The mechanisms involved for such alterations are mostly point mutations but inversion and deficiency are also indicated. Since there is a close correlation between the ct-DNA evolution and speciation in *Nicotiana* a high degree of cooperation and coordination betwen organellar and nuclear genomes is evident.

Key words: *Nicotiana* – Chloroplast DNA – Restriction fragments – Deletion – Evolution

Introduction

Nicotiana chloroplast genomes possess many distinct properties and unique features. The fragment patterns generated by restriction enzymes exhibit a high degree of diversity as well as a general similarity (Rhodes et al. 1981, Kung et al. 1981). For example, the individual restriction pattern is species specific for any given species while the overall configuration is characteristic of the genus *Nicotiana* (Kung et al. 1981). In many species, even variation of a fragment contains sufficient information to indicate its taxonomic position or to reflect its evolutionary relationship. Therefore, restriction patterns of *Nicotiana* chloroplast DNA (ct-DNA) can be used in this context to identify species in a manner similar to the taxonomic, cytogenetic (Goodspeed 1954) and biochemical (Chen et al. 1976) classifications. This is due mainly to the existence of the unusual taxonomic and evolutionary relationships among *Nicotiana* species. Many of the 64 *Nicotiana* species have originated through interspecific hybridization followed by doubling of the chromosomes (Goodspeed 1954). In each hybridization the ct-DNA is generally inherited maternally (Wildman et al. 1974) with only infrequent alteration (Kung et al. 1981). Thus, the present-day species with 24-pairs of chromosomes are more advanced than those possessing 12-pairs. Consequently, if a 12-paired chromosome species shares an identical ct-DNA fragment pattern with that of a 24-paired chromosome species a close phylogenetic relationship between them is implied. It is likely that the former is an ancestor (female) of the latter. The best studied case is the origin of *N. tabacum* (N = 24) in which *N. sylvestris* (N = 12) is the female parent in the original cross (Gray et al. 1974) and both of these species possess identical ct-DNA (Rhodes et al. 1981) as directed by the mode of maternal inheritance.

This study is designed to investigate the restriction pattern of ct-DNA from many closely related species having different levels of ploidy. From these results the sequence of ct-DNA evolution in *Nicotiana* is formulated. This is accomplished by a simple procedure using a single restriction enzyme without radiolabelling the fragments. In this paper we present our findings and discuss their significance.

Materials and Methods

Plant Material

Tobacco seedlings of approximately 1-inch in height were transplanted into 5-inch soil pots in the greenhouse. Plants

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were allowed to grow for 4 to 8 weeks depending on the season after transplanting and then covered with black cloth 48 hours before the leaves were harvested.

Purification of Chloroplasts

Chloroplasts were purified by the procedure previously described by Rhodes et al. (1981). Freshly harvested leaves were homogenized in a metal Waring blender with liquid nitrogen (Rhodes and Kung 1981). When the liquid nitrogen had evaporated and the temperature had warmed to −5 °C at room temperature, three weight volumes of isolation buffer (Walbot 1977) were added to the powder with rapid mixing. The suspension was filtered through miracloth and centrifuged at 1,500 × g for 15 min. The crude chloroplast pellets were gently resuspended in the same buffer and purified through a discontinuous silica sol gradient (Walbot 1977) containing 0.24 M sucrose throughout.

Preparation of Chloroplast DNA

Chloroplast DNA was prepared from the purified chloroplasts according to the method of Kolodner and Tewari (1975). The chloroplasts were lysed in 2% sarkosyl and DNA was fractionated by CsCl density gradient centrifugation.

Restriction Enzyme Analysis of Chloroplast DNA

Chloroplast DNA was digested with EcoRI, Bam HI, Hind III, Xho I, Pst I, Kpn I, Sma I, or Sal I as directed by the supplier, New England Biolabs. The restriction fragments were seperated by electrophoresis in a 0.8 or 1.5% agarose slab gel according to the procedure of Helling et al. (1974). The fragments produced by Pst I, Kpn I, Sma I, and Sal I were analyzed in 0.8% agarose slab gels. Ethidium bromide-stained bands were visualized with a Model C-62 Chromatovue transilluminator (Ultra-Violet Product, Inc.). The EcoRI and Hind III single or double digest fragments of Lambda phage DNA were used as molecular weight markers.

Results and Discussion

Figure 1 illustrates the restriction patterns of *N. tabacum* ct-DNA generated by eight different enzymes. It is evident that EcoRI produces the largest number of fragments among the enzymes used and therefore has the highest resolving power to uncover differences. As represented in Fig. 2 A, of the 40 fragments generated by the EcoRI enzyme, the first 10–13 in regions I and II are worth noting. In many species there are six fragments in each of these two regions. Fragments 1–3 and 6 in region I are stable and present in all 35 *Nicotiana* species examined so far. In contrast, fragments 4 and 5 are extremely variable. One or both can be altered; one or both may be absent. Unlike region I, the six fragments in region II are stable with only a few exceptions. The constant fragments may indicate that a mutation in this region is not likely to survive because of the essential function of the fragment. Indeed, the fragments 6 and 9 are known to contain the 23 s and 16 s rRNA genes respectively (Sugiura and Kusuda 1979).

In region III only half of the fragments are constant. The variability increases in the remaining regions (IV–VI). It is such a combination of diversity and similarity in fragment pattern that forms the basis of species specificity as well as the overall identity of *Nicotiana* ct-DNA. The EcoRI patterns in Fig. 2 A contain essential information reflecting many important evolutionary events. For example, the ct-DNA of *N. noctiflora* bears close resemblance to that of *N. gossei* because they are historically related (Fig. 2 A lane c and d). The same information exists among the numerous fragments generated by Bam HI (Fig. 2 B) and many other restriction enzymes. Among the seven *Nicotiana* species analyzed in Fig. 2 B, ct-DNAs of *N. longiflora, N. sylvestris* and *N. tabacum* (lane c, e, and f) have an identical Bam HI fragment pattern. Although *N. longiflora* and *N. langsdorffii* ct-DNAs (lanes c and g) bear a close resemblance they are clearly different. *N. glauca* ct-DNA (lane b) is quite similar to that of *N. plumbaginifolia* (not shown) but is distinguishable from that of both *N. gossei* and *N.*

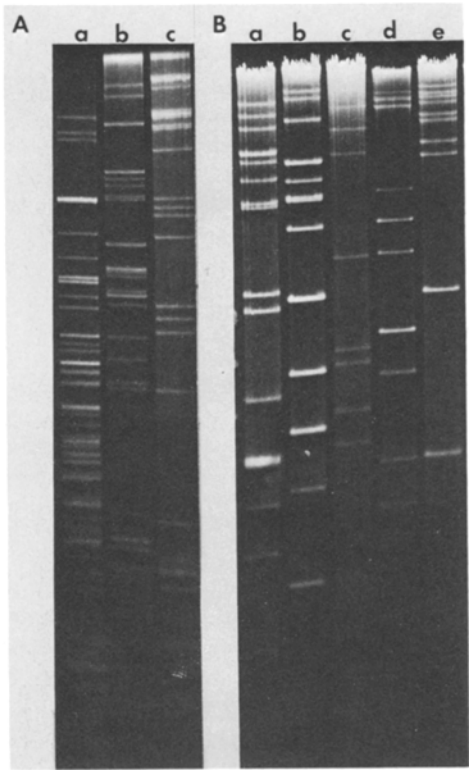

Fig. 1. A and B. Restriction fragment patterns of *N. tabacum* ct-DNA digested with several enzymes. In **A:** EcoRI (a); Bam-HI (b); and Hind III (c). In **B:** Xho I (a); Sma I (b); Kpn I (c); Pst I (d); and Sal I (e). Fragments were seperated by electrophoresis for 42 h in 1.5% **(A)** and 0.8% **(B)** agarose gels

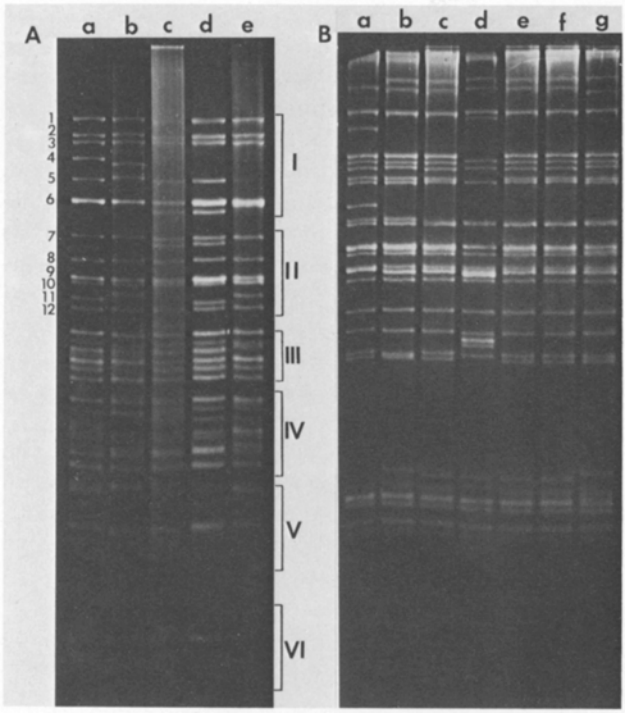

Fig. 2A and B. Restriction fragment patterns of ct-DNA from several *Nicotiana* species digested with Eco RI **(A)** and Bam HI **(B)**. In **A:** the Eco RI fragments are grouped into 6 regions (I–IV) and the fragments of *N. otophora* in the first two regions are numbered (1–12). The species are *N. otophora* (a); *N. africana* (b); *N. noctiflora* (c); *N. gossei* (d); and *N. tabacum* (e), In **B:** *N. gossei* (a); *N. glauca* (b); *N. otophora* (c); *N. longiflora* (d); *N. sylvestris* (e); *N. tabacum* (f) and *N. langsdorffii* (g). Fragments were seperated by electrophoresis for 42 h in 1.5% agarose gels

otophora (lane a and d). The difference of ct-DNA between *N. gossei* and *N. otophora* is remarkable. They differ in 13 out of the 27 Bam HI bands. This matches the extent of variation in their EcoRI fragments in which 18 of the 40 bands are different (Fig. 2A lanes a and d). Therefore, both EcoRI and Bam HI can be used to measure accurately the degree of variability of ct-DNA in *Nicotiana* species.

In the case of restriction enzymes which generate only a small number of fragments (Fig. 1B) Sal I and Sma I were selected for this study because they have been frequently used to construct restriction maps of higher plant ct-DNA (Bedbrook and Bogorad 1976; Jurgenson and Bourque 1980; Herrmann et al. 1980).

Fig. 3A is a typical Sal I restriction pattern of *Nicotiana* ct-DNA in which there are 9–11 bands. There is no difference in Sal I pattern among many *Nicotiana* species and only in four species closely related to *N. tomentosa* are some small fragments combined to form a large one. In these species (*N. tomentosa, N. tomentosiformis, N. knightiana* and *N. paniculata*) fragments 7 and 10 are replaced by fragment 6 which is now represented as doublet (Fig. 3A, lane b).

In contrast, the Sma I fragment patterns are variable and provide much more revealing information. Figures 3B and C show the Sma I pattern of ct-DNA from several key *Nicotiana* species on the evolutionary scheme. They represent six unique types of Sma I ct-DNA fragment patterns. In the 22 species examined there are a combined total of 21 distinct Sma I bands. Eight are present in every species and thirteen are variable and scattered (Table 1). On the average there are 13–15 bands in each species. The fragments 17 and 20 are doublets in all species whereas fragment 15 exists in duplicate in most species but is missing in *N. tomentosa, N. tomentosiformis, N. knightiana* and *N. paniculata.* Only in *N. tabacum, N. sylvestris* and *N. longiflora* are there two copies of fragment 18 (Table 1). Since the molecular weight of each fragment is known (Table 1) it is therefore possible to trace the origin and conversion of each variable band in all six species. For example, frag-

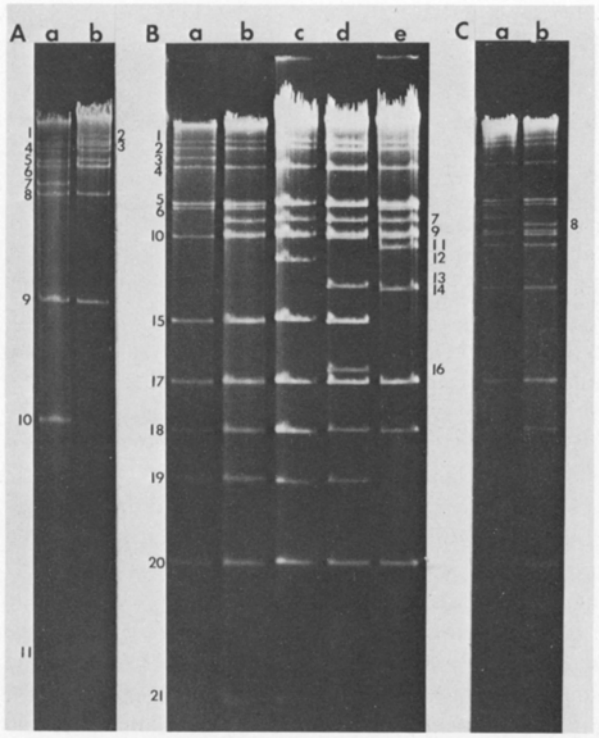

Fig. 3A–C. Restriction fragment patterns of ct-DNA from several *Nicotiana* species digested whith Sal I **(A)** and Sma I **(B** and **C).** In **A:** there are a total of 11 Sal I fragments in *N. tabacum* (a) and 9 in *N. tomentosa* (b). In **B** and **C:** there are a combined total of 21 Sma I fragments (as numbered) found in these species. The molecular weights and distribution of each fragment are listed in Table 1. The species are *N. plumbaginifolia* (a); *N. langsdorffii* (b); *N. sylvestris* (c); *N. otophora* (d); and *N. tomentosa* (e). In **C:** *N. tomentosiformis* (a) and *N. paniculata* (b). Fragments were seperated by electrophoresis in 0.8% agarose gels for 24 h in **A** and **B,** 42 h in **C** in order to achieve better seperation between fragments 9 and 10

Table 1. Restriction fragment size generated by Sma I from six *Nicotina* ct-DNA

Fragment Number	Molecular Weight X 10^6	*N. plumbaginifolia*	*N. langsdorffii*	*N. tabacum*[a]	*N. otophora*	*N. tomentosa*	*N. paniculata*
1	21	+	+	+	+	+	+
2	17	+	+	+	+	+	+
3	12.5	+	–	–	–	–	–
4	11.5	+	+	+	+	+	+
5	7.2	++	++	++	++	++	++
6	6.9	+	+	–	–	+	+
7	6.3	–	+	+	+	+	–
8	6.0	–	–	–	–	–	+
9	5.7	–	+	+	+	+	+
10	5.6	+	+	+	+	+	+
11	5.1	–	–	–	–	+	+
12	4.9	–	–	+	–	–	–
13	4.1	–	–	–	+	–	–
14	4.0	–	–	–	–	+	+
15	3.5	++	++	++	++	–	–
16	2.6	–	–	–	+	–	–
17	2.4	++	++	++	++	++	++
18	1.95	+	+	++	+	+	+
19	1.55	+	+	+	+	–	–
20	1.15	++	++	++	++	++	++
21	0.61	+	+	+	+	–	–

[a] Data from Rhodes and Kung (1981); ++ = two copies

ment 3 is unique and found only in *N. plumbaginifolia* (Fig. 3B, lane a). It is replaced in *N. langsdorffii* by fragments 7 and 9 (Fig. 3B, lane b). The combined molecular weight of fragment 7 and 9 (12.0×10^6) is practically the same as that of fragment 3 (12.5×10^6). Since all the other fragments are constant the conversion from fragment 3 of *N. plumbaginifolia* to 7 and 9 of *N. langsdorffii* is clearly demonstrated. It is thus deduced that ct-DNA of *N. langsdorffii,* a more advanced species, has evolved from *N. plumbaginifolia* by the introduction of a single Sma I site into fragment 3 of *N. plumbaginifolia* (Fig. 3B, lanes a, b; Fig. 4). Likewise, a gain of a single Sma I site into fragment 6 of *N. langsdorffii* ct-DNA at different positions has generated the ct-DNAs of *N. sylvestris* and of *N. otophora* (Fig. 3B, lanes c–d and Fig. 4). The two new Sma I fragments generated in each case are fragments 12 and 18 for *N. sylvestris,* 13 and 16 for *N. otophora.* Their combined molecular weights are 6.85×10^6 and 6.7×10^6 which is essentially equal to the original size of 6.9×10^6 for fragment 6 (Table 1). This is confirmed by nick translation and hybridization experiment (data not shown). The alternative interpretation in this case is that initially both *N. sylvestris* and *N. otophora* could have identical Sma I sites but subsequently an inversion occurred in one of them.

In *N. tomentosa, N. tomentosiformis, N. knightiana* and *N. paniculata* elimination instead of gain of the Sma I site seems to be the mechanism of the evolution of their ct-DNA. As illustrated in Fig. 3B and C, the unique fragment 11 (5.1×10^6) of *N. tomentosa* appeared which has a combined molecular weight (5.1×10^6) of fragments 15 and 19 not found in this species (Table 1). The formation of fragment 14 (4.0×10^6) resulted from the combination of the missing fragments 15 (second copy, 3.5×10^6) and 21 (0.61×10^6). Therefore, the elimination of Sma I sites between fragments 15 and 19, 15 and 21, is clearly demonstrated (Fig. 4). Since all other fragments are equal, the reduction of fragment size from 7 to 8 in *N. paniculata* (Fig. 3C, lane b; Fig. 4) may indicate a deletion. To date, no polymorphism for a restriction site has been observed in this study although such intraspecific variability was reported in *N. debneyi* (Scowcroft 1979) and in animal mitochondrial genomes (Castora et al. 1980).

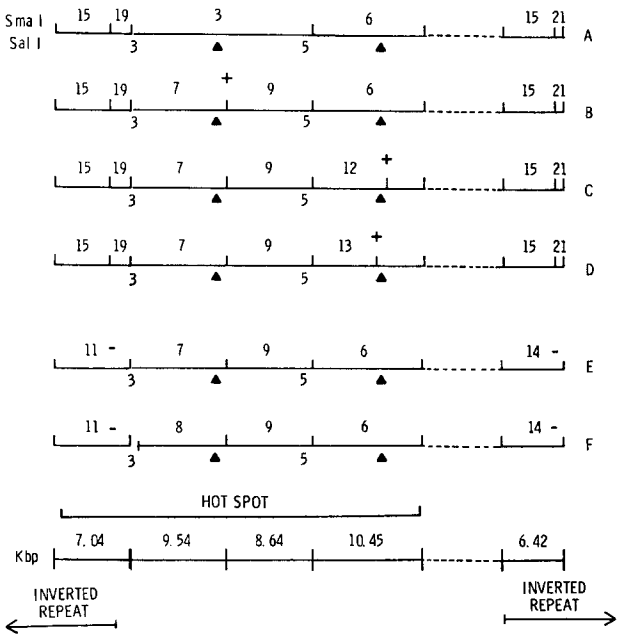

Fig. 4. A diagramatic representation of restriction maps of two segments of *Nicotiana* chloroplast genome showing the locations of gain (+) and elimination (–) of Sma I (1) and Sal I (▲) sites. The relative positions of the inverted repeats and the suggested "hot spot" are also indicated. (*A*), *N. plumbaginifolia* has the unique Sma I fragment 3; (*B*), an gain of a Sma I site at (+) position converted fragment 3 into fragments 7 and 9 in *N. langsdorffii;* (*C*), a second gain of Sma I site at (+) position divided fragment 6 into fragments 12 and 18 in *N. sylvestris* or 13 and 16 in *N. otophora* (*D*). The elimination of Sma I sites between fragments 15 and 19, 15 and 21 at (–) positions generated fragments 11 and 14. A possible deletion of a segment of Sma I fragment 8 of *N. paniculata* (*F*) as compared with that 7 *N. tomentosa* (*E*) is marked by a small breakage. All fragment numbers used here are based on Figure 3 and Table 1

From the combination of gain and elimination of Sma I sites it is established that fragments 7 and 9, 12 and 18, 13 and 16, 15 and 19 and 15 and 21 are linked. This is in agreement with the Sma I restriction map of *N. tabacum* ct-DNA (unpublished data) on which fragments 15, 19, 7, 9, 12 and 18 are linked together and arranged in this order. On the basis of this information a sequence of evolution of ct-DNA from several species in *Nicotiana* is proposed. Figure 4 is a diagramatic representation indicating the sequential addition of three Sma I sites and the deletion of Sma I and Sal I sites during the course of ct-DNA evolution. Because all of these observed changes are clustered together in one region they may represent a "hot spot" as defined by Benzer (1961). This region or at least the Sma I site seems to mutate much more frequently than others.

Figure 5 is a phylogenetic tree of ct-DNA evolution within this genus. Goodspeed (1954) assummed that the ancestral stocks having 5 pairs of chromosomes had diverged into several branches. It appears the present-day species *N. plumbaginifolia* represents one of them at the 12-pair level. In this proposed scheme of evolution of the *Nicotiana* chloroplast genome, *N. plumbaginifolia*

Table 2. The relationship of ct-DNA from five closely related species determined by restriction enzyme analysis

Enzymes	Distribution[a]	*N. tabacum*	*N. sylvestris*	*N. longiflora*	*N. langsdorffii*	*N. plumbaginifolia*
Sal I	T	11	11	11	11	11
	C		11	11	11	11
	S		0	0	0	0
Sma I	T	14	14	14	14	13
	C		14	14	13	11
	S		0	0	1	2
Bam HI	T	27	27	27	27	27
	C		27	27	24	24
	S		0	0	3	3
EcoRI	T	40	40	40	39	38
	C		40	39	31	28
	S		0	1[b]	8	10

[a] The total (T), common (C) (with *N. tabacum*), and species specific (S) fragments

[b] This difference is so small that it can be considered identical

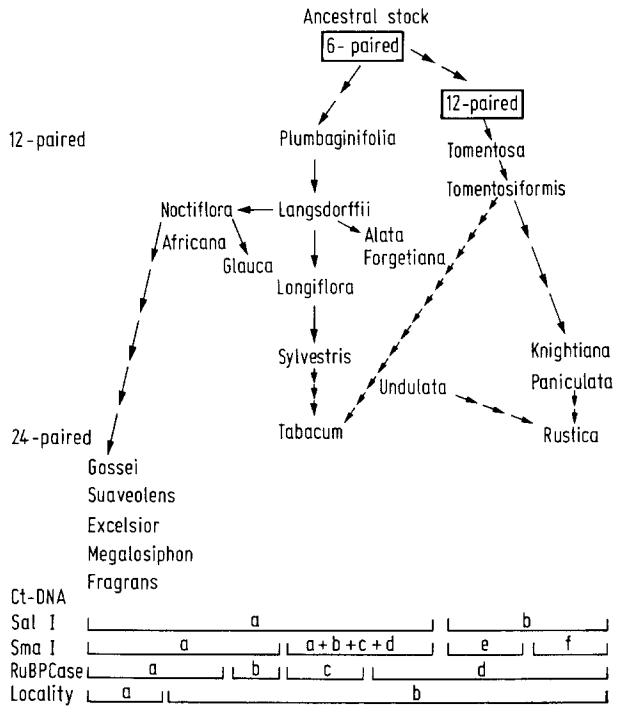

Fig. 5. A phylogenetic tree of ct-DNA evolution in *Nicotiana* based on the restriction pattern of ct-DNA supported by other biochemical (RuBPCase) and distributional data. Arrows indicate the sequence of evolution. *N. tabacum* originated from a cross between *N. sylvestris* and *N. tomentosiformis* whereas *N. rustica* was a product between *N. paniculata* and *N. undulata.* (a to f indicates differences)

may have served as the ancestral stock from which all the Australian and some of the American species were derived. It is proposed here that ct-DNA of *N. langsdorffii* originated directly from *N. plumbaginifolia.* There is adequate cytogenetic evidence to support this claim (Goodspeed 1954). *N. langsdorffii* may in turn have given rise to *N. noctiflora, N. longiflora* and *N. alata.* The evidence that *N. noctiflora* includes *N. langsdorffii* elements is available (Goodspeed 1954). At this point of evolution the ancestor of *N. noctiflora* had migrated to Australia and formed the current Australian population. *N. noctiflora* ct-DNA has similar or identical restriction fragment patterns generated by Sal I, Sma I, Bam HI and EcoRI, to that of *N. langsdorffii* and many Australian species including *N. suaveolens, N. gossei, N. excelsior, N. megalosiphon* and *N. fragrans.* The evidence is therefore very convincing that the ancestor of *N. noctiflora* is indeed the link between these two populations of *Nicotiana* as often suggested (Goodspeed 1954; Chen et al. 1976). The restriction enzyme analysis of *N. africana* ct-DNA also indicate that it may have been derived from an ancestor similar to *N. noctiflora.*

The proposed sequence of ct-DNA evolution from *N. plumbaginifolia* to *N. langsdorffii, N. longiflora, N. sylvestris* and *N. tabacum* deserves special treatment. These are very closely related species and all belong to the same section Alatae except *N. tabacum* (Goodspeed 1954) which is the progeny of a cross between *N. sylves-*

tris and *N. tomentosiformis* (Gray et al. 1974). This proposal is based on the restriction analysis of their ct-DNAs but is strongly supported by evidence obtained from taxonomic and biochemical studies (Goodspeed 1954; Chen et al. 1976). The recent biochemical evidence is particularly convincing. All five species in this sequence contain the identical isoelectric focusing pattern of the large subunit of RuBPCase, a product of the chloroplast genome (Chen et al. 1976). Their differential relationship is clearly illustrated in Table 2 which strongly supports this proposed sequence.

Chloroplast DNAs from *N. tomentosa, N. tomentosiformis, N. knightiana* and *N. paniculata* are clearly separated from all the other species studied here. Their immediate ancestor is different from but probably parallel to *N. plumbaginifolia.* However, the close relationship of this group is highly supported by other evidence (Goodspeed 1954). Furthermore, the evidence obtained from amino acid analysis and peptide mapping of the large subunit of RuBPCase (Kung et al. 1976) and the current analysis of restriction fragments of ct-DNA (Fig. 3 B–C, Fig. 4) further divide these species into two subgroups; *N. tomentosa* and *N. tomentosiformis* for one and *N. knightiana* and *N. paniculata* for the other (Fig. 5). This is in agreement with the results obtained previously (Rhodes et al. 1981).

It is interesting to note that in this proposed scheme of evolution (Fig. 5) there is a close correlation between the restriction pattern of ct-DNA and the isoelectric focusing pattern of the large subunit of RuBPCase. It is well established that the large subunit of RuBPCase is coded by ct-DNA (Kung 1976). Whether such a correlation bears any functional relationship is currently under investigation.

Conclusions

Several remarkable features are evident from this study. First, a single restriction enzyme (Sma I) can uncover sufficient variation of ct-DNA among *Nicotiana* species to construct a sequence of its evolution. It is a simple procedure and does not need to radioactively label the fragments or a second enzyme digestion. Second, a sequential gain, as well as elimination, of the Sma I site is a unique feature of ct-DNA evolution in *Nicotiana.* Third, the great majority of mutations are clustered in one region to form a "hot spot". Fourth, most of the variability is due to point mutation but deletion and inversion are also implicated. However, recombination has been suggested but only as a rare event (Kung et al. 1981). Finally, there is a close correlation between the ct-DNA evolution and speciation in *Nicotiana* indicating a high degree of cooperation between organelle and nuclear genomes in evolution as well as in expression (Kung 1977).

Nicotiana species studied here cover a wide range with respect to their biochemistry, cytogenetics, morphology and distribution. They include many key species having 12 pairs and 24 pairs of chromosomes indigenous to both America and Australia. Particular attention was focused on the ancestral linkage between those two continents as well as the parentage and descendant relationship during the course of ct-DNA evolution. Results obtained from the restriction fragment analysis of ct-DNA clearly demonstrate that the ancestor of *N. noctiflora* is the bridging species between the Western Hemisphere and Australian populations.

Results presented here also demonstrate that restriction fragment analysis of ct-DNA is a powerful tool and can provide important information in the study of evolution that exceeds many other means available today (Brown et al. 1979). This is demonstrated by the formulation of a sequence of ct-DNA evolution involving several key *Nicotiana* species (Fig. 4). Moreover, by selecting proper restriction enzymes such as Sma I to treat several *Nicotiana* species a restriction map of ct-DNA can be constructed in a manner similar to using several enzymes to digest a single type of organelle DNA.

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