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Species relationships in the *Hordeum murinum* aggregate viewed from chloroplast DNA restriction fragment patterns

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Summary. Three annual widespread species of *Hordeum* were investigated by the fragment pattern method on their chloroplast (cp) DNA. The species were *H. glaucum, H. leporinum* and *H. murinum; H. vulgare* was surveyed for comparison. Twelve restriction enzymes were used, nine recognizing 6 bp, one 5 bp and two 4 bp, thus, randomly surveyed, a total of 2,113 bp or 1.6% of the cp genome. Differences in patterns were found in three enzymes, HindIII, CfoI and MspI. CfoI characterizes *H. glaucum* from the other two species. HindIII and MspI revealed polymorphisms within species. These results confirm previous numerical taxonomic relationships among these three closely related species. Furthermore, cpDNA polymorphism in *Hordeum* is discussed in view of earlier reports on cpDNA polymorphism in *H. vulgare*. The taxonomic implications of cpDNA polymorphism are discussed after reviewing several articles using the fragment pattern method on cpDNA. The importance of using material from several populations representative of a species is stressed.

Key words: *Hordeum* – Chloroplast DNA – Restriction fragments – Polymorphism – Taxonomy

Introduction

The utility of chloroplast DNA (cpDNA) as a phylogenetic marker has been clearly established (Palmer 1987). Because of the slow rate of evolution of the cpDNA relative to the nuclear genome, it is fast becoming recognized as a useful tool available to taxonomists in their studies of species delimitation, and primarily as a tool for the study of transpecific cladistic relationships. Presently, comparative analysis of cpDNA variation is far from being simple, quick and inexpensive enough for plant systematists untrained in molecular technology to readily use the techniques for their own purpose, a goal enunciated by Palmer (1987).

There are three approaches to cpDNA comparison in chloroplast DNA systematics: the fragment pattern method, the cleavage-map method and the method of DNA sequence analysis of a defined portion of the genome (Palmer 1987). The cost, time and labor-intensiveness increase drastically from the first approach to the third. The first method is relatively simple but is limited in resolution and to non-complex situations. The majority of recent DNA systematic studies have used the first approach or a combination of the first and second approaches (Vedel et al. 1978; Timothy et al. 1979; Vedel and Lebacq 1980; Palmer and Zamir 1982; Kung et al. 1982; Ogihara and Tsunewaki 1982; Berthou et al. 1983; Palmer et al. 1983; Clegg et al. 1984b; Buckner and Hyde 1985; Enomoto et al. 1985; Baatout et al. 1985; Sytsma and Schaal 1985; Sytsma and Gottlieb 1986a, b; Hosaka 1986; Yanagino et al. 1987; Coates and Cullis 1987; Lehvaslaiho et al. 1987; Jansen and Palmer 1988). We decided to introduce the fragment pattern method into our taxonomic studies of *Hordeum*.

Three substantial studies of organellar DNA variation in *Hordeum* have been published so far (Clegg et al. 1984a; Holwerda et al. 1986; Kataoka et al. 1987). The first two studies focused on infraspecific polymorphism present in organellar DNA in cultivated barley (*H. vulgare* L. ssp. *vulgare*) and its wild progenitor [*H. vulgare* ssp. *spontaneum* (C. Koch) Thell.], whereas the last one dealt with interspecific relationships of the chloroplast genome between seven *Hordeum* species. The purpose of this study is to find evidence from cpDNA for the de-

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limitation of *Hordeum glaucum* Steudel, *H. murinum* L., and *H. leporinum* Link, three morphologically and genetically similar species (Baum and Bailey 1984a, b), and to provide evidence for relationships among the three species. This study is the first of a series of cpDNA phylogenetic investigations of the entire genus *Hordeum*.

Materials and methods

The source of plant material is given in Table 1. Samples constitute vegetatively cloned individuals or, when not available, the progeny of a single plant. The provenance of the accessions is listed in Baum et al. (1984), but the identifications to species were based on Baum and Bailey (1984a, b). Chromosome numbers were determined on most accessions by fixation of root tips in ethyl alcohol – acetic acid (3:1) for 24 h, then stained in Snow's (1963) solution for 7–8 days, and squashed in 45% acetic acid.

Chloroplast isolation. Plants 14–60 days old were subjected to 36–48 h complete darkness prior to harvesting. The following DNA isolation procedure was modified from that of Holwerda et al. (1986) and other details were modified from Maniatis et al. (1982). Leaf samples, 22–75 g, were collected, chilled and briefly washed in cold distilled water. Coarsely cut leaves were homogenized in a 1 l Waring blender for 3 × 7 second bursts in 500 ml ice cold H buffer (50 m*M* TRIS pH 8.0, 25 m*M* EDTA, 1.25 *M* NaCl, 10 m*M* 2-mercaptoethanol, 0.1% Bovine albumin). The homogenate was filtered through four layers of cheesecloth and squeezed dry. The liquid containing chloroplast was sieved through 125, 60, and 26 μm sterile nylon meshes, respectively, and centrifuged for 8 min at 1,500 g in a swinging bucket rotor. After decanting the supernatant, the pellets were resuspended in 25 ml of H buffer by gentle pipetting, respun, and the chloroplast pellet was suspended in 2 ml I buffer (50 m*M* TRIS pH 8.0, 25 m*M* EDTA). Proteinase K (Boehringer Mannheim) to 50 μg/ml was added to the sample, which was incubated at room temperature for 20 min. A 3/10 vol of 5% sarkosyl in I buffer was added and incubated at 65 °C for 10 min to lyse chloroplasts. All steps above were carried out in a cold room at 4 °C unless otherwise specified.

Table 1. Source of plant material used in cpDNA study. For the CHC accessions, see Baum et al. (1984)

Species	Accession no.	Country of origin	Chromosome no.
H. glaucum	CHC 602	Iran	2n = 14
	CHC 901	Iran	2n = 14
	CHC 980	Iran	2n = 14
	CHC 2288	Spain	2n = 14
	CHC 3426	USA	2n = 14
H. leporinum	CHC 103	Turkey	2n = 28
	CHC 3407	USA	2n = 28
	CHC 3408	USA	2n = 28
H. murinum	CHC 2677	Turkey	2n = 28
	CHC 3486	Spain	2n = 28
	PGRC 5025	unknown	2n = 28
H. vulgare	cv 'Bedford' [a]	Breeder's seed	2n = 14
	cv 'Argyle' [b]		2n = 14
	cv 'Acton' [b]		2n = 14
	cv 'Bonanza' [b]		2n = 14

[a] PGRC is the Plant Gene Resource Office of Canada, Ottawa
[b] Breeder's seed of these cultivars is maintained by Plant Products Branch, Agriculture Canada, Ottawa

DNA isolation. The necessary amount of I buffer was added to bring each sample volume to 10.5 ml, and 9.9 g CsCl was dissolved therein. Samples were loaded into a 13.0 ml Beckman Quick-Seal centrifuge tube through a wide-bore syringe, with 0.5 ml of 10 mg/ml EtBr added to each sample. Sealed tubes were spun at 50,000 rpm for 20 h with a 70.1 Ti rotor, and the resulting single cpDNA band was removed by side puncture of the tube. Chloroplast DNA was extracted 4–5 times with equal volumes of isopropanol, after which 2 vol of dH_2O and 4 vol of 95% EtOH were added and allowed to stand at room temperature for 8–16 h to precipitate DNA. After being spun and carefully decanted, 100 μl dH_2O, 5.0 μl 10 *M* NH_4OAc and 1.5 ml 95% EtOH were added to each cpDNA pellet before being respun and dried. The DNA pellet was dissolved in dH_2O or TE.

Restriction enzyme digestion, end-labelling, and electrophoresis. The following enzymes were used: HindIII, MspI, CfoI, BclI, ClaI, BamHI, MluI, NcoI, PvuII, SalI, Sau96I, XbaI. To a 50-ng DNA aliquot was added 1.0 μl of the appropriate 10 × core buffer, 1.0 μl BSA, a 40 U of enzyme/5 g DNA and dH_2O to a volume of 10 μl. Digestion reaction was allowed to proceed for 1 h (or more, according to the enzyme) at 37 °C (or differently, according to manufacturer's specifications) and interrupted by immersion in a 65 °C water bath for 10 min. To end-label 50 μ*M* of the required deoxynucleotides, 1.05 Ci ^{32}P dCTP and 0.5 U Klenow enzyme (BRL) were added and incubated at room temperature for 10 min and stopped by the addition of STE (10 m*M* TRIS pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA) to a total volume of 50 μl. The resulting samples were twice put through Sephadex G 50 spun-columns (Maniatis et al. 1982), and isotope incorporation was assessed by a photoscintillation counter. DNA samples representing 5,000–15,000 CPM per lane were used for agarose gel electrophoresis. A.B.R.L. horizontal apparatus was used at 50–60 V for 16–20 h. Sea-Kem agarose (FMC Bioproducts) at 1%–1.5% was selected to best resolve banding patterns. Fragments in resulting gels were immobilized by soaking for 1 h in a CTAB (1% Cetyltrimethylammonium bromide-hexadecyltrimethylammonium bromide, 50 m*M* NaOAc, pH 5.5), according to Cockerill (1988) and rinsed in dH_2O prior to drying in a BioRad slab dryer at 80 °C. Autoradiography was carried out using Kodak X-OMAT film.

In cases where initial samples of the three species showed identical patterns, no further accessions were investigated. Where variation was detected, more representatives from each species were included to give a better indication of the degree of variation.

Results

No discernible differences in the restriction enzyme fragment patterns for *H. glaucum*, *H. leporium* and *H. murinum* were found in the cpDNA digested with BclI, ClaI, BamHI, MluI, NcoI, PvuII, SalI, Sau96I and XbaI. When the enzymes HindIII, MspI and CfoI were used, differences in banding patterns were found both within and between species.

An example of identical banding patterns is shown in Fig. 1. Note that although PvuII digestion does not offer

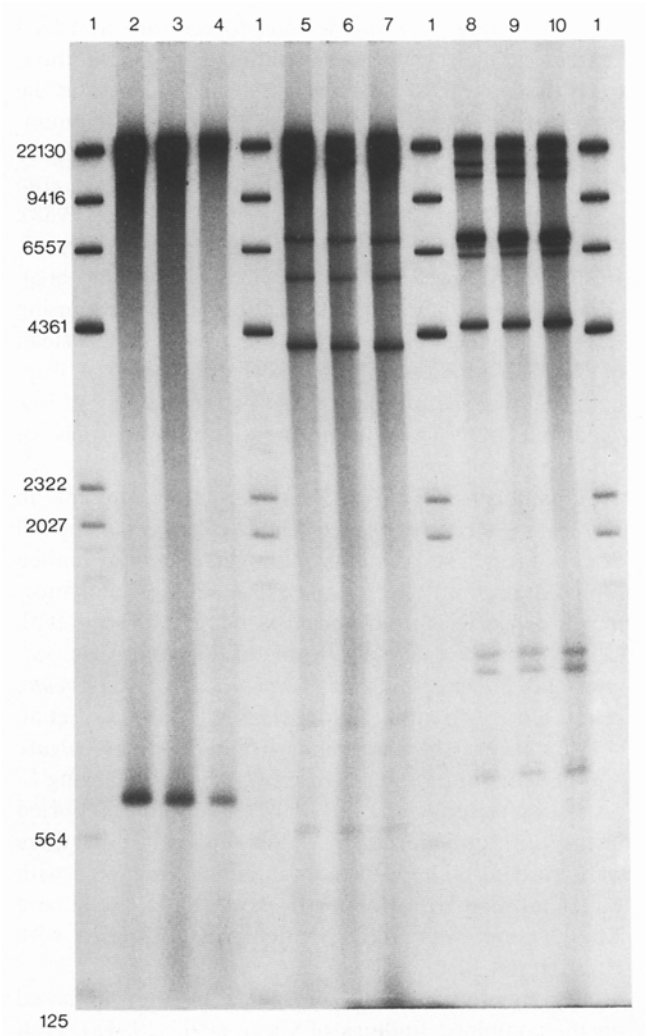

Fig. 1. Autoradiogram of digestions with MluI (*2–4*), PvuII (*5–7*) and SalI (*8–10*) of cpDNA end-labelled with ^{32}P and electrophoresed with a 1% agarose gel. *Numbers* to the left indicate base pairs; species are: Lambda HindIII (*1*), *H. glaucum* CHC602 (*2, 5, 8*), *H. leporinum* CHC3408 (*3, 6, 9*), *H. murinum* CHC2677 (*4, 7, 10*)

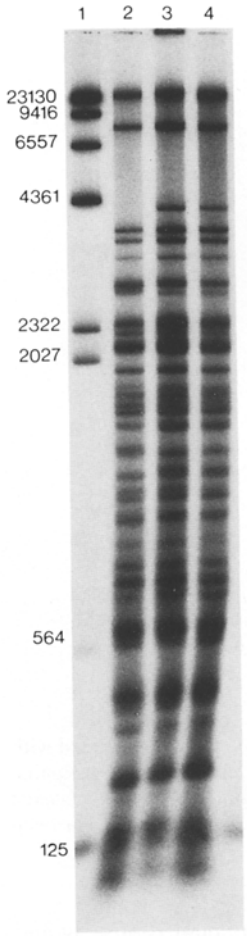

Fig. 2. Digestions with CfoI of cpDNA electrophoresed with a 1.5% agarose gel. *Numbers* to the left indicate base pairs; species/accessions are: *1* – Lambda HindIII (molecular weight marker), *2* – *H. glaucum* (CHC602), *3* – *H. leporinum* (CHC3408), *4* – *H. murinum* (CHC2677)

a recessed 3′-end for labelling, as do the other enzymes, labelling was successful. The precise reason for this was not pursued; presumably its star radioactivity causes nicking along its length suitable for labelling. In Fig. 2 we see an autoradiogram of representatives of the three species with the enzyme CfoI and the invariable pattern given for each of them, whereby the *H. glaucum* pattern is distinct from the *H. leporinum-murinum* pattern. A sample run on a 1.5% agarose gel to resolve the smallest yielded fragments resulting from a HindIII digestion is shown in Fig. 3. A summary of all the resulting banding patterns is shown in Fig. 4. Of the 12 enzymes used, the 9 which exhibited identical patterns for the three species are shown on the right of Fig. 4. The differences in pattern were found in the three enzymes on the left in Fig. 4. HindIII exhibited two patterns: one (pattern no. 1 in Fig. 4) shared by all *H. glaucum* accessions, *H. vulgare* 'Bonanza' and one accession of *H. murinum* CHC3486; the other (no. 2 in Fig. 4) is shared by all *H. leporinum* accessions and by all *H. murinum* accessions, except for CHC3486. In pattern no. 2, the 6.9-kb fragment is missing, but its components are found, one being a fragment of about 6.5 kb and the other of approximately 0.4 kb. Accession CHC3486 of *H. murinum* displayed patterns identical to the other accessions of *H. murinum* with all other enzymes tested, most notably CfoI, except for HindIII, where it fell in with the *H. glaucum* pattern. With MspI, three patterns were found: the first was exhibited by all three species except for two variant patterns exhibited by two accessions. One (no. 2) was found in *H. glaucum* CHC602, and the other (no. 3) in *H. leporinum* CHC103. The CfoI restriction fragment patterns separate clearly *H. murinum* and *H. leporinum* (no. 1 in Fig. 4) from *H. glaucum* (no. 2 in Fig. 4) in all the acces-

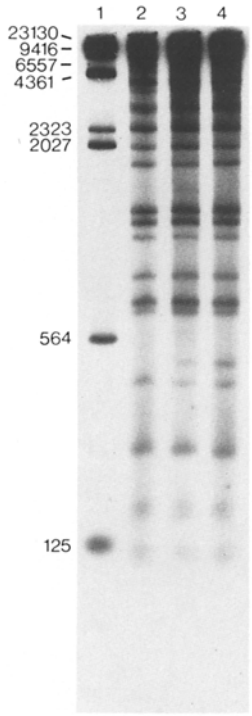

Fig. 3. Digestions with HindIII of cpDNA electrophoresed with a 1.5% agarose gel. *Numbers* to the left indicate base pairs; species/accessions are: *1* – Lambda HindIII, *2* – *H. glaucum* (CHC2288), *3* – *H. leporinum* (CHC3408), *4* – *H. murinum* (PGRC 5025)

sions. In *H. glaucum*, the 4.2-kb fragment is missing and its components are detected as two additional bands of nearly 2.8 and 1.4 kb.

It is notable that MluI produced two discernible fragments. The 12 endonucleases have randomly surveyed a total of 2,113 bp; 9 recognize 6 bp, 1 recognizes 5 bp and 2 recognize 4 bp. Since the barley chloroplast genome has about 134 kbp (Poulsen 1983), this represents a random survey of 1.6% of each cp genome.

Discussion

Initial difficulties were encountered at the early stages of this study because of the low yield of cpDNA. Poulsen (1983) used 1 kg leaf material from seedlings, and found that the uppermost 5–8 cm of the seedling leaves gave best results. Furthermore, he found it necessary to combine five homogenates at the initial stages of cp DNA isolation. Poulsen used the very commonly available cultivated barley and for different purposes. A number of the *Hordeum* species studied consisted of plants diminutive in size with sparse foliage, and the number of seeds available per accession was limited, thus providing only small amounts of leaf material. Furthermore, one of our aims was to taxonomically characterize each species in the same manner that one would proceed for traditional characters, viz. to check for variation and to select those traits that vary least within species. This necessitates the examination of single plants as far as possible. In a number of grasses it has been found difficult to extract pure and intact cpDNA such as in wheat, maize, *Agropyron repens* and other grasses (Rogers and Bendich 1988); *Hordeum, Dactylis, Festuca, Lolium* (Lehväslaiho et al. 1987). We followed the approach of Lehväslaiho et al. (1987) to end-label the restriction fragments using Klenow enzyme, but used ^{32}P-labelled nucleotide instead of ^{35}S. The added sensitivity in detection of DNA fragments allowed us to compensate for the problem of low yield of cpDNA by using nanogram quantities of cpDNA.

Polymorphism of cpDNA is clearly present in *Hordeum glaucum, H. murinum* and *H. leporinum,* the full extent of which still remains unknown, due to the limited representation of these widespread species. Polymorphism of cpDNA in *Hordeum* is not new. Clegg et al. (1984a) analysed nine accessions of *H. vulgare* ssp. *vulgare* and 11 accessions of *H. vulgare* ssp. *spontaneum,* using ten restriction endonucleases. Holwerda et al. (1986) analysed 14 accessions of *H. vulgare* ssp. *vulgare* and 11 accessions of *H. vulgare* ssp. *spontaneum* using 17 restriction endonucleases. Both of these studies reported infraspecific polymorphism. In this study, although there were no detectable differences in cpDNA digested with BclI, ClaI, BamHI, MluI, NcoI, PvuII, SalI, Sau96I, and XbaI, there was infraspecific polymorphism with HindIII and MspI.

The significance of polymorphism in *Hordeum* based on the combined findings of Clegg et al. (1984a), Holwerda et al. (1986) and of this study has implications for the use of cpDNA restriction fragments to taxonomy in *Hordeum,* and undoubtedly other genera, especially for studies of the phylogenetic relationships between species within a genus. In this connection, it is of particular interest to point out that in a number of cpDNA studies, only one accession per species was used – *Brassica* and allies (Yanagino et al. 1987), *Clarkia* and *Heterogaura* (Sytsma and Gottlieb 1986a), ten cereals including *Hordeum* (Enomoto et al. 1985), cereals (Vedel and Lebacq 1980), *Nicotiana* (Kung et al. 1982), *Linum* (Coates and Cullis 1987), *Festuca-Dactylis-Lolium-Hordeum* (Lehväslaiho et al. 1987) and *Papaver* (Milo et al. 1988).

In those studies where two or more accessions were used, cpDNA polymorphism was often detected, as in *Lycopersicon* (Palmer and Zamir 1982), *Zea* (Timothy et al. 1979), *Brassica* (Palmer et al. 1983), *Hedysarum* (Baatout et al. 1985) and *Lisianthus* (Sytsma and Schaal 1985). The occurrence of the polymorphisms was not appreciated in these phylogenetic studies. Cladistic computations used in such phylogenetic studies are very sen-

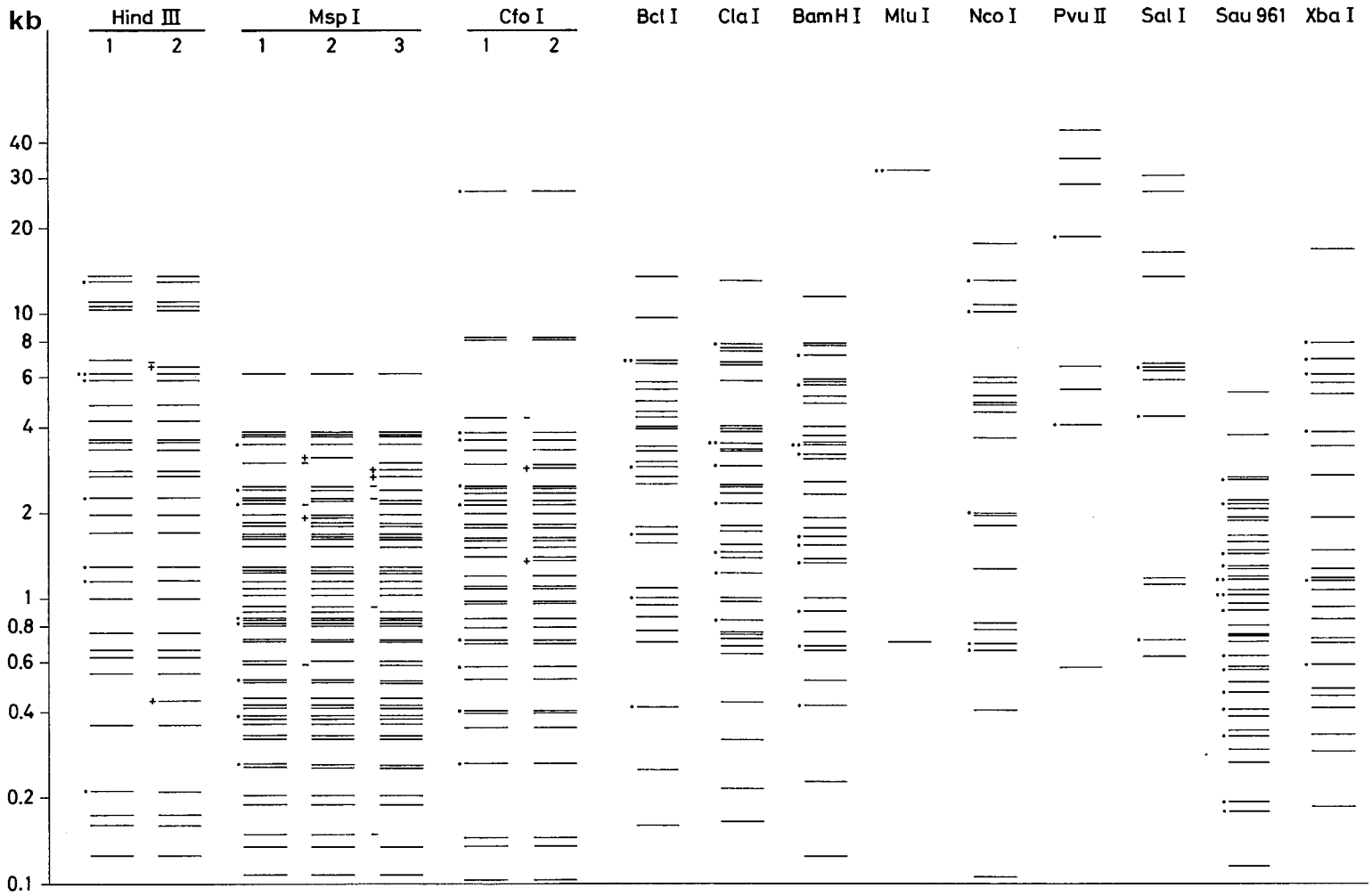

Fig. 4. Restriction enzyme patterns in *Hordeum* cpDNA, drawn from the gels. Enzyme names appear at top with *track numbers* underneath. Bands present/absent in different tracks are indicated by a + sign and multiple bands shown by *double dots*, all to the left of the bands. *Numbers* to the left indicate kilobase pairs

sitive to data including character state changes (Fitch 1984). Thus, the use of only one individual or accession may considerably misrepresent phylogenetic relationships obtained from such studies. An important factor is sample size, in that the probability that a variant will not be detected in a small sample is very high. Swofford and Berlocher (1987) have shown that traditional approaches of sampling in enzyme electrophoretic studies often fail to take adequate sampling to detect the full extent of polymorphism. A detailed study on *Lupinus texensis,* based on 100 individuals from 21 populations using seven restriction enzymes, revealed that 88 plants had identical cpDNA restriction patterns but 12 plants from three populations exhibited two restriction-site mutations and one deletion of 0.1 kb (Banks and Birky 1985).

Despite these considerations, available resources can limit the degree of sampling and the methodology of analysis. In this respect our study was a compromise and is limited in scope, not only because even larger sampling would have been desirable, but because the fragment patter method used is limiting; we were unable, without further more detailed study, to determine the cause of the differences between *H. glaucum* and the other two species as revealed by CfoI and HindIII digestion patterns. We were able to detect modifications within up to 49 restriction enzyme recognition sites of 4–6 bp, but could not determine whether a substitution or an addition/deletion had occurred. The chloroplast genome evolves at a slow rate in general, but it is base substitutions that are infrequent, while the addition/deletion events are a more common feature of the non-coding regions of this genome (Zurawski and Clegg 1987). The two previous studies (Clegg et al. 1984a; Holwerda et al. 1986) and this study have not dealt with the mechanism of these events in *Hordeum,* whereas Kataoka et al. (1987) identified by cleavage-map method 12 base substitutions and seven insertion/deletion events among 17 accessions in a total of seven *Hordeum* species.

Based on the samples used in this study, CfoI can be used as a marker to discriminate between *H. glaucum* from the other two species, as can HindIII to some extent, since only one accession of *H. murinum* exhibited the same pattern as that of *H. glaucum*. With MspI, polymorphism in *H. glaucum* and in *H. leporinum* were revealed. Different taxonomic studies of *H. glaucum, H. leporinum* and *H. murinum* based on morphological characters have historically given various renditions of the degrees of relationship (see review in Baum and Bailey 1984a). We have previously presented morphological data and numerical analyses (Baum and Bailey 1984a, b) to conclude that *H. glaucum, H. leporinum* and *H. murinum* are distinct, identifiable and warrant specific status. The present study using cpDNA underlines the close proximity of *H. leporinum* and *H. murinum* and the more distant *H. glaucum*. This is evident even without numerical analysis applied to the endonuclease restriction fragment data, which will be reserved for a future study that encompasses more species of *Hordeum*. This genus is very difficult to elucidate morphologically, especially for phylogeny reconstruction, as comparable characters are hard to find across all the species. We anticipate that cpDNA will provide such characters.

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