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Comparison of Chloroplast DNAs by Specific Fragmentation with EcoRI Endonuclease

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Summary. A technique has been developed whereby chloroplast DNA can be digested with EcoRI endonuclease without prior isolation of the DNA from the organelle. The specific fragmentation patterns produced show that chloroplast DNAs from different families or genera of plants have few if any bands in common, and it is not until comparisons are made between species of the same genus that similarities become apparent. The number of bands in common between species varies considerably from genus to genus and the degree of similarity of chloroplast DNAs may be correlated with how closely related the species are as judged by their ability to form viable hybrids.

Introduction

Previous studies of chloroplast DNA from a variety of plant species have indicated a high degree of similarity suggesting the possibility of conservation of chloroplast DNA during evolution. Examples of this can be seen in the similarity of the GC content of all higher plant chloroplast DNAs (see Kirk, 1971, for references) and in the uniform size of chloroplast DNA molecules which have all been found to be circles approximately 45 μM long (Herrmann et al., 1975; Kolodner and Tewari, 1972). However, such techniques are relatively insensitive methods for comparing DNA molecules, and even major differences in actual base sequence may not be revealed. Further support for the concept of the high conservation of chloroplast DNA has come from comparative studies of individual cistrons within chloroplast DNA. Thomas and Tewari (1974) have shown by hybridization techniques that the genes for chloroplast ribosomal RNA have been highly conserved during evolution. Similarly, studies on the structure of the large subunit of Fraction I protein by Wildman et al. (1973) have indicated little change in this protein. However, these methods of comparison also have a disadvantage in that only small regions of the DNA molecule are examined and the regions studied so far are probably less able to tolerate any alteration because of their importance to chloroplast function.

To study effectively the evolution of the whole chloroplast DNA molecule a method intermediate between these two extremes would be desirable. The development of restriction enzyme techniques for the specific cleavage of DNA molecules provides such an approach. Restriction enzyme fragmentation patterns have been used for the comparison of viral DNAs (Landy et al., 1974), plasmid DNAs (Thompson et al., 1974) and mitochondrial DNAs (Potter et al., 1975). Because of the specificity of the cleavage site of a restriction enzyme, the pattern produced is characteristic for a specific DNA molecule and small random changes in nucleotide sequence in the DNA may be reflected in changes in the fragmentation pattern. Studies by Thompson et al. (1974) have shown that this technique is more sensitive for detecting mutations than the technique of molecular hybridization.

In this paper comparisons of chloroplast DNAs from a wide range of plant species are presented. Comparisons have been made of the fragmentation patterns produced by digestion with EcoRI endonuclease which cleaves at the sequence GAATTC (Hedgpeth et al., 1972). In order to facilitate the analysis, a procedure has been developed whereby digestion of the DNA is effected without prior isolation of the DNA from the chloroplast.

Materials and Methods

Growth of Plants

Spinach plants (*Spinacia oleracea*) were grown in water-culture in a glasshouse (Spencer and Whitfeld, 1967). All other plants

were grown in vermiculite under low-intensity fluorescent lights (770 lx), in order to minimize starch production.

EcoRI Endonuclease Purification

The enzyme was purified from *Escherichia coli* strain RY13 by ammonium sulphate precipitation and DEAE-cellulose column chromatography according to the method of Hamer and Thomas, Jr. (1975). Stock samples of the enzyme were stored in 50% glycerol, 0.15 M NaCl, 5 mM potassium phosphate, pH 6.5, 0.5 mM EDTA, 0-05% Triton X-100, and had an activity of about 1 unit/μl (one unit of activity is defined as the amount of enzyme required to completely fragment 1 μg of λDNA in 1 h at 25° C in a standard reaction of 35 μl of 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8).

Spinach Chloroplast and Nuclear DNA

Chloroplasts and nuclei were prepared by razor chopping approximately 200 g spinach leaves (3–6 cm) in a Ficoll-dextran medium (Honda et al., 1966) and then fractionating the organelles on a sucrose density stepgradient (Tewari and Wildman, 1969). DNA was extracted by lysis of the separated organelles with 2% sarkosyl and purified by banding in preparative CsCl gradients. Analysis of the DNA by equilibrium buoyant density centrifugation in CsCl showed that less than 5% of the DNA was of mitochondrial origin.

EcoRI Endonuclease Treatment of Isolated Chloroplasts

Chloroplast suspensions for endonuclease digestion were prepared by a rapid blending procedure in sorbitol-containing medium and partially purified by differential centrifugation (Bottomley et al., 1974). Aliquots of chloroplast suspension equivalent to 300 μg of chlorophyll (about 4 μg DNA) were centrifuged at 3000 × g for 5 min and then resuspended in 0.2 ml of 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. After 15 min at 25° C, 30 μl of EcoRI endonuclease were added and incubation was continued at 25° C for 4 h. Chloroplasts were then lysed by adding 20 μl of 20% sodium dodecyl sulphate, mixed with 0.35 g of CsCl and warmed to 50° C for 2 min. After centrifugation at 3000 × g for 5 min, the clear fluid below the green pellicle was collected and added to 0.8 ml of water. Starch could then be removed by centrifugation at 3000 g for 5 min and the nucleic acids precipitated from the supernatant by the addition of 1 ml of ethanol and storage at −20° C overnight. The nucleic acids were collected by centrifugation at 8000 × g for 5 min, and washed once with ethanol. The dried pellet was dissolved in 25 μl of electrophoresis buffer containing 50% glycerol.

Electrophoresis

Prior to electrophoresis the nucleic acid sample was treated with 0.4 μg of DNase-free RNase for 5 min at room temperature. Tracking dye (bromo-phenol blue) and sodium dodecyl sulphate (0.2% final concentration) were added and the sample was layered onto a gel of 1% agarose (6 mm diameter × 11 cm length) in electrophoresis buffer (40 mM Tris-HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA). Electrophoresis was carried out at 4 mA/gel for 3.5 hrs. Gels were then stained for 30 min with ethidium bromide (5 μg/ml) and destained in water for 30 min. Photography was performed with a short-wave ultraviolet lamp using a red filter and Panatomic-X film.

Results

When purified spinach chloroplast DNA was digested with EcoRI endonuclease and the resultant fragments were electrophoresed on agarose gels, a characteristic pattern of approximately 16 distinct bands was ob-

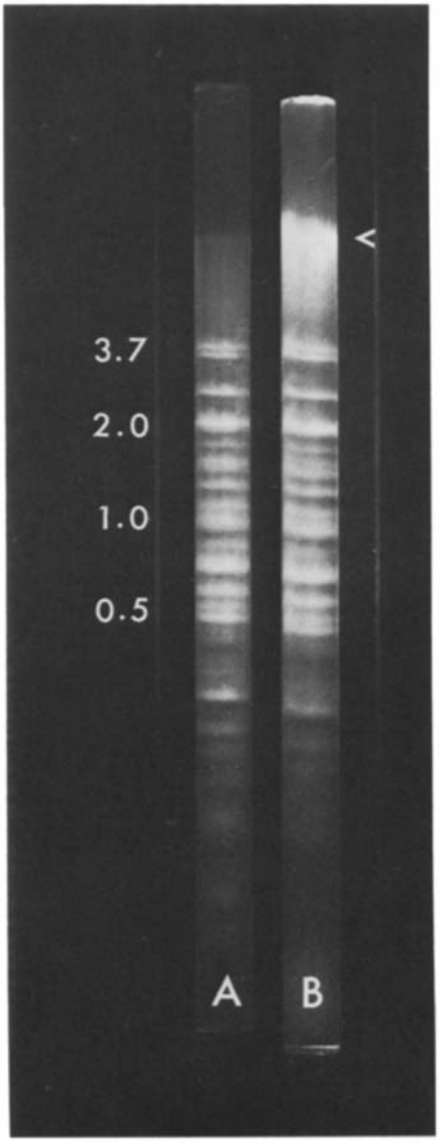

Fig. 1 A and B. Fragmentation patterns of spinach chloroplast DNA digested with EcoRI endonuclease. **A** Purified chloroplast DNA (3 μg) digested for 1 h at 37° C with 10 units of EcoRI in 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. **B** Crude preparation of chloroplasts prepared by blending leaves in sorbitol medium and then treated with an excess of EcoRI in 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8 as described in Materials and Methods. The molecular weight of the DNA fragments, estimated by comparison with λDNA and ϕ 80 DNA digests, is shown by the figures on the left (in Daltons × 10^{-6}). The arrow indicates DNA at the limiting-size of the gel (approximately 10×10^6 to 80×10^6 Daltons) present in samples resulting from treatment of crude chloroplast preparations

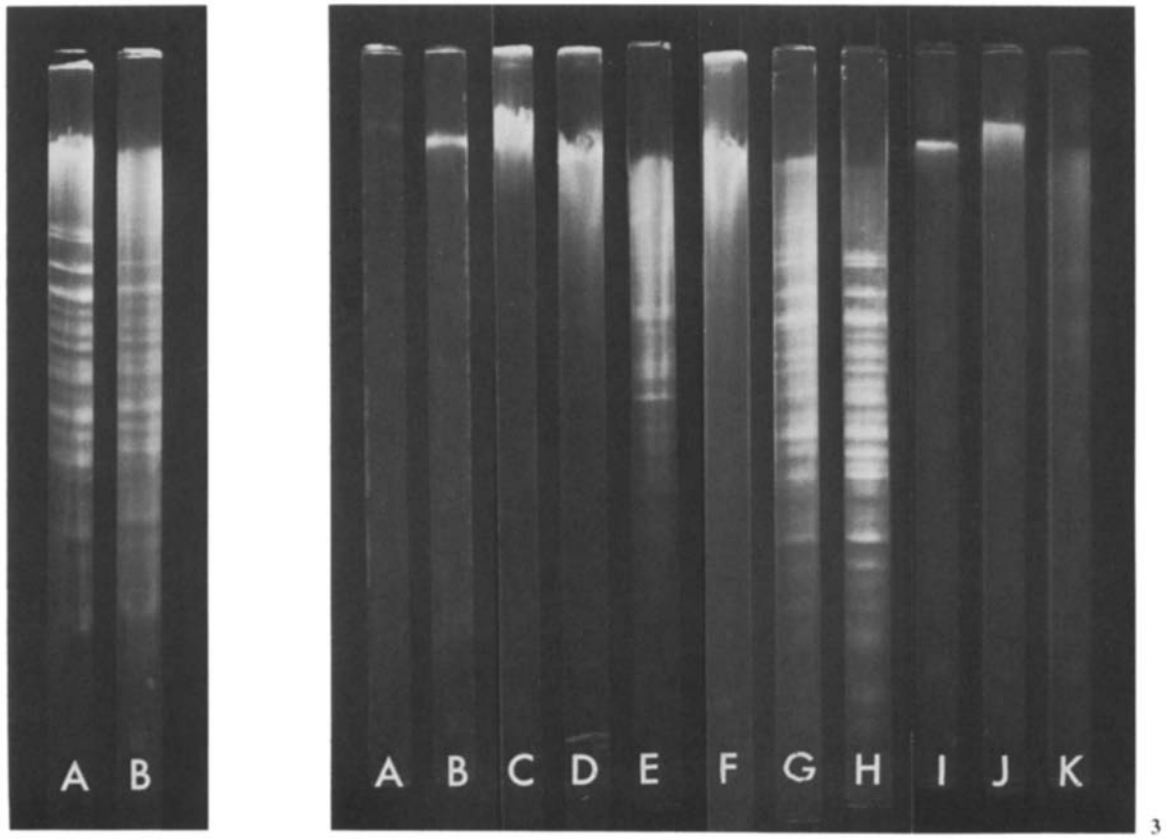

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Fig. 2A and B. Effect of Triton X-100 on EcoRI treatment of chloroplasts. **A** Crude chloroplasts in 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8, digested with EcoRI for 4 h at 25° C. **B** Chloroplasts treated as in (A) but with 2% Triton X-100 present in the digestion mixture. In the latter the two highest molecular weight fragments appear only faintly in the photograph although they were clearly visible in the stained gel

Fig. 3A–K. Digestion of nuclear and chloroplast DNA with EcoRI. **A** Spinach nuclei (approximately 4 μg DNA) incubated for 4 h at 25° C in 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8, without EcoRI present. Nuclei were lysed with 2% SDS and layered directly onto the gel for electrophoresis. **B** Spinach nuclei incubated as in (A) but with 30 units of EcoRI added. **C** Purified spinach nuclear DNA (4 μg) without treatment prior to electrophoresis. **D** Purified spinach nuclear DNA (4 μg) digested for 4 h at 25° C with 5 units of EcoRI in 35 μl 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. **E** Purified spinach nuclear DNA (4 μg) digested as in (D) but without 0.2 M NaCl in the medium. **F** Purified spinach chloroplast DNA (4 μg) without treatment prior to electrophoresis. **G** Purified spinach chloroplast DNA (4 μg) digested for 4 h at 25° C with 5 units of EcoRI in 35 μl 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. **H** Purified spinach chloroplast DNA (4 μg) digested as in (G) but without 0.2 M NaCl in the medium. **I** Limit band DNA present in gels resulting from the digestion of crude chloroplast preparations. This DNA had been eluted from the gels by the "freeze-squeeze" method of Thuring et al., and electrophoresed without treatment. **J** Sample of limit band DNA treated with 10 units of EcoRI for 1 h at 37° C in 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. **K** Sample of limit-band DNA treated as in (J) but without 0.2 M NaCl in the medium

tained (Fig. 1A). Additional faint bands were apparent in the low molecular weight region of the gel in some experiments but they were not reproducible. As judged by the relative intensities of the ethidium bromide staining, many of the bands seemed to consist of several fragments of the same molecular weight. By comparing the electrophoretic mobilities of the chloroplast DNA fragments with those fragments produced by EcoRI cleavage of λDNA and Ø80 DNA, the molecular weights of the DNA fragments were estimated to fall in the range of 4×10^6 to 2×10^5 daltons (Fig. 1A).

Analysis of purified chloroplast DNA necessitates the processing of relatively large amounts of leaf material, and lengthy procedures for the extraction and purification of the organelle DNA with the attendant risk of introducing random breaks into the molecules. For the ready comparison of chloroplast DNAs from many species such an approach was considered cumbersome and attention was therefore directed towards the possibility of digesting the DNA while it was still in the organelle. Preliminary experiments in which spinach chloroplasts were incubated at 37° C for 2 hrs in 0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-

HCl, pH 7.8, indicated that such preparations did not contain significant amounts of endogenous deoxyribonuclease (results not shown). Thus, providing conditions could be devised such that the DNA in the chloroplast was efficiently digested by the restriction enzyme, the resulting fragmentation pattern should be equivalent to that obtained by digestion of purified chloroplast DNA.

Optimum Conditions for Chloroplast Treatment

EcoRI endonuclease digestion of chloroplasts suspended in sorbitol medium (0.3 M sorbitol, 50 mM HEPES-NaOH, pH 7.6, 10 mM $MgCl_2$) failed to fragment the chloroplast DNA. If the chloroplasts were allowed to swell by suspending them in 0.2 M NaCl, 10 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, for 15 min at 25° C and then incubated a further 2 hrs at 25° C in the presence of EcoRI, digestion of the DNA resulted (Fig. 1B). Increasing the incubation time to 20 h or doubling the amount of enzyme added did not alter the fragmentation pattern and, in all subsequent experiments, digestions were carried out for 4 h at 25° C with an excess of enzyme. The optimal concentration of NaCl in the incubation medium was found to be 0.2 M NaCl, lower concentrations giving rise to smeared, less distinct fragmentation patterns, and higher concentrations of NaCl inhibiting the activity of the EcoRI endonuclease.

Comparison of the electrophoretic patterns of EcoRI restricted chloroplasts and purified chloroplast DNA showed them to be identical with the exception that in the patterns produced using chloroplasts a variable amount of DNA migrated at the limiting size of the gel (Fig. 1B) (limiting-size DNA is defined as DNA of molecular weight between 10×10^6 and 80×10^6 which tends to move as a single band in 1% agarose gels (Helling et al., 1974)). As the amount of this component was substantial in many instances it was considered necessary to ascertain its origin.

Nature of the Limiting-Size DNA

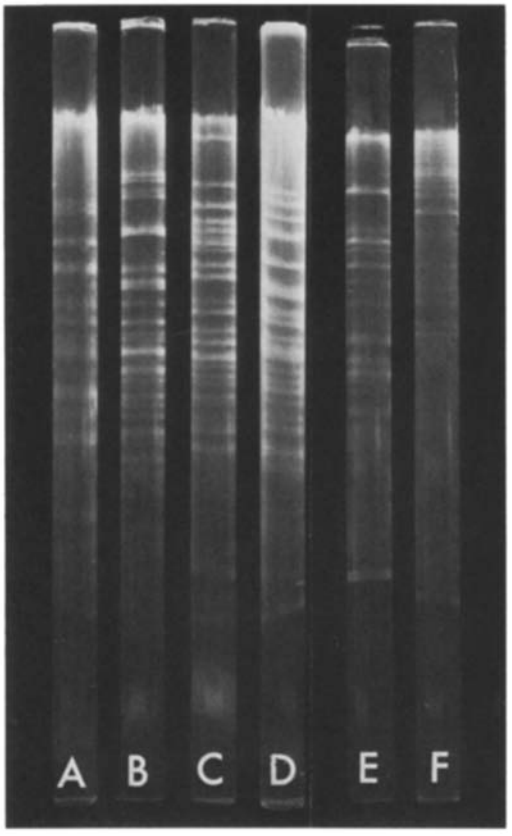
The limiting-size DNA produced during the endonuclease digestion of chloroplasts was not due to incomplete digestion of the DNA because long term (20 h) digestion failed to eliminate it. Nor was it due to the fact that some chloroplasts did not swell in the incubation medium thereby preventing access of the endonuclease to the DNA. Chloroplasts completely disrupted with 2% Triton X-100 still produced limiting-size DNA (Fig. 2). EcoRI activity was not affected by the addition of 2% Triton X-100, as judged by the digestion of λDNA.

The possibilities remained that the limiting-size band was a large fragment of chloroplast DNA which was protected from endonuclease digestion by virtue of its association with some chloroplast protein, or that it was contaminating nuclear DNA. The following observations led to the conclusion that it was nuclear DNA. The proportion of DNA migrating in the limiting-size region of the gel varied from one chloroplast preparation to another and was least in preparations which had been purified by centrifugation on a sucrose-gradient. Spinach nuclei which had been digested with EcoRI endonuclease in buffer containing 0.2 M NaCl, lysed with 2% SDS and electrophoresed on an agarose gel, gave only one band which migrated at the limiting-size of the gel (Fig. 3B). DNA released from nuclei incubated without EcoRI endonuclease in the medium failed to enter the gel (Fig. 3A), presumably because of its large size.

Purified nuclear DNA was only slightly fragmented below the limiting-size if digested with EcoRI in the presence of 0.2 M NaCl, but was cleaved to much smaller fragments if digested in the absence of NaCl (Fig. 3D,E). The pattern obtained in the absence of salt was mainly a broad smear but discrete fragment sizes could be seen, presumably arising from repetitive sequences within the nuclear DNA (Mowbray and Landy, 1974). Chloroplast DNA yields the same basic pattern whether digested in the presence or absence of salt (Fig. 3G, H) although it should be noted that the presence of salt in the sample has a slight effect on the migration rate of the DNA bands. When the limiting-size DNA fragment arising from digestion of chloroplasts with EcoRI was eluted from the gel (Thuring et al., 1975), and redigested with EcoRI, it was found to be unchanged if 0.2 M NaCl was present during digestion but degraded to smaller fragments in the absence of NaCl (Fig. 3I, J, K). From these results it was concluded that the limiting-size band of DNA resulting from restriction of chloroplasts was nuclear in origin and that the presence of contaminating nuclei in a chloroplast preparation would not affect the restricted chloroplast-DNA pattern, provided digestion with EcoRI was done in the presence of 0.2 M NaCl.

Comparison of Chloroplast DNAs from Different Plant Species

Chloroplasts from a range of plant species were treated with EcoRI endonuclease in the presence of 0.2 M NaCl under conditions determined to give complete



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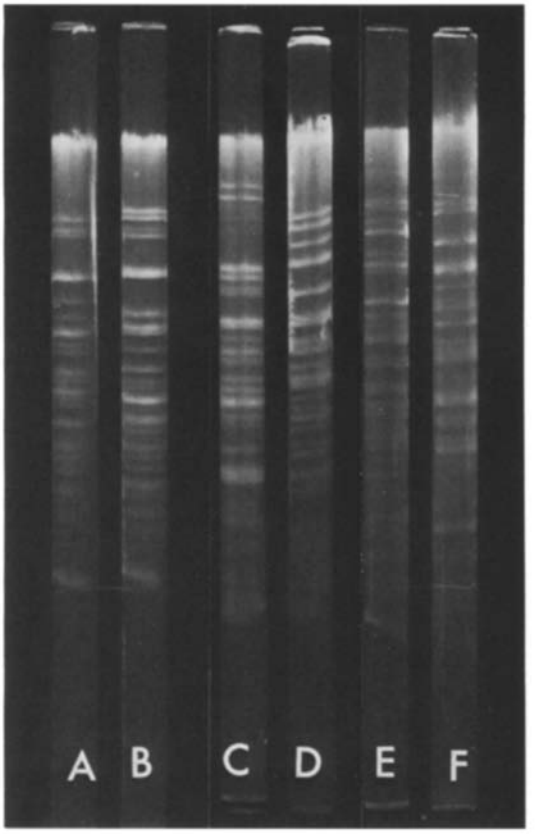

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Fig. 4A–F. Digests of chloroplasts DNA—comparison of Families. **A** *Spinacia oleracea* (Chenopodiaceae). **B** *Lycopersicum esculentum* (Solanaceae). **C** *Cucumis sativa* (Cucurbitaceae). **D** *Pisum sativum* (Leguminosae). **E** *Raphanus sativus* (Cruciferae). **F** *Euglena gracilis* (Euglenaceae)

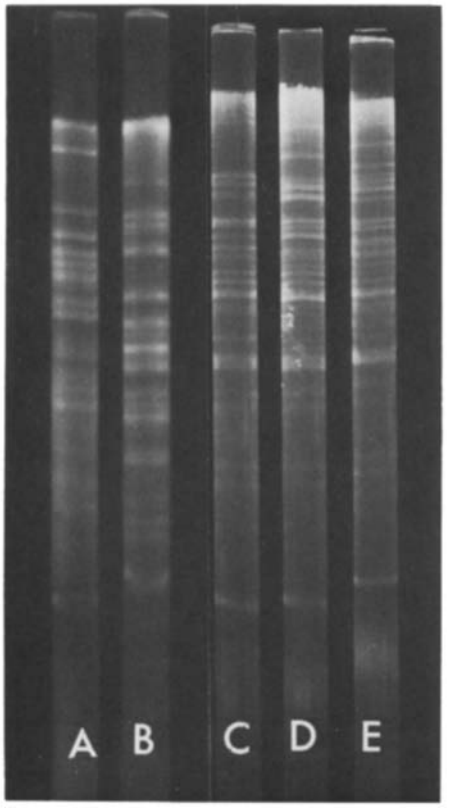
Fig. 5A–F. Digests of chloroplast DNA—comparison of Genera. **A** *Nicotiana tabacum* (Solanaceae). **B** *Lycopersicum* (Solonaceae). **C** *Lupinus angustifolius* (Leguminosae). **D** *Pisum sativum* (Leguminosae). **E** *Beta vulgaris* (Chenopodiaceae). **F** *Spinacia oleracea* (Chenopodiaceae)

digestion and the fragmented DNAs electrophoresed on agarose gels. The species have been arranged into taxonomic groups for ease of comparison.

Chloroplast DNA of different plant families produced markedly different fragmentation patterns (Fig. 4). All of the higher plant species examined yielded DNA fragments of similar size range (10^5 to 6×10^6 Daltons) but those from *Euglena* were, on average, clustered more in the higher molecular weight region of the gel. Comparison of different genera within a family also showed that there were few, if any, bands in common (Fig. 5).

It was not until comparisons were made within a genus that similarities in the restricted chloroplast DNA patterns became apparent and even then the degree of similarity varied considerably from genus to genus. The two *Cucumis* species which were examined showed few bands in common (Fig. 6A, B). EcoRI chloroplast DNA patterns of three *Lupinus* species showed a grouping of bands of similar molecular weight which leads to an impression of overall similarity (Fig. 6C, D, E). Closer examination, however, revealed that only a few bands are common to all three species.

The most striking result obtained in this study was that with the genus *Nicotiana*. This genus was originally examined because it is possible to readily cross different species within the genus thus making it suitable for studies on the inheritance of chloroplast DNA (Wildman et al., 1973). As can be seen in Fig. 7 (A, B, C) the three *Nicotiana* species native to Australia which were analysed had identical chloroplast DNA restriction patterns. Similarly, three *Nicotiana* species from America gave identical patterns except for one pair of bands missing in the *N. tabacum* pattern (Fig. 7D, E, F). This difference may be due to variation amongst cultivars of *N. tabacum*, a species which has undergone extensive hybridization during its cultivation by man. Between the Australian and American *Nicotiana* species only five band differences



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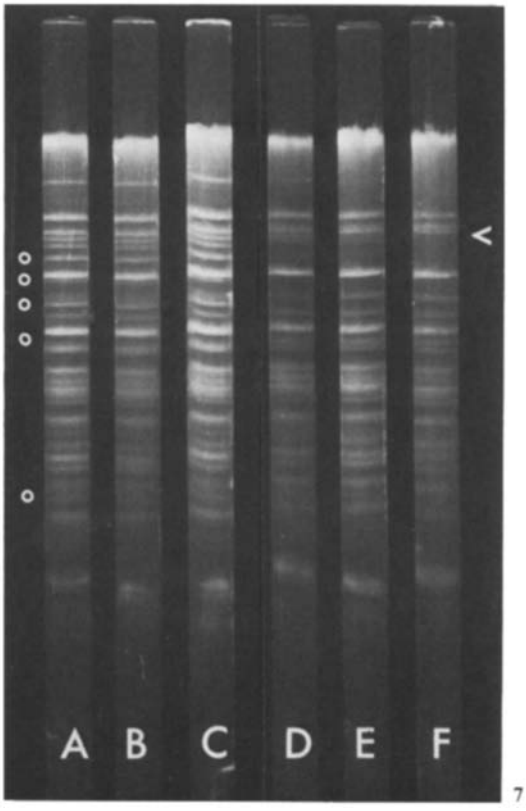

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Fig. 6A–E. Digestion of chloroplast DNA—comparison of species. **A** *Cucumis sativa*. **B** *Cucumis melo*. **C** *Lupinus angustifolius*. **D** *Lupinus luteus*. **E** *Lupinus albus*

Fig. 7A–F. Digestion of chloroplast DNA—comparison of species. **A** *Nicotiana excelsion* (Australian species). **B** *Nicotiana suaveolens* (Australian). **C** *Nicotiana gossei* (Australian). **D** *Nicotiana langsdorfii* (American). **E** *Nicotiana bonariensis* (American). **F** *Nicotiana tabacum* (American). Differences between the Australian and American species are denoted by the white dots. The arrow indicates the position of two bands missing from the *N. tabacum* fragmentation pattern compared with the other two American species

were observed, even though the two groups have been separated for over 150 million years (Tarling, 1971).

Discussion

The procedure described in this paper for the endonuclease restriction analysis of chloroplast DNA provides a simple means for comparing the DNAs from many plant species. Only small amounts of leaf material are required for the analysis and contamination of the chloroplast preparation with nuclei does not constitute a real problem. We have had some difficulty in obtaining distinct fragmentation patterns from a few plant species (*Zea mays, Atriplex, Hordeum vulgare*), but this may reflect the difficulty of preparing intact chloroplasts from these species rather than the failure of the restriction enzyme to digest the DNA in the chloroplast. If the chloroplasts are damaged when the leaf tissue is first disrupted then deoxyribonucleases in the cytoplasm may be able to partially degrade the DNA before the organelles are purified.

It is not clear why nuclear DNA is not cleaved to fragments smaller than the limiting-size DNA when digestion is carried out in the presence of 0.2 M NaCl but it is when NaCl is absent. However, it is known that under some conditions EcoRI will cleave at the sequence -AATT-, whereas under conditions of different pH or ionic strength, the sequence -GAATTC- is required for cleavage (Polisky et al., 1975). It may be that in nuclear DNA there are a number of sites which are cleaved by EcoRI in the absence of salt but which are resistant in the presence of 0.2 M NaCl.

Analysis of chloroplast DNA structure by restriction enzyme fragmentation has shown that the organelle DNAs from most plant species are clearly different. Although chloroplast DNAs are similar at the gross level of GC content, they have few sequences in common unless they come from very closely related plants. It would appear from this rather limited survey that the similarity of chloroplast DNA restriction-

fragmentation patterns produced from two species may be related to the ability of the species to form viable hybrids. Interspecific hybridization cannot be carried out between the three species of the genus *Lupinus* which were examined (Hackburth and Troll, 1959), nor between the two species of the genus *Cucumis* (Deakin et al., 1971), and the species within these genera possessed markedly different chloroplast DNAs as judged by the fragmentation patterns. At the other extreme, *Nicotiana* species especially those of similar geographical distribution, can be readily crossed (Goodspeed, 1954), and appear to possess identical chloroplast DNAs. American and Australian *Nicotiana* species can be hybridized less successfully, which also correlates with the relatively small differences in fragmentation pattern of these two groups.

Because of the unexpected similarity of *Nicotiana* fragmentation patterns it is necessary to consider the taxonomy of these species. Taking the Australian species as a group, the three species examined have been classified as separate species on the basis of morphological differences, differences in chromosome number, and their geographical distribution (Burbidge, 1960). The morphological features used in the classification of *Nicotiana* species are presumably controlled by nuclear genes. Thus, it would appear that evolutionary change in the chloroplast genome has occurred at a slower rate than the nuclear genome. This conclusion is in accord with that resulting from a study of the structure of Fraction I protein of *Nicotiana* by Wildman et al. (1973). However, it is apparent from the fragmentation patterns of the various species so far examined that it is not possible to generalise about the rate of evolution of chloroplast DNA, since the chloroplast DNA of *Nicotiana* species is highly conserved while others have changed markedly.

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