Theor Appl Genet (1987) 74:298–302

Chloroplast DNA variation in the grass tribe Festuceae

H. Lehväslaiho, A. Saura * and J. Lokki

Department of Genetics, University of Helsinki, Arkadiankatu 7, SF-00100 Helsinki 10, Finland

Received January 26, 1987; Accepted February 23, 1987
Communicated by P. M. A. Tigerstedt

Summary. Six grasses, *Hordeum sativum, Dactylis glomerata, Festuca arundinacea, F. pratensis, F. rubra* and *Lolium multiflorum* were subjected to chloroplast DNA analysis based on restriction endonuclease digestion fragments and end labeling with ^{35}S nucleotides. This method is compared with others in general use. The results indicate that *Lolium multiflorum* is closely affiliated with *Festuca pratensis* and *F. arundinacea;* in fact much closer than *F. rubra* is to any of them.

Key words: Chloroplast DNA – Restriction enzymes – Taxonomy – Festuca – Lolium

Introduction

Organelle DNA studies have become commonplace in tracing the phylogenetic relationships of animals (e.g. Wilson et al. 1985). For natural reasons, these studies concentrate on mitochondrial DNA. Chloroplast DNA has also been used in establishing the taxonomy of plants (e.g. Palmer and Thompson 1982; Bowman et al. 1983; Hosaka et al. 1984; Terachi et al. 1984; Palmer et al. 1985). Chloroplast DNA (cpDNA) is transmitted in a fashion different from genomic DNA; it is considered to be of maternal origin (Sears 1980). It has been subject to an evolution different and independent from the main genome. In studying chloroplast DNA differentiation, it is advisable to concentrate on a well-known group of plants which allows direct comparison of new and established data.

In this paper we present a sensitive method for restriction analysis of cpDNA and a way to use fragment patterns in taxonomy. The power of the method is demonstrated on the monocotyledonous grass tribe Festuceae where the location of the genus *Lolium* (ryegrasses) has been a matter of dispute (Stebbins 1956).

* Present address: Department of Genetics, University of Umeå, S-90187 Umeå, Sweden

Materials and methods

Plant material

The following species were studied: barley (*Hordeum sativum*), cocksfoot (*Dactylis glomerata*), tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*), red fescue (*F. rubra*), and Italian ryegrass (*Lolium multiflorum*, var. Prego Daehnfeldt). The plants were grown on vermiculite under a 16 h light/8 h dark period at 18 °C for 10–20 days.

Isolation of chloroplasts

The plants were kept for 1 day in complete darkness before isolation of chloroplasts. Then 10 g samples of leaf material were homogenized with a Waring Blendor for 2×5–7 s in a batch of 80 ml H buffer (50 mM Tricine-KOH, pH 7.9; 330 mM sorbitol; 2 mM EDTA; 1 mM $MgCl_2$; 0.1% BSA. Mills and Joy 1980). The homogenate was sieved through one layer of Miracloth (Calbiochem) into a 100 ml centrifugation flask and 10 ml of 40% Percoll in H buffer was pipetted under the homogenate. The flasks were centrifuged for 12 min at maximum of 3,200 g. The pellet was resuspended with a Pasteur pipette in a 2 ml of 40% Percoll in H buffer. Different runs of a single sample were pooled and pipetted into a 10 ml centrifuge tube over 1 ml of 80% Percoll in H buffer. The tubes were centrifuged for 15 min at 3,200 g. Intact chloroplasts accumulate at the border layer between the liquid phases.

The chloroplasts were diluted with a minimum of 2 volumes H buffer and pelleted (3 min, 3,200 g). The pellet was resuspended into 1 ml of NET buffer (150 mM NaCl, 15 mM EDTA, 40 mM Tris-Cl, pH 7.9), pelleted (8 s, Eppendorf Minifuge) and resuspended to attain the final volume (0.4 ml NET buffer). All phases of this work were done in a cold room at 4 °C and/or on ice.

Lysis of chloroplasts and cpDNA isolation

The chloroplasts were lysed immediately by adding 80 µl 20% Triton X-100 (final concentration 4%) and were incubated for

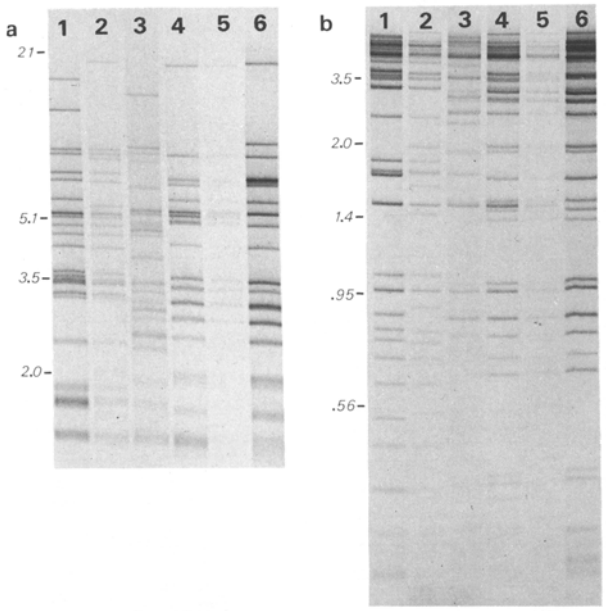

Fig. 1. BamH1 digestions of cpDNAs fractionated by **a** 0.5% and **b** 1.5% agarose gel. The species are *1: Hordeum sativum; 2: Festuca rubra; 3 F. pratensis; 4: F. arundinacea; 5: Lolium multiflorum; 6: Dactylis glomerata*

30–60 min at room temperature thereafter. After 10 s centrifugation (Eppendorf Minifuge), 25 µl 20% SDS (final concentration 1%) was added to the supernatant. Following 10 min incubation, the lysate was extracted twice with phenol and twice with ether. Then 10 µl (20 µg) of RNase solution was added to the solution and it was incubated for 15 min at 37 °C. DNA was precipitated by adding 2 volumes of absolute ethanol, then being pelleted, washed and dried in the usual way. The DNA pellet was dissolved into 10–100 µl TE buffer (6 mM Tris-Cl, pH 7.9; 1 mM EDTA).

Restriction enzyme digestion, labelling and electrophoresis

The volume of a 40 ng cpDNA sample was increased to 10 µl by adding TE buffer. The buffer was modified by adding 1.1 µl 10×CA (200 Tris-HCl, pH 7.5, 1,000 mM KCl, 70 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 1 mg/ml BSA). Then 3–7 units of appropriate restriction enzyme were added and the solution was incubated for 2 h at 37 °C. Following that, it was made up to 200 nM with the necessary deoxynucleotides and 0.16 pmol of ^{35}S dATP (>600 Ci/mmol) (Amersham) and 0.12 units of Klenow enzyme (Boehringer Mannheim) was added. After an incubation of 30 min, the reaction was interrupted. Electrophoresis was done in a Bethesda Research Laboratories horizontal apparatus. The agarose used was BioRad "Ultra Pure DNA grade". The digested and labelled cpDNA sample was divided into 2 equal volumes and run in 0.5% and 1.5% agarose gel to allow separation of large (2–20 kb) and small (0.2–2 kb) restriction fragments, respectively. After the run, the gel was dried and autoradiographed on Sakura X ray film.

Results

Five restriction enzymes were used, namely: BamH1, EcoR1, Hind3, Pvu2 and Sal1. The restriction fragment pattern of BamH1 is shown in Figs 1 and 2a. Each species shows a unique pattern. *Festuca pratensis, F. arundinacea* and *L. multiflorum* appear to share many features in comparison to the other species (Figs 1, 2a).

The outcome is essentially similar for EcoR1, Hind3, Pvu1 and Sal1 (Fig. 2). *F. pratensis, F. arundinacea* and *L. multiflorum* group together and the other species diverge from this cluster and each other. Sal1 and also Pvu2 restriction fragments of barley are identical to those reported by Poulsen (1983).

As shown above, each restriction enzyme gives a species-specific pattern. They also reveal a different amount of variation, so that Sal1 and Pvu2 are relatively insensitive, and BamH1, Hind3 and EcoR1 give many restriction fragments.

Discussion

CpDNA isolation and detection. The use of Percoll gradients in the isolation of intact chloroplasts is fairly well established (Herrman 1982). Their greatest shortcoming in cpDNA evolution studies is a low yield of DNA from certain species. We have overcome this problem by direct end labelling of restriction fragments using the Klenow enzyme. The label used is ^{35}S-dATP, which is much more convenient (longer half life and thus improved economy; shorter radiation range resulting in sharper bands) than the commonly used ^{32}P-based label. It combines the advantages of labelling to the high resolution of ethidium bromide, but minimizes the hazards. The only inconvenience is reduction in sensitivity, which increases the exposure time by an order of magnitude.

The nonaqueous method (Bowman and Dyer 1982) has been reported to give so far the highest yield of wheat cpDNA: 18 µg from 10 g fresh plant. Ethidium bromide detection requires 0.75 µg cpDNA per digestion; i.e. out of 10 g wheat one gets 24 digestions. The Percoll technique gives approximately 0.5 µg cpDNA from 10 g wheat and as 20 ng (or as little as 10 ng) suffice for one digestion with end labelling, this gives 25 digestions per 10 g wheat. Thus our method gives at least as good a yield as the nonaqueous method. Of course, nonaqueous isolation and end labelling together would give superior results.

Hybridization techniques have been used to study cpDNA (Day and Ellis 1984; Palmer et al. 1985). They have visualized cpDNA restriction patterns with a specific probe. This approach uses as little DNA as direct labelling but is less sensitive to changes in fragment length: the bands become diffuse during blotting. Hybridization is also a much more laborious

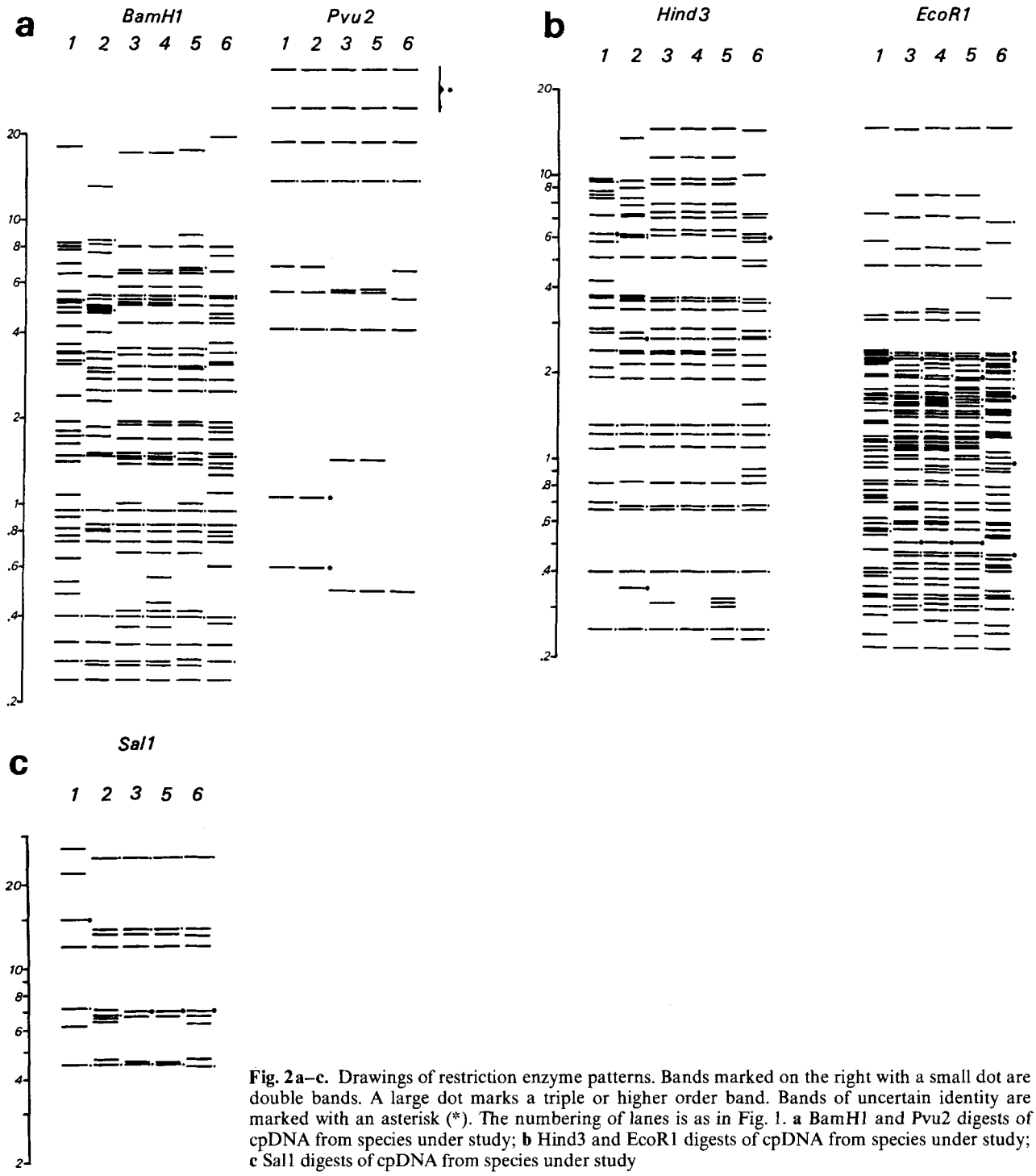

Fig. 2a–c. Drawings of restriction enzyme patterns. Bands marked on the right with a small dot are double bands. A large dot marks a triple or higher order band. Bands of uncertain identity are marked with an asterisk (*). The numbering of lanes is as in Fig. 1. **a** BamH1 and Pvu2 digests of cpDNA from species under study; **b** Hind3 and EcoR1 digests of cpDNA from species under study; **c** Sal1 digests of cpDNA from species under study

technique than direct labelling. In cases where the correct restriction map is not known, the use of hybridization techniques is dubious because it is difficult to exclude bands resulting from promiscuous DNA. A fragment of spinach cpDNA has been shown to have homologous counterpart in mitochondrial DNA (Timmis and Scott 1983).

General considerations

The method of Nei and Li (1979) approaches the problem of species differentiation without constructing a detailed restriction enzyme map. A distance matrix is formed entirely from changes in DNA fragment length. The theory underlying this is based on the assumption

that DNA diverges through a substitution of one base pair for another. The cleavage sites are expected to be randomly distributed and the method of detection should allow the separation of nonhomologous fragments from each other.

The assumption of random distribution of cleavage sites within cpDNA is apparently not satisfied. Adams and Rothman (1982) have extensively studied these distributions in a wide range of DNA's from different organisms. The distributions deviate in most cases significantly from random expectations. The nonrandom distribution in the animal mitochondrial DNA is based mainly on an unequal presence of transitions and transversions (Nei and Tajima 1985), i.e. there is a high bias favouring transitions. Zurawsky et al. (1984) have, however, shown that this bias is low in cpDNA. This increases the value of cpDNA in phylogenetic studies.

It has become apparent that the evolution of cpDNA higher plants is based on rather rare, large inversions and numerous small deletions. Substitutions are, in fact, relatively uncommon (Curtis and Clegg 1984; Zurawsky et al. 1984; Palmer 1985).

Scoring as a substitution a fragment change which results from a deletion underestimates differentiation. This is, however, less severe than when the fragment change is not included in the estimate. Many deletions (or insertions) make it difficult to identify substitutions properly. We found it most convenient to include all fragment changes into an estimate of differentiation. The direct labelling technique used here allows recognition of small fragments and thus decreases the underestimation of differentiation.

The inverted repetition within the cpDNA undergoes concerted evolution; a change in one arm results in an identical change in another (see Palmer 1985). It has, therefore, been suggested (Banks and Birky 1985) that double bands should be excluded from diversity estimates. However, our material does not include a single case in which a set of double band had together moved an equal distance in either direction. We have, therefore, felt that the inclusion of double bands does not result in any significant error.

We should like to stress that the numerical values obtained from restriction data are not, for the reasons above, straightforward values of genetic distance, but also depend greatly on the choice of enzymes.

The most recent observations on paternal transmission of chloroplasts in *Nicotiana* (Medgyesy et al. 1986) further support not drawing very precise conclusions.

The evolution of cpDNA in Festuceae

All restriction enzymes used recognize a unique, non-overlapping nucleotide sequence. This, with the assumptions mentioned above, allows the pooling of data in the construction of a between species distance matrix that gives a measure of chloroplast nucleotide diversity (P, the probability that two randomly chosen cpDNA molecules will have different nucleotides at a given nucleotide site; Nei and Li 1979). The distance matrix is given in Table 1.

To convert the distance matrix into a visual form, phylogenetic trees were constructed using different methods. Figure 3 shows phylogenetic trees constructed with the UPGMA (Sneath and Sokal 1973) and the method of Li (1981). In addition, the methods of Fitch

Table 1. Divergence matrix showing nucleotide divergence (upper half), with p, calculated by Nei and Li's (1979) approach and number of total and shared fragments, separated by commas (lower half); 1 to 6 refer to plant species in the same order as Fig. 1

	1	2	3	4	5	6
1	–	0.217	0.181	0.184	0.204	0.173
2	272,37	–	0.163	0.204	0.181	0.180
3	338,57	276,52	–	0.039	0.029	0.154
4	314,52	252,37	318,126	–	0.066	0.140
5	340,50	278,47	344,144	320,108	–	0.153
6	344,60	282,48	348,69	264,57	350,70	–

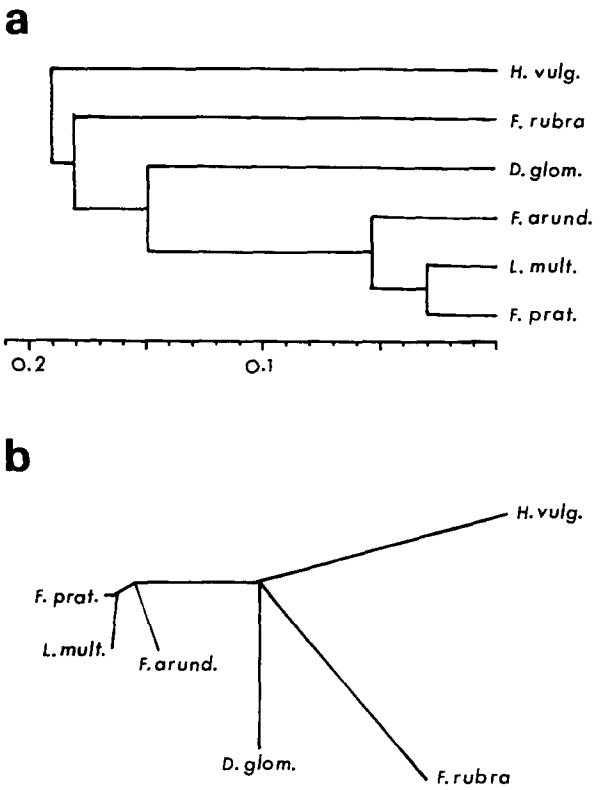

Fig. 3. Phylogenetic trees of Festuceae constructed according to **a** UPGMA and **b** Li (1981). Standard deviations from the original distance matrix are 13% and 6.6%, respectively

and Margoliash (1967) and Farris (1972) were applied to the data. The two latter methods gave larger percentage standard deviations (calculated according to Fitch and Margoliash 1967) from the starting matrix (SD is 39% and 14%, respectively). The method of Farris (1972) gave a result essentially similar to that obtained with method of Li (1981). Each method gives a slightly differing solution but the essential features of phylogeny are unchanged. The absence of *F. arundinacea* from two digestions (Sall, Pvu2) does not affect the structure of phylogenetic trees.

The phylogenies in Fig. 3 show that barley (*H. sativum*), which belongs to the tribe Hordeae (Triticeae), clearly differs from the other species. Within the tribe Festuceae an interesting pattern emerges: *Festuca rubra* appears to be isolated from the other species of *Festuca*. *Lolium multiflorum* groups consistently together with *F. arundinacea* and *F. pratensis*. *F. rubra* is, in fact, about equally distant from the other two fescue species and ryegrass as *Dactylis glomerata*, which belongs to an other genus.

Taken together, the above results suggest that the genus *Festuca* is an artificial construction. The rubra group within the genus seems to differ widely from the other species. The position of *Lolium multiflorum* is most interesting: it is closely related to the two species of the pratensis group (*F. pratensis* and *F. arundinacea*).

Stebbins (1956) pointed out that the placement of *Lolium perenne* into the tribe Hordaceae is incorrect. As evidence for this view he marshalled data on hybridization and morphology. *L. perenne* and *L. multiforum* have been known to hybridize with *F. pratensis* and *F. arundinacea* (Tutin et al. 1980). Nevertheless, the division into the genera *Festuca* and *Lolium* is maintained on morphological evidence. The cpDNA data suggest revision of the situation.

References

Adams J, Rothman ED (1982) Estimation of phylogenetic relationships from DNA restriction patterns and selection of endonuclease cleavage sites. Proc Natl Acad Sci USA 79:3560–3564

Banks JA, Birky CW Jr (1985) Chloroplast DNA diversity is low in a wild plant, *Lupinus texensis*. Proc Natl Acad Sci USA 82:6950–6954

Bowman CM, Dyer TA (1982) Purification and analysis of DNA from wheat chloroplasts isolated in nonaqueous media. Anal Biochem 122:108–118

Bowman CM, Bonnard G, Dyer TA (1983) Chloroplast DNA variation between species of *Triticum* and *Aegilops*. Location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm. Theor Appl Genet 65:247–262

Curtis SE, Clegg MT (1984) Molecular evolution of chloroplast DNA sequences. Mol Biol Evol 1:291–301

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. Cell 39: 359–368

Farris J (1972) Estimating phylogenetic trees from distance matrices. Am Nat 106:645–668

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284

Herrman RG (1982) The preparation of circular DNA from plastids. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in chloroplast molecular biology. Elsevier/North Holland, Amsterdam, pp 259–280

Hosaka K, Ogihara Y, Matsubayashi M, Tsunewaki K (1984) Phylogenetic relationship between the tuberous *Solanum* species as revealed by restriction endonuclease analysis of chloroplast DNA. Jpn J Genet 59:349–369

Li W-H (1981) Simple method for constructing phylogenetic trees from distance matrices. Proc Natl Acad Sci USA 78: 1085–1089

Medgyesy P, Páy A, Márton L (1986) Transmission of paternal chloroplasts in *Nicotiana*. Mol Gen Genet 204:195–198

Mills WR, Joy KW (1980) A rapid method for isolation of purified, physiologically active chloroplast, used to study the intracellular distribution of amino acids in pea leaves. Planta 148:75–83

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Nei M, Tajima F (1985) Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. Genetics 105:207–217

Palmer JD (1985) Comparative organization of chloroplast genomes. Ann Rev Genet 19:325–354

Palmer JD, Thompson WF (1982) Chloroplast DNA rearrangements are more frequent when a large inverted repeat is lost. Cell 29:537–550

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

Poulsen C (1963) The barley chloroplast genome: physical structure and transcriptional activity in vivo. Carlsberg Res Commun 48:57–80

Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plasmid 4:233–255

Sneath PHA, Sokal RR (1973) Numerical taxonomy. Freeman, San Francisco (USA)

Stebbins GL (1956) Taxonomy and the evolution of genera, with special reference to the family Gramineae. Evolution 10:235–245

Terachi T, Ogihara Y, Tsunewaki K (1984) The molecular basis of genetic diversity among cytoplasms of *Triticum* and *Aegilops*. III. Chloroplast genomes of the M and modified M genome-carrying species. Genetics 108:681–695

Timmis JN, Scott NS (1983) Sequence homology between spinach nuclear and chloroplast genomes. Nature 305: 65–67

Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA (1980) Flora Europaea, vol 5. Cambridge University Press, Cambridge (UK)

Wilson AC, Cann RL, Carr SM, George M, Gyllensten UB, Helm-Bychowski KM, Higuchi RG, Palumbi SR, Prager EM, Sage RD, Stoneking M (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. Biol J Linn Soc 26:375–400

Zurawsky G, Clegg MT, Brown AHD (1984) The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. Genetics 106:735–749