Genetic analysis and molecular characterization of RFLP DNA markers in barley (Hordeum Vulgare L.)
by Jeong Sheop Shin

A thesis submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Crop and Soil Science
Montana State University
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Abstract:
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Seventeen polymorphic DNA sequences were mapped relative to seventeen previously mapped marker loci. Genotypes of 34 loci in 100 mapping lines were characterized and described to simplify the mapping of additional RFLP loci. Twelve of seventeen RFLP loci showed codominant segregation. Four of the five loci which demonstrated dominance were from genomic clones which hybridized to several bands in each lane of the Southern blot. The probes and markers utilized in this mapping project span 680 recombination units of the barley genome, approximately 50 percent of its estimated recombinational length.

Detailed physical maps of fifteen polymorphic DNA fragments that were mapped in barley were developed using several restriction endonucleases. All fifteen DNA clones were well characterized by one or several restriction enzymes. In the Southern blot analysis of double digested genomic DNA probed with one of these clones, one allele was found to contain about 200 base pair inserted sequences compared with an alternate allele. The polymorphic region of this clone was sequenced using dideoxy chain termination reaction.

Polymorphic DNA markers were also utilized to identify barley cultivars. Some cultivars undifferentiated by hordeins were well discriminated using a subset of the DNA markers.

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of

Doctor of Philosophy

in

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APPROVAL

of a thesis submitted by

Jeong Sheop Shin

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Single or low copy number DNA clones from random genomic DNA libraries using the plasmid vector pBR322 and the phage EMBL4 were constructed using DNA from barley (Hordeum vulgare L.). This work was done to provide a relatively large number of genetic markers and to characterize the level of genetic variation in the barley genome. Selected genomic clones and cDNA clones were used to probe the barley genome for the presence of restriction fragment length polymorphisms (RFLPs). This methodology is based upon fragment size differences of defined length that are produced when DNA is cleaved by restriction endonucleases. A multiple recessive marker stock and a relatively distantly related cultivar 'Apex' were selected as parents in a cross to map the genomic location of seventeen RFLP loci. Nine genomic clones and seven cDNA clones produced clear polymorphisms using at least one restriction endonuclease. The majority of selected genomic clones showed polymorphisms using two or more restriction endonucleases. This suggests that the variation observed among barley lines is due to insertion/deletion or rearrangement events rather than point mutations. Utilizing selected single or low copy clones as probes, it was confirmed that polymorphisms are readily detectable among cultivars of barley.

Seventeen polymorphic DNA sequences were mapped relative to seventeen previously mapped marker loci. Genotypes of 34 loci in 100 mapping lines were characterized and described to simplify the mapping of additional RFLP loci. Twelve of seventeen RFLP loci showed codominant segregation. Four of the five loci which demonstrated dominance were from genomic clones which hybridized to several bands in each lane of the Southern blot. The probes and markers utilized in this mapping project span 680 recombination units of the barley genome, approximately 50 percent of its estimated recombinational length.

Detailed physical maps of fifteen polymorphic DNA fragments that were mapped in barley were developed using several restriction endonucleases. All fifteen DNA clones were well characterized by one or several restriction enzymes. In the Southern blot analysis of double digested genomic DNA probed with one of these clones, one allele was found to contain about 200 base pair inserted sequences compared with an alternate allele. The polymorphic region of this clone was sequenced using dideoxy chain termination reaction.

Polymorphic DNA markers were also utilized to identify barley cultivars. Some cultivars undifferentiated by hordeins were well discriminated using a subset of the DNA markers.

CHAPTER 1

INTRODUCTION

The lack of available genetic markers in cutivated genotypes has limited the development of saturated genetic linkage maps in plant species. Analysis of restriction fragment length polymorphisms (RFLPs) will provide a relatively unlimited number of genetic markers and permits the construction of detailed genetic linkage maps in eukaryotic species. The studies reported here focussed on the recombinational location of selected RFLP DNA markers relative to previously mapped marker loci in the nuclear genome of barley (Hordeum vulgare L.).

The goals of the first part of this investigation were to screen single or low copy number DNA probes selected from random genomic DNA libraries and to identify genomic RFLPs. The objectives of the second part of this study were to utilize the selected barley DNA clones as genetic markers and to locate them in barley chromosomes relative to previously mapped morphological and biochemical markers. In the third part of this study, the genotypes of thirty-four loci in 100 mapping lines were described and their application as a template to simplify the mapping of additional RFLP loci was discussed. Fifteen out of seventeen mapped DNA clones were also characterized by restriction mapping analysis and the polymorphic region of one interested polymorphic probe was sequenced. The objective of the final part of the study was to determine the relative utility of RFLP markers in cultivar identification.

CHAPTER 2

MOLECULAR CLONING AND EVALUATION OF BARLEY GENOMIC LOW COPY NUMBER DNA CLONES AS GENETIC MARKERS

Introduction

Restriction fragment length polymorphism (RFLP) analysis probes specific regions of the genome for the presence of variation at the DNA level (Grodzicker et al., 1974; Botstein et al., 1980). RFLPs were first identified in temperature sensitive mutations of adenoviruses (Grodzicker et al., 1974). This methodology is based on DNA fragment size differences of defined length that are produced by cleavage of DNA with restriction endonucleases and that are identified by Southern (1975) blot analysis. The use of RFLPs was proposed as a new source of genetic markers for the human genome in 1980 (Botstein et al., 1980; Bishop and Skolnick, 1980). These studies demonstrated the basic principle of using random single copy DNA probes to detect DNA sequence polymorphisms among different genotypes. Gusella et al. (1983) identified polymorphic DNA marker loci associated with the mutant allele causing Huntington's disease using this technology.

In basic plant genetics as well as in plant breeding, RFLPs have been suggested as potent tools (Tanksley, 1983; Beckman and Soller, 1983; Burr et al, 1983; Soller and Beckman, 1983; Evola et al., 1986; Helentjaris et al., 1985; Landry and Michelmore, 1987). The promising potential for this technology in plants is based on the practically unlimited amount of variability at the DNA level in their genomes.

Recently RFLPs were utilized to saturate the genetic linkage maps in maize and tomato (Helentjaris et al., 1986), in tomato (Bernatzky and Tanksley, 1986) and in lettuce (Landry et al., 1987).

The objectives of this study were to select single or low copy number DNA clones from barley random genomic DNA libraries using the plasmid vector pBR322 and the phage EMBL4, and to use these clones to identify RFLPs in the barley genome. In order to detect these RFLPs, selected single copy DNA probes were hybridized to Southern blots containing restriction endonuclease-digested barley DNA from a multiple recessive marker stock and the European 2-rowed cultivar 'Apex'.

Materials and Methods

Plant DNA Extraction

Leaf and stem tissues of barley seedlings were freeze-dried in a VirTis freezedryer for 3-4 days. Total plant DNA was extracted from the lyophilized tissue using modifications of the method of Murray and Thompson (1980) suggested by Saghai-Maroof et al. (1984).

Buffers and Abbreviations

Stock solutions and working solutions utilized in this study were prepared as followed:

20 x SSPE : 3.6 M NaCl, 0.2 M sodium phosphate (pH 7.0), 0.2 M EDTA

20 x SSC : 3 M NaCl, 0.3 M trisodium citrate

10 % Blotto : 10 % non-fat powdered milk, 0.2 % sodium azide

5 x TBE (Tris-Borate) Buffer : Tris base 54 g, boric acid 27.5 g, 0.5 M EDTA (pH 8.0) 20 ml, water up to 1 L

Gel loading Dye Solution : 0.25 % bromophenol blue, 0.25 % xylene cyanol, 40 % (w/v) sucrose

10 x Ligation Buffer : 0.66 M Tris-HCl (pH 7.5), 50 mM MgCl , 50 mM dithiothreitol, 10 mM ATP

10 x Nick-translation Buffer : 0.5 M Tris-HCl (pH 7.2), 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 ug/ml bovine serum albumin (BSA)

Prehybridization Solution (nitrocellulose) : 5 x SSC, 5 x Denhardt's reagent, 20 mM Na-phosphate (pH 6.5), 50 % formamide, 100 ug/ml denatured salmon sperm DNA

Hybridization Solution (nitrocellulose) : 5 x SSC, 1 x Denhardt's reagent, 20 mM Na-phosphate (pH 6.5), 50 % formamide, 100 ug/ml denatured salmon sperm DNA

1 x Denhardt's Solution : 0.02 % Ficoll 400, 0.02 % polyvinyl-pyrrolidone, 0.02 % BSA

Library Construction and Evaluation

Barley genomic DNA libraries were constructed in the phage vector EMBL4 (Frischauf et al., 1983) and the plasmid vector pBR322 using total DNA from the barley cultivars 'Betzes' and 'Traill', respectively. Phage and plasmid clones were randomly selected and amplified using the methods of Maniatis et al. (1982). Plasmid DNA was isolated from E. coli hosts using the mini-prep procedure of Birboim and Doly (1979). Phage clones were randomly picked from the library

and cloned DNAs were prepared following the procedure of Maniatis et al. (1982). Isolated phage DNA was digested with restriction endonucleases, Eco RI, Hind III, Bam HI or Sal I. For plasmid clones, prescreening was first performed by colony hybridization (Grunstein and Hogness, 1975). Selected plasmids were digested with Bam HI and electrophoresed in 0.8 %, 110 x 135 mm horizontal agarose gel using 1 x TBE buffer at 2 V/cm overnight.

Gels were then stained with ethidium bromide and photographed under UV light. Restriction fragments were transferred to either nitrocellulose (Southern, 1975) or Zeta-probe nylon membrane (Reed and Mann, 1985). Nitrocellulose filters were baked at 80 °C in a vacuum oven for 2 hours after transfer was completed. Filters were hybridized with total DNA from 'Betzes' barley which had been radioactively labeled by nick-translation (Rigby et al., 1977). Single and low copy number barley inserts were identified as those which bound low or undetectable amounts of the total barley DNA probe (Figures 1 and 2). Selected phage fragments were subcloned into the plasmid vector pBR322. Selected clones from the plasmid library were utilized directly.

Genomic Blot Preparation

The parents utilized in the mapping study were a multiple recessive marker stock (MMS) developed by Dr. R.W. Wolfe (1984) (discussed in Chapter 3) and the European 2-rowed cultivar 'Apex'. Isolated DNA from these lines was quantified by fluorometry using the DNA specific fluorescent dye Hoechst 33258. Fifteen ug aliquots of DNA were digested with the restriction endonucleases Bam HI, Hind III, Eco

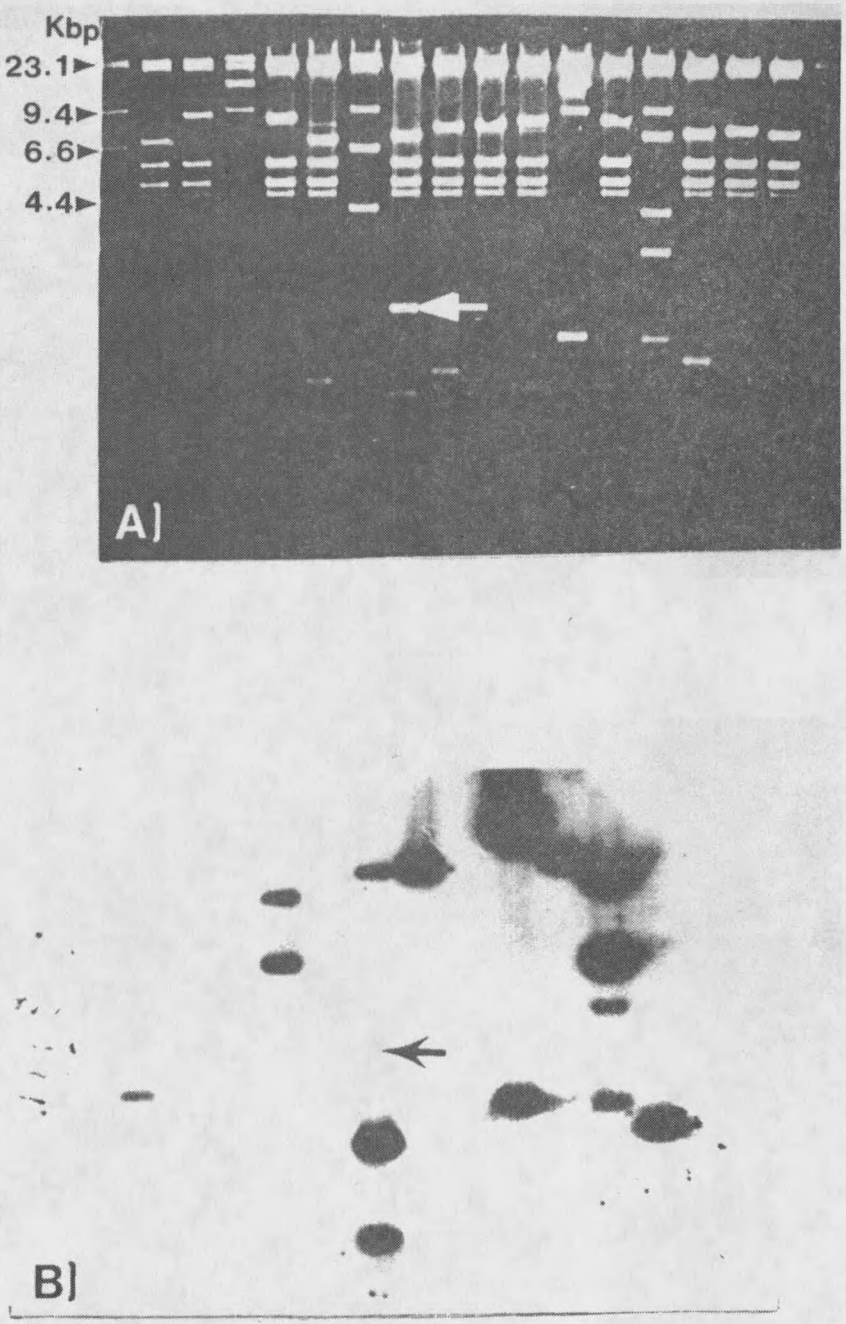
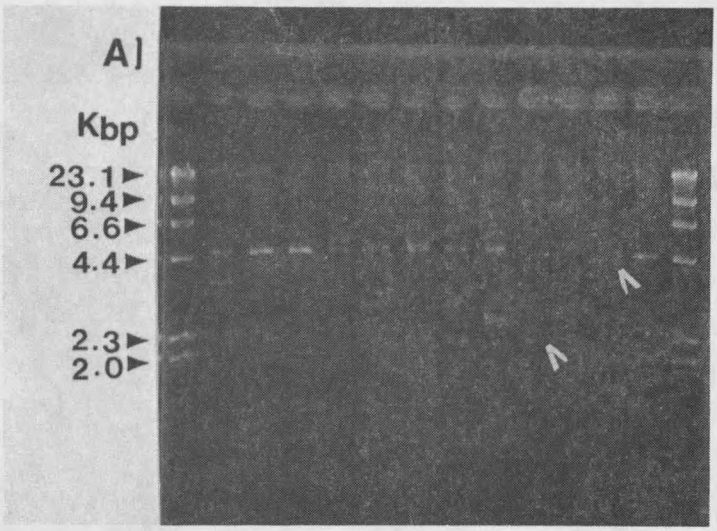


Figure 1. Identification of low copy number genomic clones using EMBL4. A: Gel of 14 EMBL4 clones containing random barley fragments digested with Hind III. B: Autoradiograph of blot of A probed with total nick-translated barley DNA.

Estimated molecular weights in kilobase pairs listed at left. Arrows indicate low copy number fragments tested for identification of polymorphisms between 'Apex' and 'MMS'.



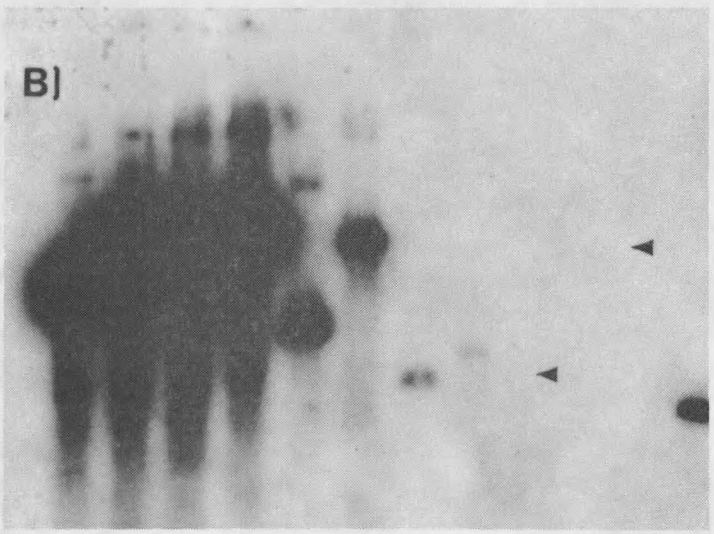


Figure 2. Identification of low copy number genomic clones using a plasmid vector. A: Gel of 12 plasmid vector pBR322 clones containing random barley DNA fragments digested with Bam HI. B: Autoradiograph of blot of A probed with nick-translated total barley genomic DNA.

Estimated molecular weights in kilobase pairs listed at left. Arrows indicate single or low copy number barley DNA fragments.

RI, Eco RV, and Dra I, separated by gel electrophoresis (Figure 3), and transferred to Zeta-probe nylon membrane as indicated above.

Probe Labeling

Approximately 0.1 ug of cloned DNA fragment or total barley DNA was labeled with ^{32}P dNTPs using nick-translation (Rigby et al., 1977). The labeled DNA probes were separated from unincorporated nucleotides using centrifuged Sephadex G-50 1 cc columns. Alternatively, agarose gel slices containing the fragment of interest were labeled by primer extension (Feinberg and Vogelstein, 1984) and utilized without removing the unincorporated nucleotides. Prior to hybridization, the labeled probes were mixed with 0.2 ml of 0.2 N NaOH and denatured by heating to 100°C for 10 minutes.

Hybridization

Nitrocellulose filters were prehybridized and hybridized at 42°C using 50 % formamide according to the method of Spruill et al. (1981). Zeta-probe nylon membrane was prehybridized and hybridized in 15-20 ml of 1.5 x SSPE, 1.0 % SDS and 0.5 % Blotto solution at 68°C in a water incubator with gentle shaking for 4-24 hours (Reed and Mann, 1985). The carrier salmon sperm DNA (5 mg) and radioactive-labeled probe were denatured immediately before adding it to the hybridization solution.

Washing and Autoradiography

Three washes for 15 minutes at room temperature in 300 ml of 2 x SSC/0.1 % SDS, 0.5 x SSC/0.1 % SDS and 0.1 x SSC/0.1 % SDS solutions

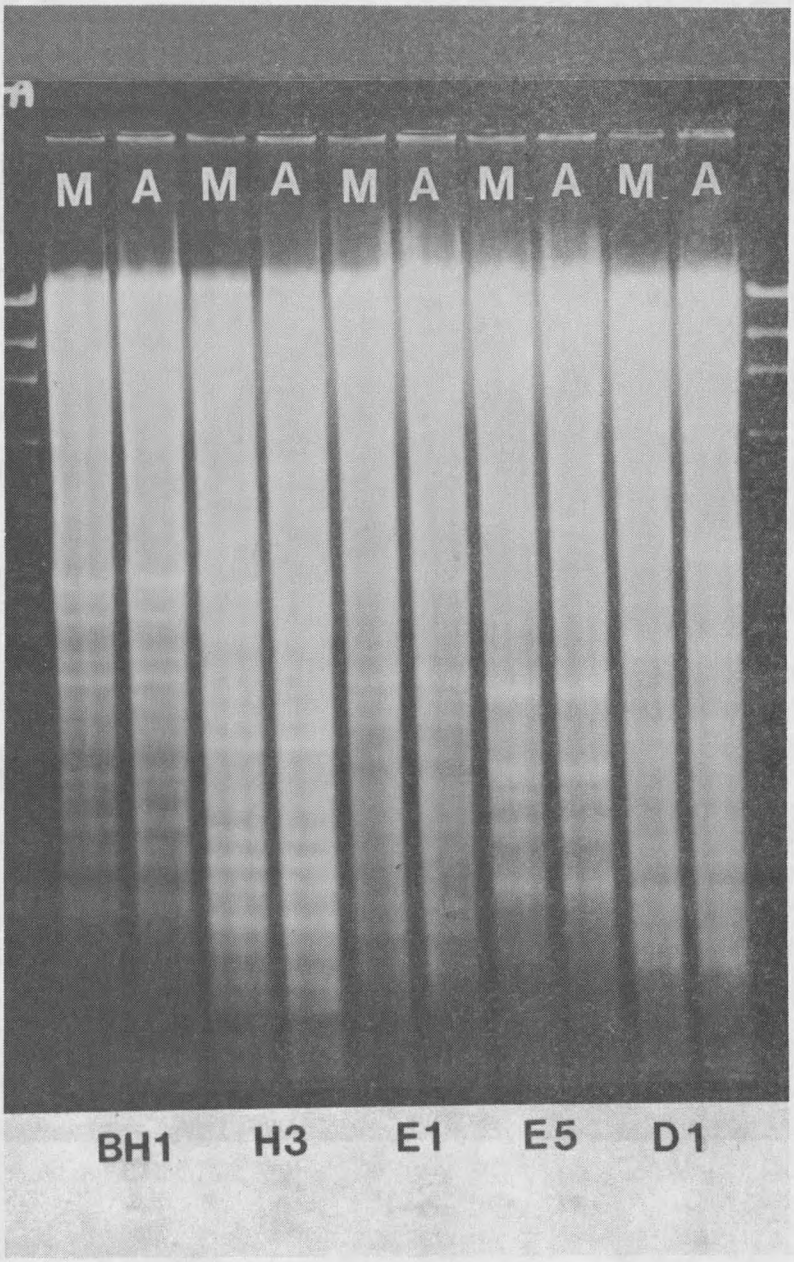


Figure 3. Digestion of barley genomic DNA with restriction endonucleases. Fifteen micrograms of total genomic DNA from the lines 'MMS' and 'Apex' was digested with five different restriction endonucleases. Letters indicate following DNA samples and restriction endonucleases; M: multiple recessive marker stock. A: Apex. BH1: Bam HI. H3: Hind III. E1:Eco RI. E5: Eco RV. D1: Dra I.

successively were followed by two or three final washes with prewarmed solutions of 0.1 x SSC/1.0 % SDS at 65°C.

Filters were wrapped in plastic wrap and placed adjacent to a sheet of X-ray film in an exposure cassette with one or two intensifying screens. Autoradiography was performed at -70°C for 5-7 days.

Filters were reused by removing the hybridization probe. Two methods were used with equal success. Filters were either washed two times of washing in an initially boiling solution of 0.1 x SSC/0.5 % SDS which was allowed to cool over a 20 minute period or washed in 0.2 N NaOH for 20 minutes followed by 0.5 M Tris-HCl (pH 7.5)/0.1 x SSC/ 0.1 % SDS wash solution. Both methods completely removed the hybridization probe.

Results and Discussion

Low Copy Number Clone Selection

In order to determine which clones contained single or low copy number sequences, each clone was hybridized to the ^{32}P-labeled total plant DNA (Figures 1 and 2). The duration of autoradiography and the specific activity of the total DNA probe were critical factors in recognizing repeat-free fragments. Bands showing very faint or no hybridization signal after 5 days autoradiography were selected as potential unique or low copy number sequences.

Fifty phage containing unique barley DNA inserts were screened. In the library using plasmid pBR322, 141 colonies out of 1361 total transformed cells (about 10 %) were observed as Amp^r Tet^s showing

recombinants. Of the 141 pBR322 recombinants, 39 single or low copy number probes (29 single and 10 low copy number clones) were identified and advanced to further screening.

Restriction Fragment Length Polymorphisms

The selected single or low copy number DNA probes were hybridized to Southern blots containing five different restriction endonuclease-digested barley genomic DNAs from a multiple recessive marker stock (MMS) and the European two-rowed cultivar 'Apex' (Figure 3). Utilizing these selected clones, it was confirmed that polymorphisms are readily detectable in barley genome. Eight single or low copy number probes using from the pBR322 library and six fragments cloned into EMBL4 were screened. Nine genomic clones of these were identified which produced clear, consistent results in Southern blot analysis (detailed in Chapter 3).

Some probes displayed different sizes of hybridization bands and the other showed presence vs. absence of detectable bands within two parental lines. Differences among higher molecular weight DNA fragments were quite difficult to identify. Most of the clones evaluated identified polymorphisms using several restriction endonucleases. The simplest explanation for this observation is that the variation observed among barley lines is due to rearrangements or insertion/deletion events, rather than point mutations.

The autoradiograph using the probe pxMSU 21 demonstrated clear polymorphisms using Bam HI and Hind III (Figure 4). Using Hind III,

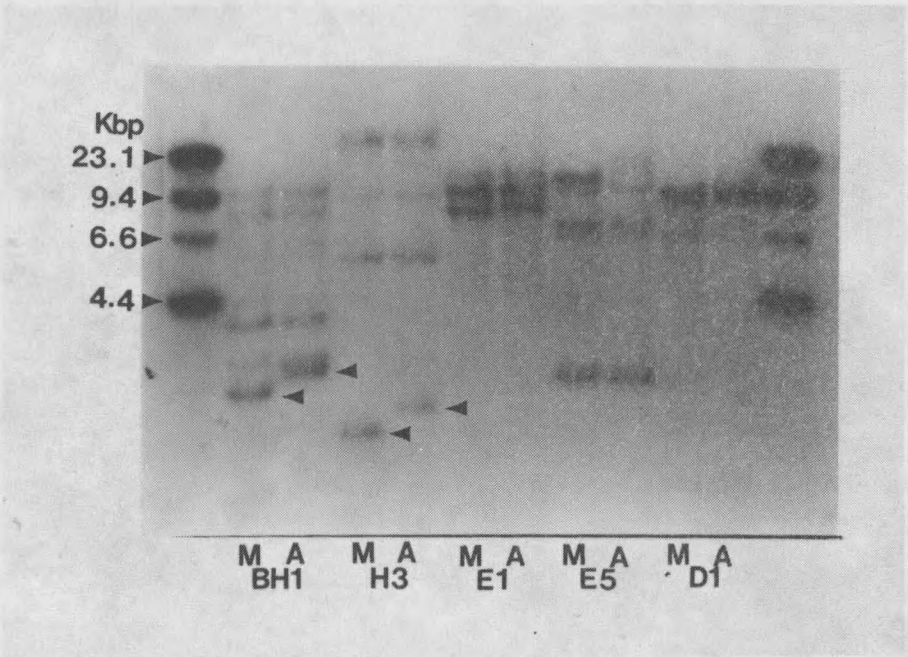


Figure 4. Screening blot of a polymorphic low copy number DNA clone using a plasmid vector. Fifteen micrograms of DNA from the lines 'MMS' and 'Apex' was digested with five different DNA restriction endonucleases and transferred to nylon membrane. Estimated molecular weights in kilobase pairs listed at left. Letters indicate the following DNA samples and restriction endonucleases; M: multiple recessive marker stock, A: Apex, BH1: Bam HI, H3: Hind III, E1: Eco RI, E5: Eco RV, D1: Dra I.

Blot was hybridized with nick translated pxMSU 21. Parental variation was identified with both Bam HI and Hind III.

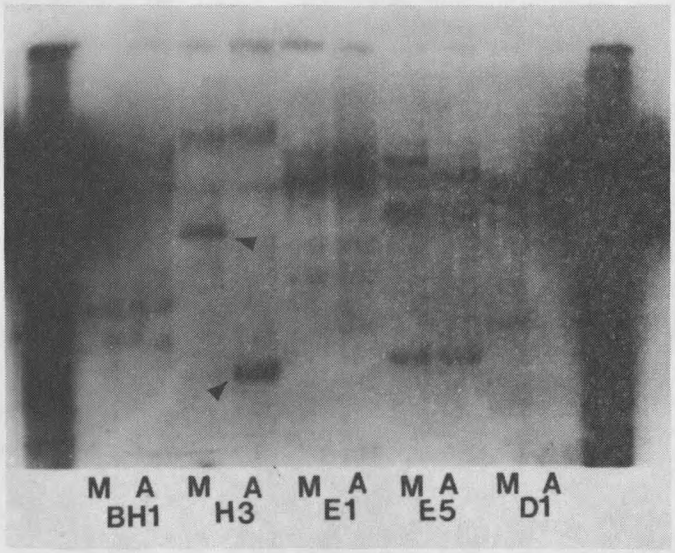


Figure 5. Screening blot of a polymorphic low copy DNA clone using EMBL4. Fifteen micrograms of DNA from the lines 'MMS' and 'Apex' was digested with five different DNA restriction endonucleases and transferred to nylon membrane. Lambda molecular weight marker lanes of left and right ends are completely identical to the cloning vector and showed darkened hybridization signal. Letters indicate the following DNA samples and restriction enzymes; M: MMS, A: Apex, BH1: Bam HI, H3: Hind III, E1: Eco RI, E5: Eco RV, D1: Dra I. Genomic DNA blot was hybridized with nick translated pxMSU 72. The clearest parental variation was identified with Hind III.

pxMSU 72 showed an approximately 3.4 Kbp size difference between 'MMS' and 'Apex' (Figure 5). These restriction endonucleases were utilized in a subsequent mapping study.

CHAPTER 3

A 34 POINT LINKAGE MAP OF THE BARLEY GENOME INCLUDING 17 RFLP LOCI

Introduction

Increasing the number of informative marker loci in crop species will improve our ability to both understand the genetic basis for complex characters and aid in the identification and characterization of germplasm (Beckmann and Soller, 1983). In many important agricultural species the lack of available genetic markers has limited the development of saturated genetic linkage maps.

Analysis of restriction fragment length polymorphisms (RFLPs) provides relatively unlimited numbers of genetic markers and permits the construction of detailed genetic linkage maps. After the use of RFLPs as genetic loci was proposed in human genetic linkage map (Botstein et al., 1980; Bishop and Skolnick, 1980), this approach was extensively utilized to saturate the genetic linkage maps in humans (Gusella, 1986), in maize and tomato (Helentjaris et al., 1986), in tomato (Bernatzky and Tanksley, 1986) and lettuce (Landry at al., 1987).

In this chapter, the recombinational location of seventeen cloned DNA sequences relative to seventeen previously mapped marker loci is described. Nine low copy number sequences which identified RFLPs in cultivated barley were selected as described in Chapter 2. The recombinational locations of seven cDNA clones and a clone of the

barley and wheat ribosomal gene clusters also were identified relative to ten previously mapped morphological marker loci, five previously mapped isozyme loci and two storage protein loci.

Materials and Methods

Plant Materials

The parents utilized in this mapping study were a multiple recessive marker stock (MMS) and the European 2-rowed cultivar 'Apex'. One hundred F_2 plants and twelve F_3 progeny from each F_2 plant were evaluated for the ten morphological characters listed in Table 1.

Table 1. Morphological and biochemical characters evaluated.

Locus Designation	Phenotype	Chromosomal Location
wx	waxy endosperm	1
n	naked caryopsis	1
lk2	short awn	1
v	six rowed	2
wst,,B	white stripe	2
al	albino lemma	3
i	fertile laterals	4
o	orange lemma base and nodes	6
s	short rachilla hairs	7
r	smooth awn	7
Per 1	peroxidase	2
Per 2	peroxidase	2
Est 1	esterase	3
Est 2	esterase	3
Pgd 2	6-phosphogluconate dehydrogenase	5
Hor 1	C hordeins	5
Hor 2	B hordeins	5

DNA Markers and biochemical loci

Nine low copy number random genomic DNA clones which identified clear polymorphisms were utilized in analysis of F_2 progeny from the

cross described above. Seven cDNA clones selected from an endosperm cDNA library (Muthukrishnan et al., 1983) and a clone of the barley and wheat ribosomal gene clusters (Gerlach and Bedrook, 1979; Saghai-Maroof et al., 1984) were also utilized. One hundred F_2 plants and six F_3 progeny each were characterized for polymorphisms esterase, peroxidase and 6-phosphogluconate dehydrogenase isozymes (Table 1 and Figure 5) using the methods described in Benito et al. (1988). Six F_3 seeds from each F_2 plant were evaluated for B and C hordein polymorphisms (Figure 6) according to the methods of Blake et al. (1982).

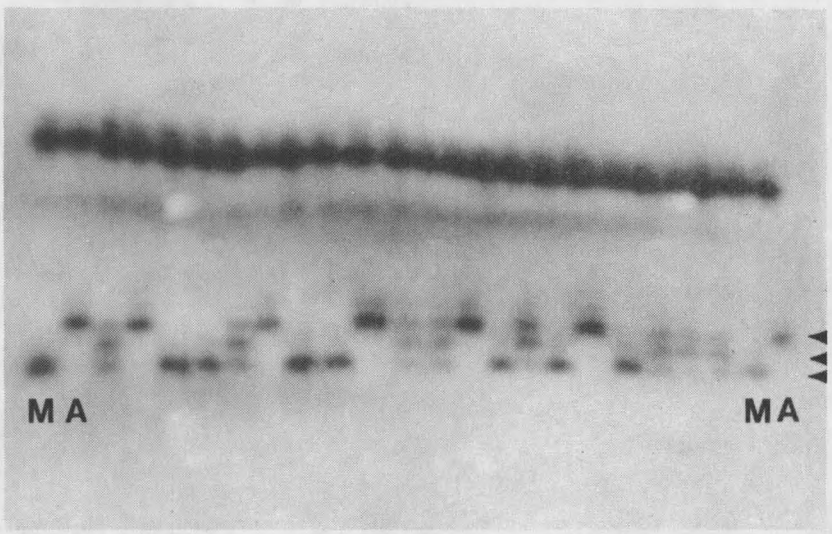


Figure 6. Starch gel of 6-phosphogluconate dehydrogenase in an F_2 population. Arrow indicates the segregating Pgd-2 locus in chromosome 5. Two samples of each left and right margin are two parents; M: MMS, A: Apex. Single band of higher and lower molecular weight are homozygous for allele from 'MMS' and 'Apex', respectively. Triple bands indicate heterozygous type individual.

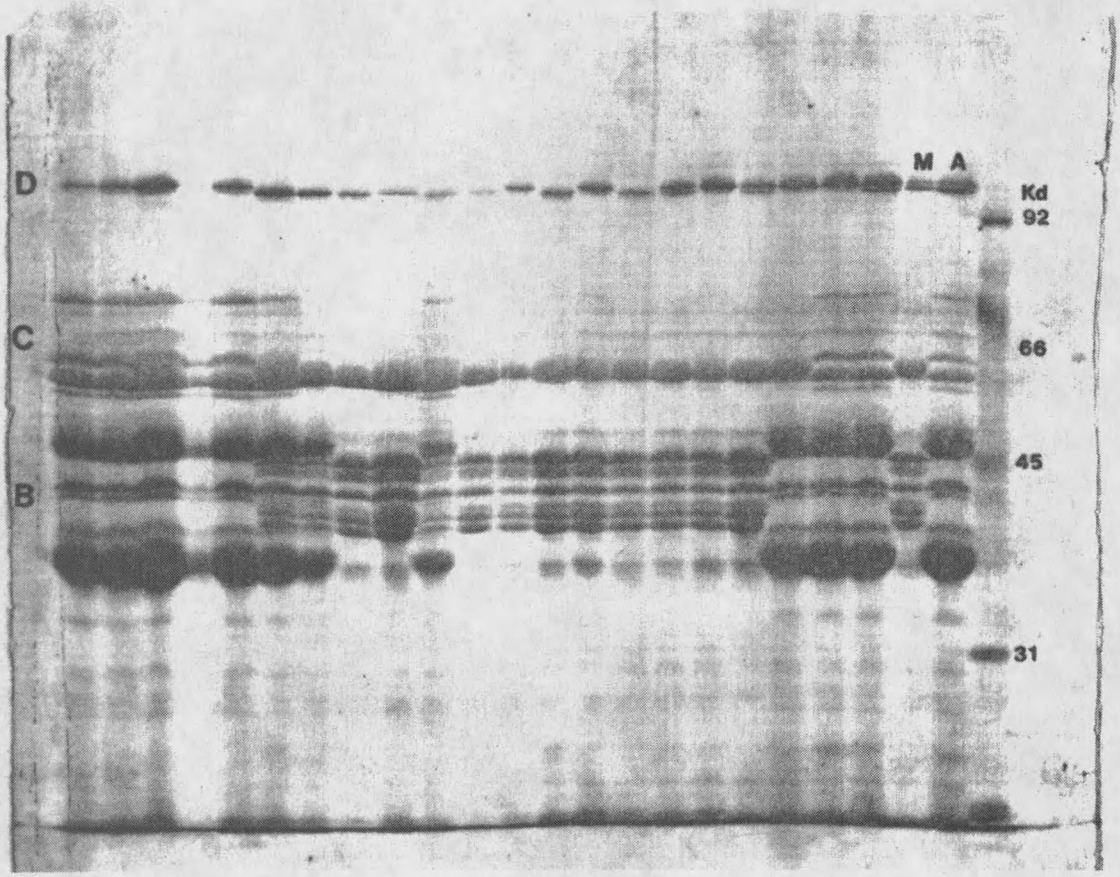


Figure 7. SDS-polyacrylamide gel of barley storage proteins. Six F_3 seeds from each F_2 plant were evaluated for B and C hordein polymorphisms designated Hor 2 locus and Hor 1 locus in barley chromosome 5, respectively. But D hordeins designated Hor 3 locus in the same chromosome did not show clear polymorphisms in these parents. Estimated molecular weights in kilodalton listed at right. Letters indicate as following; B: B hordeins, C: C hordeins, D: D hordeins.

DNA Extraction, Southern Blotting and Hybridization

DNA isolation, Southern blotting, labeling, hybridization, and autoradiography procedures used were identical to those detailed in Chapter 2.

Linkage Analysis

Recombination analyses were performed using Linkage-1 (Suiter et al., 1983) packaged for maximum likelihood linkage analysis (Allard, 1956). While gametic frequencies for isozymic and storage protein loci did not differ significantly from chi-square expectations, differencies in one class of homozygote indicated that loci associated with the morphological marker locus v and three RFLP loci appeared to have modified gametic or zygotic viability (Table 3). This has been observed frequently in the past (Heun 1987), but a clear understanding of the mechanism underlying the bias in segregation data is required before a correction can be applied to the data.

Results

Evaluation of RFLP DNA Markers

Nine genomic clones and seven cDNA clones were identified which produced clear, consistent results in Southern blot analysis of segregating progeny from mapping cross, MMS x Apex (Table 2) (Figure 8). The ribosomal clone, pTA71, also proved useful in this analysis.

This approach to maximizing the chance of observing significant linkages revolved around the use of previously mapped isozyme, storage

protein and morphological marker loci (Wolfe, 1984; Benito et al., 1988).

Table 2. Informative DNA loci in the barley linkage map

Clone name	Vector	Insertion site(s)	Size (Kbp)	Cloner	Polymorphism			Lab name
					Enzyme	MMS	Apex	
pMSU 11	pBR322	BH1	3.0	JS	H3	6.0	8.6	BS134
pMSU 12	pBR322	BH1	3.0	JS	H3	6.6	-	BS20
pKSU 11	pBR322	P1	0.5	SM	H3	6.1	8.3	MC44
pMSU 21	pBR322	BH1	3.0	JS	BH1	3.2	3.4	BS113
pMSU 22	pBR322	BH1/S1	1.0	SC	H3	9.3	11.2	BC146
pKSU 21	pBR322	P1	0.5	SM	E5	11.6	6.4	MC11
pKSU 31	pBR322	P1	0.5	SM	H3	5.5	-	MC22
pKSU 32	pBR322	P1	0.4	SM	E5	5.1	2.2	MC5
pMSU 51	pBR322	BH1	3.0	JS	BH1	20.8	32.8	BS16
pMSU 71	pBR322	BH1	4.0	JS	H3	3.8 1.9	-	BS118
pMSU 72	pBR322	BH1/S1	2.0	SC	H3	6.6	3.2	BC107
pMSU 73	pBR322	BH1	5.0	JS	BH1	5.1	9.4	BS95
pMSU 74	pBR322	BH1/S1	1.0	SC	H3	10.0	7.4	BC196
pKSU 71	pBR322	P1	0.5	SM	BH1	11.3	6.6	MC26
pKSU 72	pBR322	P1	0.2	SM	H3	6.3	4.7	MC75
pKSU 73	pBR322	P1	0.2	SM	D1	2.9	3.6	MC24
pTA71(Rrn2)	pAC184	E1	9.0	WG	T1	1.0	-	PTA71

Abbreviation used in Table: P1=Pst I, H3=Hind III, BH1=Bam HI, E5=Eco RV, S1=Sal I, T1=Taq I, D1=Dra I, E1=Eco RI; JS=J.S. Shin, SM=S. Muthukrishnan, SC=S. Chao, WG=W. Gerlach.

Twelve of seventeen RFLP loci showed codominant segregation. Four of the five loci which demonstrated as dominant allele (presence vs. absence of a band) were from genomic clones which hybridized to several bands in each lane of the Southern blot. Either comigration of bands or varying numbers of hybridizing sequences between genotypes could explain this result. Careful experiments to precisely determine sequence copy number will be required to distinguish between these hypotheses.

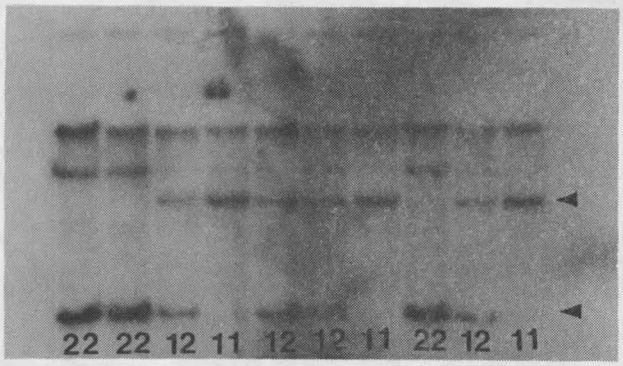


Figure 8. Blot demonstrating F_2 segregation. DNA from 10 F_2 plants was digested with Hind III, electrophoresed, blotted and probed with pxMSU 72. Simple Mendelian segregation was observed for the polymorphisms indicated by arrows. Lanes labeled '11' are homozygous for the allele from 'MMS', lanes labeled '12' are heterozygous, and lanes labeled '22' indicate homozygosity for the allele from 'Apex'.

Table 3. Segregations and chi-square goodness-of-fit analysis for 17 RFLP, 5 isozyme, 2 storage protein, and 10 morphological markers in a barley F_2 population.

Locus	Homozygous MMS	Heterozygous	Homozygous Apex	Chi-square
wx	16	56	28	3.745
n	21	48	30	1.359
lk2	22	43	34	4.025
v	15	46	38	10.732*
al	21	43	35	4.945
i	16	61	22	5.399
o	25	45	29	0.894
s	29	41	29	2.429
r	24	54	21	0.518
wst,,B	25	44	31	1.825
Est1	25	41	34	4.345
Est2	30	40	30	3.425
Per1	20	52	28	1.105
Per2	14	58	28	5.785
Pgd2	28	50	22	0.550
Hor1	17	42	33	5.156
Hor2	23	44	25	0.158
xMSU 11	22	37	17	0.454
xMSU 12	-- 45 --		17	0.086
xKSU 11	11	24	27	10.202*
xMSU 21	25	40	18	0.946
xMSU 22	14	37	5	7.759*
xKSU 21	22	42	22	0.006
xKSU 31	-- 53 --		18	0.005
xKSU 32	17	48	16	2.278
xMSU 51	29	-- 55 --		3.571
xMSU 71	-- 74 --		17	1.615
xMSU 72	22	49	21	0.245
xMSU 73	22	50	23	0.153
xMSU 74	27	24	18	7.862*
xKSU 71	27	47	15	2.949
xKSU 72	21	38	16	0.433
xKSU 73	24	45	18	0.649
Rrn2	-- 68 --		18	0.558

* indicates Chi-square value greater than would be expected by chance at the 0.05 level of significance.

Mapping of RFLP Loci

Using a multiple marker stock (MMS) as one parent in linkage analysis provided seventeen 'benchmark' loci which had been previously mapped. Figures 9, 10, 11 and Table 4 display a thirty-four point linkage map which utilized ten morphological markers, 5 isozyme loci and 2 hordeins as reference points in map construction. With the exception of chromosome four and six, significant linkages were found between marker loci and biochemical and RFLP loci. In the case of chromosome six, the previously located Rrn1 locus was not found to vary among the parents of this population with the enzymes evaluated. Eight of the sixteen clones identified polymorphisms with multiple restriction endonucleases. The simplest explanation for this observation is that the variation observed among barley lines is due to rearrangements or insertion/deletion events rather than point mutations.

Nomenclature

The clones and loci they identify are named following recommendations of several researchers compiled by Dr. Gary Hart (Hart, pers. comm.). The initial three letters indicate the clone source, the next two or three digits indicate the clone number, and in this project the first digit was assigned to the chromosome number to which the clone has been initially mapped. As none of clones have a known function, the locus names differ from the clone names only by the prefix 'x' to indicate that they are RFLP loci.

Table 4. Recombination frequencies and standard deviations.

Chromosome Number	Linked loci	No. of progeny	Recombination frequency and S.D.
1	wx - xKSU 11	62	34.88 +/- 5.60
1	xKSU 11 - n	61	38.12 +/- 5.91
1	n - 1k2	99	17.51 +/- 2.86
1	1k2 - xMSU 12	62	28.89 +/- 6.64
1	xMSU 12 - xMSU 11	53	27.51 +/- 7.03
1	wx - n	99	44.78 +/- 4.95
1	xKSU 11 - 1k2	61	38.02 +/- 5.90
1	n - xMSU 12	62	36.25 +/- 7.27
2	xMSU 22 - Per1	56	38.01 +/- 6.16
2	Per1 - Per2	100	15.34 +/- 2.81
2	Per2 - v	99	30.46 +/- 4.11
2	v - xKSU 21	85	31.63 +/- 4.53
2	xKSU 21 - xMSU 21	71	30.37 +/- 4.85
2	xMSU 21 - wst,,B	83	37.37 +/- 5.02
2	xMSU 22 - Per2	56	41.22 +/- 6.39
2	Per2 - xKSU 21	86	38.49 +/- 5.00
2	Per1 - v	99	38.37 +/- 4.65
3	al - xKSU 32	81	36.85 +/- 5.04
3	xKSU 32 - xKSU 31	64	37.35 +/- 7.23
3	xKSU 31 - Est2	71	7.22 +/- 3.17
3	Est2 - Est1	100	8.81 +/- 2.11
3	al - xKSU 31	71	43.03 +/- 7.14
3	xKSU 31 - Est1	71	13.97 +/- 4.39
4	i	no significant linkage identified	
5	Hor2 - Hor1	92	13.91 +/- 2.78
5	Hor1 - xMSU 51	78	38.81 +/- 6.63
5	xMSU 51 - Pgd2	84	18.13 +/- 4.58
5	Hor2 - xMSU 51	78	41.80 +/- 6.77
5	Hor1 - Pgd2	92	38.08 +/- 4.87
6	o	no significant linkage identified	
7	xMSU 74 - xMSU 73	68	31.61 +/- 5.07
7	xMSU 73 - r	94	23.26 +/- 3.63
7	r - xKSU 73	86	9.83 +/- 2.40

Table 4. (continued)

Chromosome Number	Linked loci	No. of progeny	Recombination frequency and S.D.
7	xKSU 73 - xMSU 72	83	8.85 +/- 2.32
7	xMSU 72 - s	91	24.36 +/- 3.78
7	s - xKSU 72	74	13.67 +/- 3.07
7	xKSU 72 - xKSU 71	72	43.50 +/- 5.75
7	xKSU 71 - Rrn2	74	7.54 +/- 3.17
7	Rrn2 - xMSU 71	73	9.64 +/- 11.57
7	xMSU 73 - xKSU 73	86	26.59 +/- 4.09
7	r - xMSU 72	91	14.93 +/- 2.90
7	xKSU 73 - s	86	27.29 +/- 4.15
7	xMSU 72 - xKSU 72	72	26.65 +/- 4.47
7	xKSU 71 - xMSU 71	82	11.58 +/- 3.72

Discussion

In this paper sixteen new informative genomic and cDNA clones for use in barley RFLP analysis were evaluated and released. These have been mapped using previously mapped marker loci and provide an initial analysis of the types of polymorphisms identified.

Four loci of the 34 loci tested, xKSU 11, xMSU 22, v, and xMSU 74, did not meet chi-square expectations for segregation of dominant alleles at a single locus. Linkage estimates with these loci are likely biased and will need confirmation with other crosses or progeny of these 100 F_2 lines. Twenty-two percent of the loci evaluated by Landry et al. (1987), and 10 percent of the maize loci and 34 percent of the tomato loci evaluated by Helentjaris et al. (1986) segregated with abnormal frequencies. Without a careful estimates of gametic or zygotic selection, it is impossible to correct for distorted gene

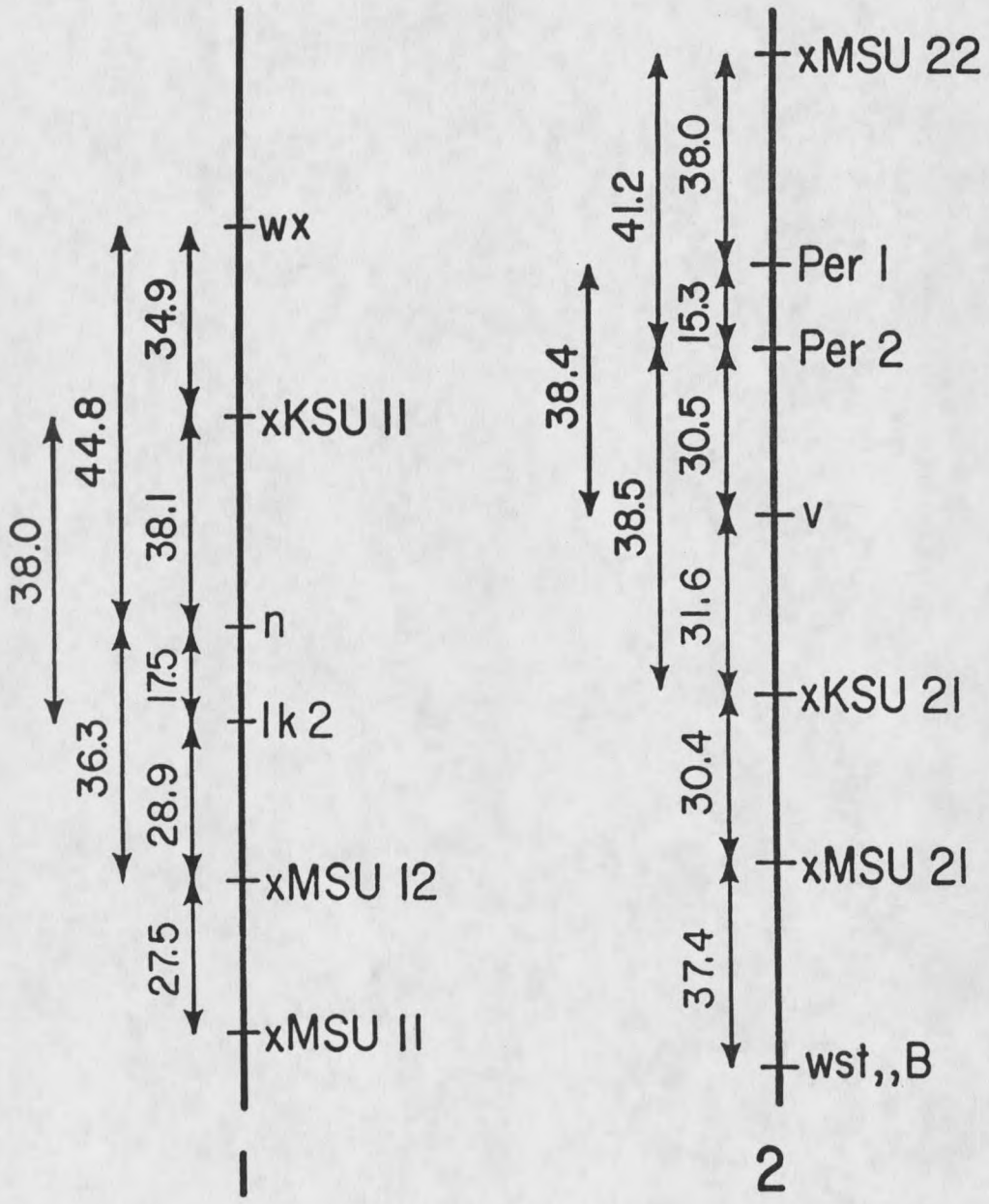


Figure 9. Barley genetic linkage maps of chromosomes 1 and 2. Distances listed are in percent recombination, not centimorgans. All the data were analyzed by the maximum likelihood method using computerized program, Linkage-1. The detailed distances show in table 4.

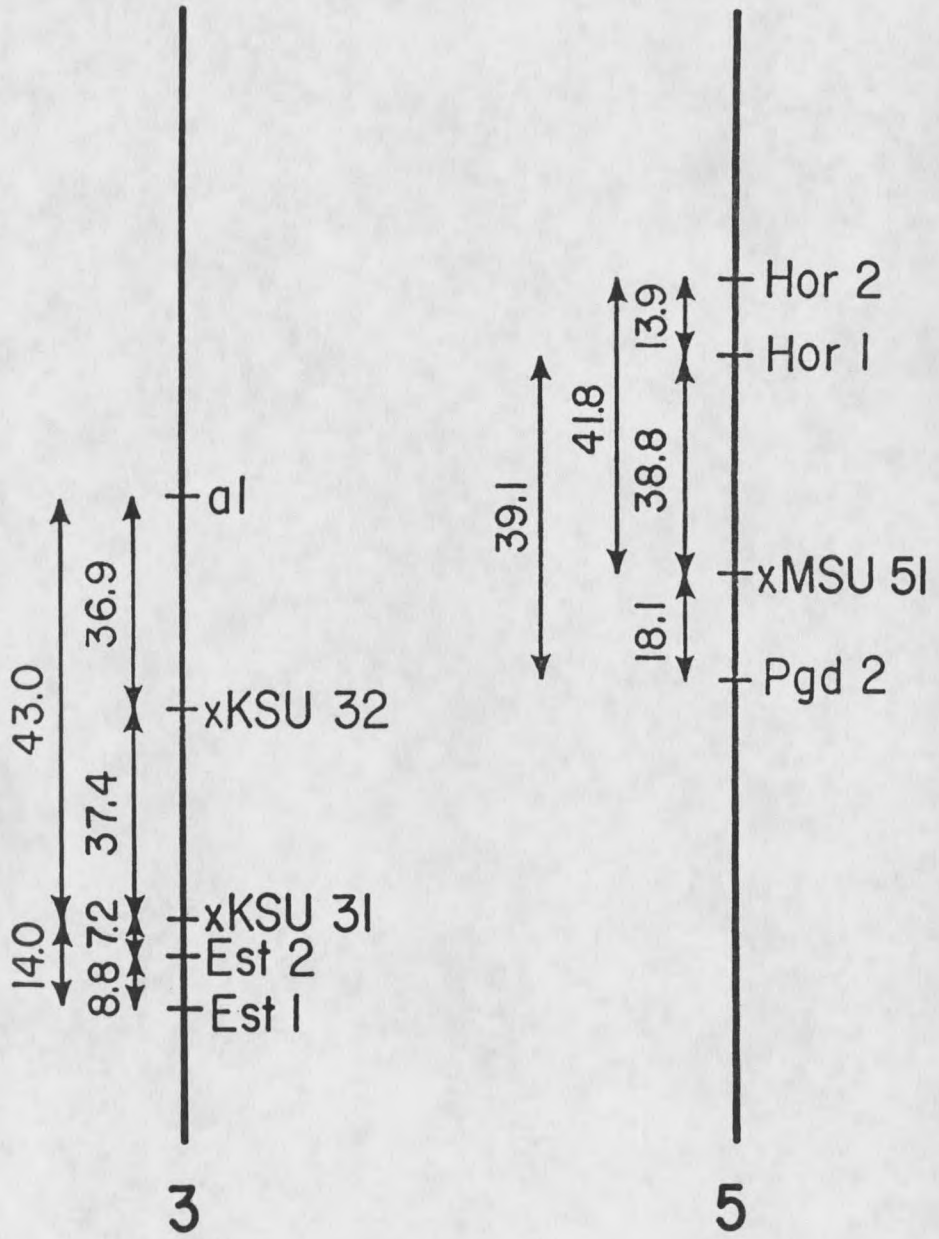


Figure 10. Barley genetic linkage maps of chromosomes 3 and 5. All the data are listed in Table 4.

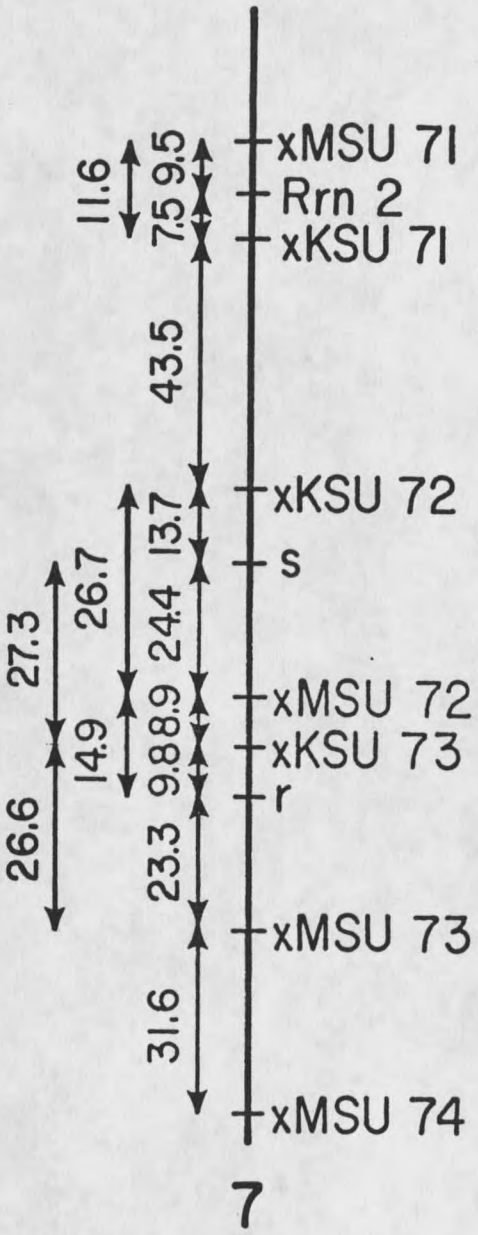


Figure 11. Barley genetic linkage map of chromosome 7. All the data are listed in Table 4.

segregation (Heun, 1987). We therefore recommend caution in utilizing data involving these loci and their probes.

The probes and markers utilized in this project span 680 recombination units of the barley genome, approximately 50 percent of its estimated recombinational length. Kleinhofs et al. (1988) recently published an RFLP map of barley chromosome 6, increasing the amount of coverage of the barley genome to well over 50 percent.

Release of these clones along with release of the 100 mapping lines (detailed in Chapter 4) produced in this effort provides two sets of tools which will simplify the mapping of additional RFLP loci.

CHAPTER 4

PHYSICAL MAPS OF SEVENTEEN INFORMATIVE DNA MARKERS AND DESCRIPTION OF 100 MAPPING LINES

Introduction

The recently developed technique, restriction fragment length polymorphism (RFLP) analysis, provides the potential for an unlimited number of genetic markers which could be utilized not only to saturate genetic linkage maps but also to estimate intervarietal and interspecific genetic relationships among plant genomes. RFLPs are dependent upon mutational events, either point mutations, insertion and deletion events, or rearrangements and inversions. To evaluate polymorphic DNA clones and identify the genomic locations of their homologous alleles in barley lines, well characterized genetic stocks are required. Recently, recombinant inbred lines (RIL) were developed in maize for the rapid mapping of molecular probes to chromosomal locations (Burr et al., 1988).

It is fortunate for barley geneticists to have available a well-marked master recessive stock (Wolfe, 1984) that provides a relatively large number of 'benchmarks'. Genotypes of one hundred F_2 lines using 10 morphological characters, 5 isozyme loci, 2 hordeins, and 17 RFLP loci were characterized (detailed in Chapter 3). In this chapter along with the genotypic matrix of 100 mapping F_2 lines and thirty-four loci, seventeen polymorphic DNA fragments that have been mapped in barley linkage groups were characterized in detail by restriction mapping

analysis. At least 8 different restriction endonucleases were utilized to digest them either singly or in combination. DNA sequence analysis was perfomed according to Sanger et al. (1977) to provide a detailed physical map of pxMSU 21.

Materials and Methods

Genotype Characterization

Plant materials, isozyme loci, hordein loci, experimental techniques for DNA work and linkage analyses were described in Chapters 2 and 3. Experimental materials were derived from the hybrids of MMS x Apex. The methods of Nilan (1964) and the USDA handbook 'Barley' (1979) were adapted to identify the genotypes for morphological characters. The pedicellate lateral selection method of Gilbertson and Hockett (1986) was used to identify genotypes for the v and i alleles.

Restriction Mapping

Plant materials, cloning, transformation, and clone selection procedures were identical to the previous chapters (Chapter 2 and 3). About 500 ng of plasmid DNA was digested with appropriate restriction endonucleases, either Bam HI, Hind III or Pst I, followed by at least 7 different restriction endonucleases either singly or in combination. DNA fragments were separated in 0.8 % agarose gel, stained by ethidium bromide, and photographed. The size of each fragment was determined using the Hind III digested fragments of the phage Lambda as standards. The exact clockwise direction of each fragment in plasmid pBR322 was also determined.

A fully detailed restriction map of pxMSU 21 was compared to double digestions of total barley genomic DNA from the cultivars 'Betzes' and 'Robust' to identify the mechanism by which allelic variation was generated at the locus xMSU 21.

Nucleotide Sequencing

The Bam HI and Sst I digested fragment of pxMSU 21 containing approximately 500 base pairs was cloned into M13mp18 and nucleotide sequenced using the dideoxynucleotide chain termination method of Sanger et al. (1977). This M13mp18 RF and the bacterial strain NM522 were utilized in this purpose as per manufacture's instruction.

Results and Discussion

Genotypes of 34 Alleles

The genotype matrix of 100 F_2 mapping lines and 34 loci is listed in Table 5. Nomenclature for RFLP loci was explained in a previous chapter (Chapter 3). Digit labeled '11' are homozygous for the allele from 'MMS', '12' is heterozyous, '22' indicates homozygosity for the allele from 'Apex', and '99' means no data identified.

All the morphological characters were relatively simply determined. For isozyme loci wheat-barley addition lines and ditelosomic series were utilized to evaluate whether the alleles at the polymorphic loci in 'MMS' and 'Apex' were identical to previously located alleles. Twelve of seventeen RFLP loci and the isozyme Pgd2 showed codominant segregation; otherwise, dominant patterns were observed in the F_2 population.

Table 5. Genotypes of 34 loci in 100 mapping lines

Plant No.	Locus Name																																
	wx	a	lk2	v	al	o	s	r	i	Est 1	Est 2	Per 1	Per 2	Pgd 2	MSU 73	MSU 21	Hor 2	Hor 1	MSU 72	MSU 22	wat ,,B	MSU 71	MSU 11	MSU 12	MSU 74	MSU 51	KSU 32	KSU 72	Rrn 2	KSU 73	KSU 11	KSU 71	KSU 21	KSU 31
1	12	11	11	22	22	11	11	11	22	12	12	12	12	22	12	22	12	12	11	12	22	11	11	11	22	22	12	11	99	11	12	11	12	11
2	12	22	22	22	22	22	22	22	12	12	22	22	22	22	22	22	12	12	22	12	22	22	22	22	12	22	99	22	11	22	11	12	22	11
3	22	12	12	11	12	12	22	12	12	11	11	11	11	22	12	12	12	12	12	11	12	11	11	11	22	22	12	22	11	12	22	12	11	11
4	12	22	22	12	22	12	12	12	12	22	22	11	12	12	12	12	12	12	12	12	12	11	11	11	12	22	11	12	11	12	22	11	22	11
5	22	12	12	22	12	11	11	11	11	11	11	12	12	12	12	11	22	22	11	12	12	22	12	11	12	22	12	11	22	11	11	22	11	11
6	12	12	12	22	11	12	11	12	11	12	12	11	11	11	11	11	12	12	11	99	12	11	12	11	22	11	99	11	11	11	22	12	11	11
7	12	22	22	22	11	12	12	12	22	12	12	22	22	12	12	12	11	12	12	12	22	11	22	22	12	11	99	12	11	12	99	12	12	11
8	12	12	22	12	12	22	11	22	12	12	12	11	12	11	22	11	22	22	22	11	12	11	11	11	11	11	99	11	99	22	99	12	11	11
9	12	11	11	22	11	12	22	22	22	22	22	22	22	12	22	12	11	11	22	12	12	11	11	11	11	22	99	22	11	22	11	11	22	22
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32	12	11	11	22	12	22	12	12	12	11	11	12	22	11	12	11	11	12	12	12	22	11	12	11	99	22	12	12	11	12	22	11	12	11
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40	22	12	11	11	12	12	11	11	12	11	11	12	22	12	11	12	11	11	11	12	22	11	12	99	99	22	99	99	11	11	99	11	99	99

Table 5. (continued)

Plant No.	Locus Name																																
	wx	n	lk2	v	al	o	s	r	i	Est 1	Est 2	Per 1	Per 2	Pgd 2	MSU 73	MSU 21	Hor 2	Hor 1	MSU 72	MSU 22	wat ,,B	MSU 71	MSU 11	MSU 12	MSU 74	MSU 51	KSU 32	KSU 72	Rrn 2	KSU 73	KSU 11	KSU 71	KSU 21	KSU 31
41	12	22	22	11	12	12	11	12	22	11	11	11	12	12	99	11	99	99	12	11	12	11	12	99	11	22	12	11	11	99	99	12	99	99
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48	12	11	12	12	22	11	11	11	12	12	22	22	12	11	11	12	12	12	11	11	22	11	11	99	11	11	11	11	11	11	99	12	12	99
49	11	12	22	22	12	12	12	12	12	22	22	12	11	12	12	12	11	11	22	12	12	22	99	99	12	22	11	12	22	12	99	22	22	99
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Table 5. (continued)

Plant No.	Locus Name																																
	wx	n	lk2	v	al	o	s	r	i	Est 1	Est 2	Per 1	Per 2	Pgd 2	MSU 73	MSU 21	Bor 2	Bor 1	MSU 72	MSU 22	wat ,,B	MSU 71	MSU 11	MSU 12	MSU 74	MSU 51	KSU 32	KSU 72	Rrn 2	KSU 73	KSU 11	KSU 71	KSU 21	KSU 31
81	22	12	12	22	11	12	12	11	12	12	12	12	12	12	11	22	22	22	11	99	22	11	99	11	11	22	12	12	11	11	99	12	22	11
82	12	11	11	22	12	22	22	12	22	12	12	12	12	11	12	11	22	22	12	99	11	99	99	11	11	22	11	22	11	12	99	12	12	11
83	12	22	22	12	22	12	11	12	12	11	11	22	12	12	12	22	12	12	11	99	22	11	99	22	11	22	12	11	11	11	12	11	22	11
84	12	12	12	11	12	12	12	12	22	11	11	11	11	22	12	99	11	12	11	99	12	22	99	99	12	22	22	12	22	11	12	22	11	11
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86	12	12	12	12	22	12	11	11	12	22	22	12	12	12	22	99	11	12	11	99	22	22	99	99	22	11	12	11	22	11	99	22	11	22
87	22	11	11	12	12	22	12	22	12	12	12	12	12	11	12	99	12	22	12	99	12	11	99	22	12	11	11	12	22	12	99	12	12	11
88	11	12	12	12	22	12	22	11	12	22	12	11	11	12	11	99	11	12	12	99	22	11	99	22	22	22	12	22	11	11	11	12	22	22
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91	22	11	11	22	22	22	22	12	12	12	12	11	11	22	11	12	22	12	22	99	22	11	99	99	99	99	99	99	11	99	99	99	99	99
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93	12	22	22	12	12	12	12	12	12	12	12	22	22	12	12	12	11	12	22	99	12	11	99	99	99	99	99	99	99	22	99	11	99	99
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95	11	99	99	99	11	99	99	99	99	11	11	22	22	22	12	12	12	12	12	99	12	22	99	99	99	99	12	22	99	12	12	11	12	11
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100	12	22	22	12	11	22	22	12	12	12	12	12	12	22	12	22	12	12	22	99	22	11	99	99	99	99	11	12	11	12	22	12	12	11

Release of these 100 mapping lines along with 17 informative DNA clones will provide a 'master template' for locating additional RFLP loci in the barley linkage map. Development of saturated genetic linkage maps with the relatively unlimited DNA markers will likely allow the identification of agronomically important quantitative trait loci.

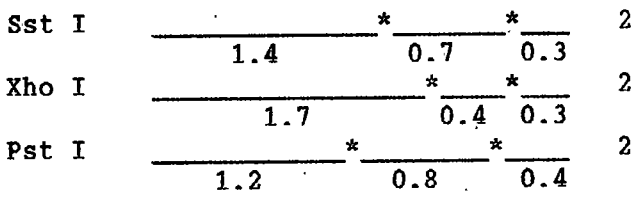
Physical Maps of DNA Markers

Fifteen clones out of seventeen polymorphic DNA markers were physically mapped using several restriction endonucleases as shown in Figure 12. Two clones out of seventeen were not utilized in this project, since pTA71 was characterized by Gerlach and Bedbrook (1979) and Saghai-Maroof (1984), and pxMSU 22 is available only in the virus vector EMBL4.

Twelve clones were well characterized by one or more restriction endonucleases (Figure 12). Three cDNA clones pxKSU 32, pxKSU 71 and pxKSU 73 had no site using 13 or 14 different restriction endonucleases, but had distinctive fragment sizes ranging from 500 to 650 base pairs.

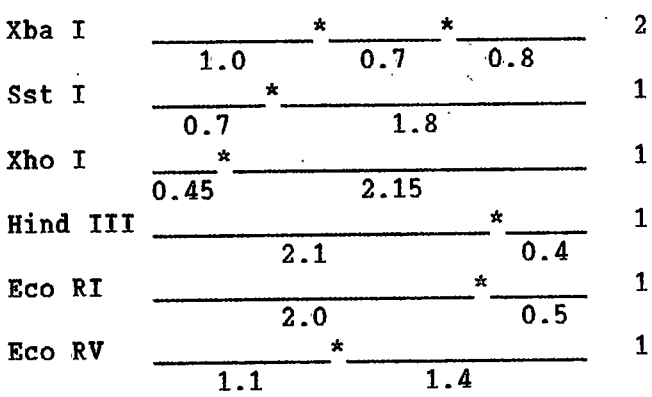
The clone pxMSU 21 was utilized to determine the mechanism by which variation was generated between the cultivars 'Betzes' and 'Robust'. Total genomic DNAs of two barley cultivars, 'Robust' and 'Betzes', were doubly digested with combinations of Bam HI and either Sst I or Hind III, electrophoresed and transferred to a nylon membrane.

pxMSU 11 2.4 Kbp with Bam HI



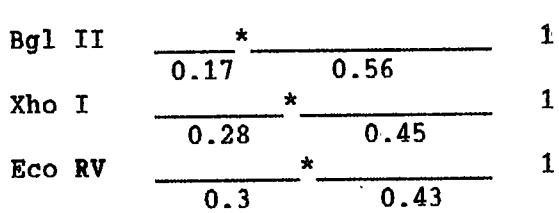
(No site identified)
Bgl II, Xba I, Hind III, Eco RI, Eco RV, Dra I, Kpn I, and Bcl I

pxMSU 12 2.5 Kbp with Bam HI



(No site identified)
Bgl II and Dra I

pxKSU 11 0.73 Kbp with Pst I

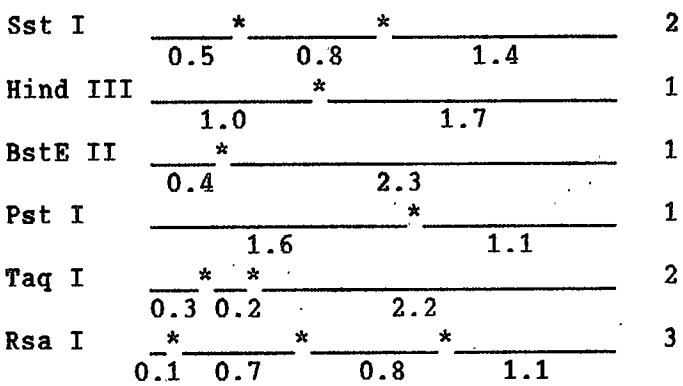


(No site identified)
Xba I, Sst I, Hind III, Eco RI, Dra I, Kpn I, and Bcl I

Figure 12. Restriction maps of 15 informative DNA clones which have been mapped in barley chromosomes. All the sizes of fragments are in kilobase pairs. Total size of each clone is described with the cloned-site restriction endonuclease

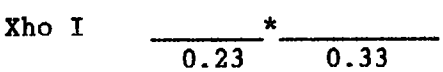
Figure 12. (continued)

pxMSU 21 2.7 Kbp with Bam HI



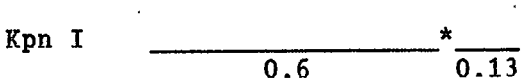
(No site identified)
Bgl II, Xho I, Eco RI, Eco RV, Dra I, Kpn I, Bcl I, and Sal I

pxKSU 21 0.56 Kbp with Pst I



(No site identified)
Bgl II, Xba I, Sst I, Hind III, Eco RI, Eco RV, Dra I, Kpn I, and Bcl I

pxKSU 31 0.73 Kbp with Pst I



(No site identified)
Bgl II, Xba I, Sst I, Xho I, Hind III, Eco RI, Eco RV, Dra I, and Bcl I

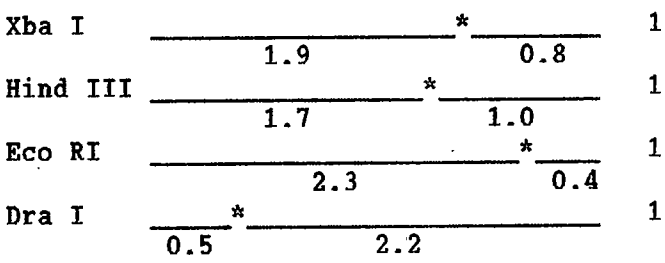
pxKSU 32 0.65 Kbp with Pst I

None of the following enzymes identified restriction site.

Bgl II, Xba I, Sst I, Xho I, Hind III, Eco RI, Eco RV, Dra I, Kpn I, Bcl I, and Bam HI

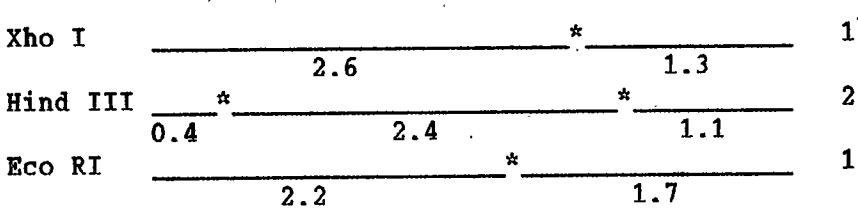
Figure 12. (continued)

pxMSU 51 2.7 Kbp with Bam HI



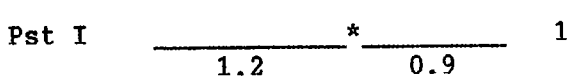
(No site identified)
Bgl II, Sst I, Xho I, and Eco RV

pxMSU 71 3.9 Kbp with Bam HI



(No site identified)
Bgl II, Xba I, Sst I, Eco RV, and Dra I

pxMSU 72 2.1 Kbp with Hind III



(No site identified)
Bgl II, Xba I, Sst I, Xho I, Eco RI, Eco RV, Dra I, Bam HI, Kpn I, and Bcl I

pxMSU 73 5.8 Kbp with Bam HI

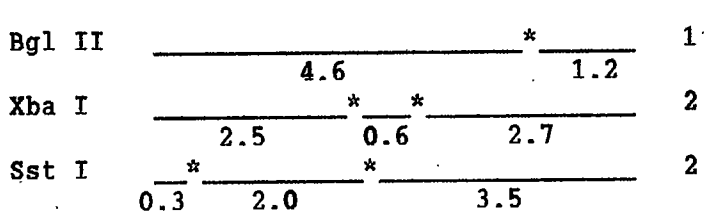
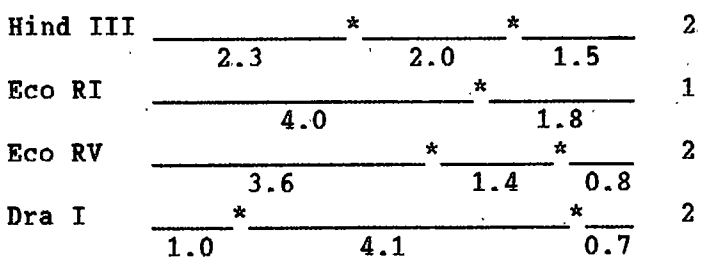
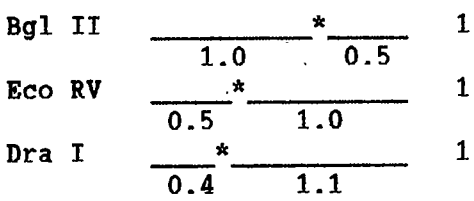


Figure 12. (continued)



(No site identified)
Xho I

pxMSU 74 1.5 Kbp with Hind III



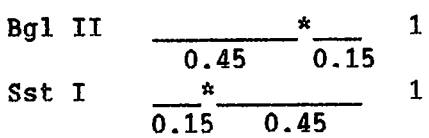
(No site identified)
Xba I, Sst I, Xho I, and Eco RI

pxKSU 71 0.65 Kbp with Pst I

None of the following enzymes identified restriction site.

Bgl II, Xba I, Sst I, Xho I, Hind III, Eco RI, Eco RV, Dra I, Kpn I, and Bcl I

pxKSU 72 0.6 Kbp with Pst I



(No site identified)
Xba I, Xho I, Hind III, Eco RI, Eco RV, Dra I, Kpn I, and Bcl I

Figure 12. (continued)

pxKSU 73 0.56 Kbp with Pst I

None of the following enzymes identified restriction site.

Bgl II, Xba I, Sst I, Xho I, Hind III, Eco RI, Eco RV, Dra I, Kpn I, and Bcl I

Labeled pxMSU 21 was hybridized to the filter (Figure 13). In the Bam HI/Sst I digestion, fragments of 800 bp and 1.4 Kbp of Robust were the same size as the Betzes fragments. However, the Betzes allele contained about 200 base pairs of inserted sequence within the 500 bp fragment, as is shown in Figure 13.

Sequence of Polymorphic Region

When sequence information for allelic variants is available, allele-specific oligonucleotides (ASOs) (Erlich et al., 1986) can be synthesized that detect single base substitutions and identify allelic variants using simple dot blot procedures. In human genetics, synthetic oligonucleotides were applied to detect genetic diseases, sickle cell disease and thalassemia traits in the prenatal stage (Conner et al., 1983; Kazazian, 1985). The rationale of using short DNA oligomers as probes for the detection of oligonucleotide polymorphisms in agricultural species was reviewed and suggested by Beckman (1988).

An approximately 500 bp size fragment digested by Bam HI and Sst I, which identifies polymorphisms, was sequenced using the

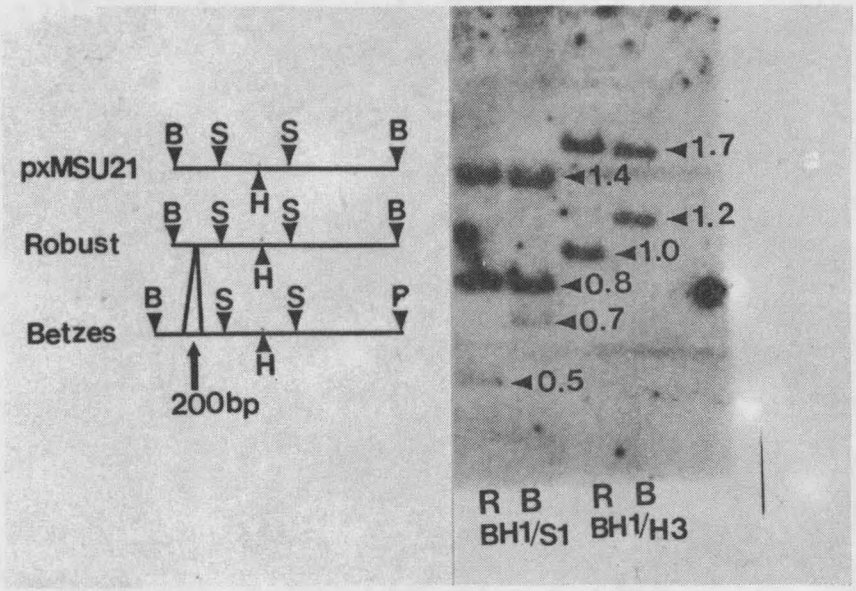


Figure 13. Autoradiograph of double digested genomic blot hybridized with probe pxMSU 21. (Right); All digits indicate kilobase pairs. Arrows indicate polymorphic bands. Letters indicate as following; BH1: Bam HI, S1: Sst I, H3: Hind III, R: Robust, B: Betzes. (Left); Betzes allele contains about 200 base pairs (bp) of inserted sequence within the 500 bp fragment. Letters indicate as following; B: Bam HI, S: Sst I, H: Hind III.

dideoxynucleotide chain termination reaction (Sanger et al., 1977) (Figure 13). A total of 474 base pairs including the cloning site were sequenced (Table 6). The sequence was AT rich (70 %) and contained inverted repeat sequences which were eight base pairs and six base pairs long. The locus xMSU 21 probably contains a deletion in the allele in 'Robust' relative to the 'Betzes' allele. To prove this hypothesis, further sequencing in the allelic polymorphic fragment in the Betzes type cultivars is required.

Table 6. Sequence data of 474 bp from sequence gel of Figure 14. Bases underlined exhibit inverted repeat sequences.

GGATCCCATA TTTATGATAT TTTGGTCTTT CATGTACCTA CCTATTATGC GTATCCATAA 60
BamH1
TAGATACATA CCAATTTATC CAAGTACATA ATCAGAAAAC GCAAGACTAA AGATGCCCAT 120
GTTGTTGGAT CAGCCATTTC TAGTTTCATC CTTGTGCCTT GTAACTCAAA ACAGTGTAAA 180
CAGCTTGGAC AACCGCACAA ACTAGCCCGG TGATACCTGC CAGGAGCCCG ATGAGCAGCC 240
AGGGATTGCT AAAGTATTTC TGCCCTAGCC ACACCATCGA TCTCCGAAAA TAAACTTGGC 300
GGATATGGCT CCGGGACCGG GACCGCATGT CTAGCTTCAA GCATGTCTCC GCAGGTAGTT 360
GGTGGCCTTG TTGTTAGGGT TGAACAAGAT CCCTTGCAAG GTCACCGAAG CACTCGGCCA 420
CCTCGTCATT GTTGCGGTGG TTGTGCTTGA TGACACATTC CTCGACAGGA GCTCGAATTC 480
Sst1
GTAATCATGG TCAT 494

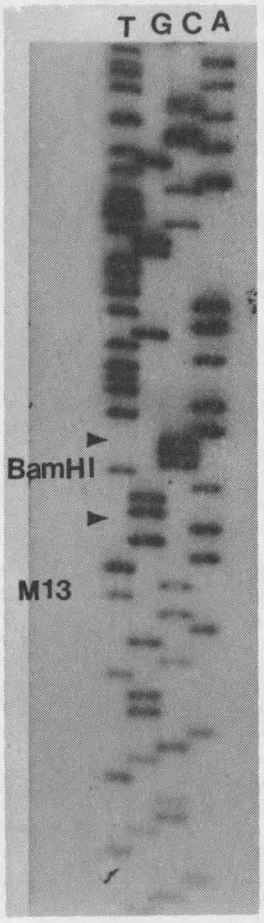


Figure 14. Sequence of Bam HI and Sst I double digested pxMSU 21 fragment that identified a polymorphism. The double digested fragment was cloned into M13mp18 and sequenced using a single-stranded DNA template. Part of sequence gel is shown. Letters indicate as following; A: Adenine, C: Cytosine, G: Guanine, T: Thymine, Bam HI: Bam HI cut sequences which are located at the cloning site.

CHAPTER 5

BARLEY VARIETAL DISCRIMINATION USING RFLP AND HORDEIN MARKERS

Introduction

Estimates of genetic relationships among cultivars provide useful information to solve the related problems of varietal identification, purity and origin. Several approaches have been utilized to provide these estimates. Common methodologies are based on pedigree analysis (Cox et al., 1985; Delannay et al., 1983; Smith and Smith, 1988), quantitative characters (Jain et al, 1975; Martinez et al., 1983; Price et al., 1984), isozyme variation (Linde-Laursen et al., 1987; Nielsen and Johansen, 1986), and polymorphisms in storage proteins (Gebre et al., 1986; Linde-Laursen and Doll, 1982; Shewry et al., 1978). The advantage of biochemical markers over most morphological markers is that molecular markers are generally selectively neutral, often large in number, and may be evaluated at many plant growth stages.

Levels of genetic diversity, geographic structure and environmental correlation with allozyme variation have been studied in natural populations of wild barley, Hordeum spontaneum (Brown et al., 1978; Brown et al., 1980; Nevo et al., 1986). Two concepts of genetic diversity were utilized. First, 'allelic richness', which is the average number of alleles per locus, indicates the number of distinct kinds of alleles encountered in a sample of a particular size. The second component of genetic diversity, 'evenness of allele

frequencies', is related to the distribution of allelic frequencies. 'Gene diversity' (Nei, 1973) is the most common measure of evenness within and between populations. This method is applicable to any population without regard to the number of alleles per locus, the pattern of evolutionary forces such as mutation, selection, and migration, and the reproductive method of the organism used.

In previous chapters, seventeen barley RFLP DNA markers were selected, characterized, and their genomic locations mapped. The probes selected identified different alleles in 'MMS' and 'Apex'. In this chapter, the number of different banding patterns identified using each probe in Southern blot hybridizations to DNA from 21 barley cultivars was estimated. The amount of genetic diversity identified with each probe was then compared to that identified by the seed storage protein loci. The objective of this chapter was to determine the relative utility of RFLP markers in cultivar identification.

Materials and Methods

Plant Materials

Twenty-one barley cultivars grown in North America and Europe for malting and feed were selected. Their parentage, origin, spike row number, and common usage is given in Table 7.

Data Collection

SDS-polyacrylamide gel electrophoresis for hordeins was performed as previously described (Blake et al., 1982). The DNA procedures for Southern blot analysis were the same as described in Chapter 2.

Table 7. Barley cultivars utilized to identify genetic relationships using polymorphisms of hordeins and RFLP DNA markers.

No.	Cutivar	Pedigree	Origin	Row type	Common use
1	Klages	Betzes/Domen	USA	2	Malting
2	Andre	Klages/Zephyr	USA	2	Malting
3	Larker	Traill/UM 570	USA	6	Malting
4	Ingrid	Balder/(Binder/Opal)	SWE	2	Feed
5	Robust	Morex/Manker	USA	6	Malting
6	Bellona	Aramir 2*/Bomi	NET	2	Feed
7	Clark	Hector/Klages	USA	2	Malting
8	Azure	Bonanza/Nordic/NBD130	USA	6	Malting/Feed
9	Piroline	W.M.C.P./Morgenrot	GER	2	Malting/Feed
10	Menuet	L92/Minerva//Emir/3/Zephyr	NET	2	Feed
11	Hazen	Glenn/4/Nordic//Dickson /Trophy/3/Azure	USA	6	Feed
12	Harrington	Klages/S72114	CAN	2	Malting
13	Morex	Cree/Bonanza	USA	6	Malting
14	Compana	from Composite Cross I	USA	2	Feed
15	Columbia		USA	6	Feed
16	Apex	Aramir/4/CB6721/3/Julia 3* /Volla/L100	NET	2	Feed
17	Moravian III	Moravian/Firlbecks III// Moravian/Saxonia	USA	2	Malting
18	Dicktoo	Winter barley field selection	USA	6	Feed
19	Hector	Betzes/Palliser	CAN	2	Feed
20	Summit	HP1203/Zephyr/Tern	ENG	2	Feed
21	Traill	Kindred/Titan	USA	6	Malting

Abbreviation used in origin; USA: United States of America, SWE: Sweden, NET: Netherlands, GER: Germany, CAN: Canada, ENG: England

The products of the Hor-1 and Hor-2 have been well characterized by several authors (Shewry et al., 1978; Marchylo and Laberge, 1981; Gebre et al., 1986; McCausland and Wrigley, 1977). Each B or C hordein variant identified was given a number. The banding patterns identified among the Southern blots of the 21 cultivars evaluated were also each given a number and different banding patterns assumed equivalent to different alleles.

Statistic Analysis

Allelic frequency for each locus and estimates of gene diversity were calculated (Brown and Weir, 1983; Nei, 1973) using following equations;

(1) Allele frequency at a locus:

$$x = \frac{\text{frequency of an allele at a locus}}{\text{total number of tested samples}}$$

(2) Gene diversity:

$$H = 1 - \text{Gene Identity} = 1 - \Sigma x^2$$

A data matrix of scored 'alleles' was utilized to construct a dendrograph using the phylogenetic analysis program, PAUP (Swofford, 1985). This dendrograph provided a graphic representation of the estimated intervarietal genetic distances among these 21 barley cultivars.

Results and Discussion

Polymorphic banding patterns for each of the listed RFLP loci are shown in Figures 15 - 21. Using the data matrix described in Table 8, allele number for each locus (ranging from 2 to 10) and allele frequency at each locus (ranging from 0.05 to 0.95) are presented in Table 9. The average number of alleles per locus which was calculated has the obvious merit of emphasizing one component of diversity, namely allelic richness.

Hordeins showed much more richness of mean alleles (= 9.00) than of polymorphic DNA markers (= 3.571). The high allelic richness of

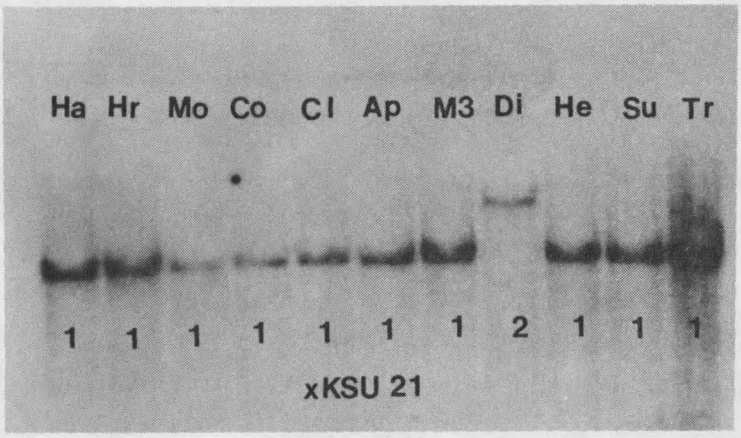


Figure 15. Polymorphic allelic patterns of pxKSU 21. Fifteen micrograms of total barley DNA of 11 cultivars were digested with Eco RV restriction endonuclease, transferred to nylon membrane and hybridized by probe pxKSU 31. Digits indicate cultivars identical to numbers of Table 8. Digits indicate polymorphic alleles identified. Letters indicate as following; Ha: Hazen, Hr: Harrington, Mo: Morex, Co: Compana, Cl: Columbia, Ap: Apex, M3: Moravian III, Di: Dicktoo, He: Hector, Su: Summit, Tr: Traill.

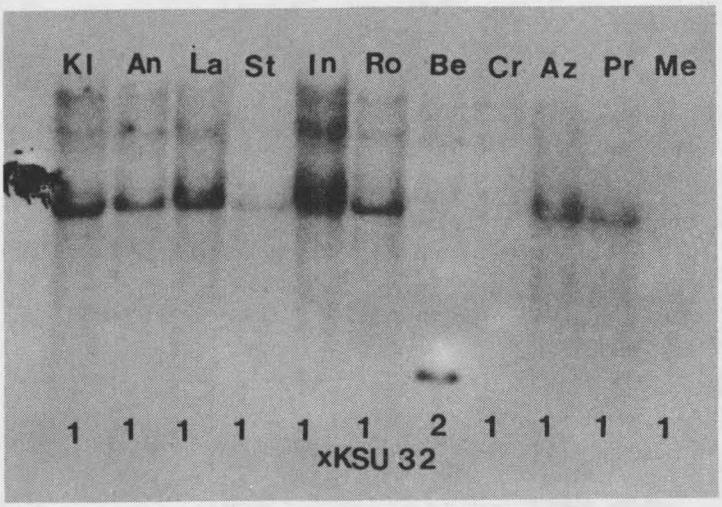


Figure 16. Polymorphic allelic patterns of pxKSU 32. DNA from 11 barley cultivars were digested with Eco RV restriction endonuclease. Digits indicate polymorphic alleles identified. Letters indicate as following; Kl: Klages, An: Andre, La: Larker, St: Steptoe, In: Ingrid, Ro: Robust, Be: Bellona, Cr: Clark, Az: Azure, Pr: Piroline, Me: Menuet.

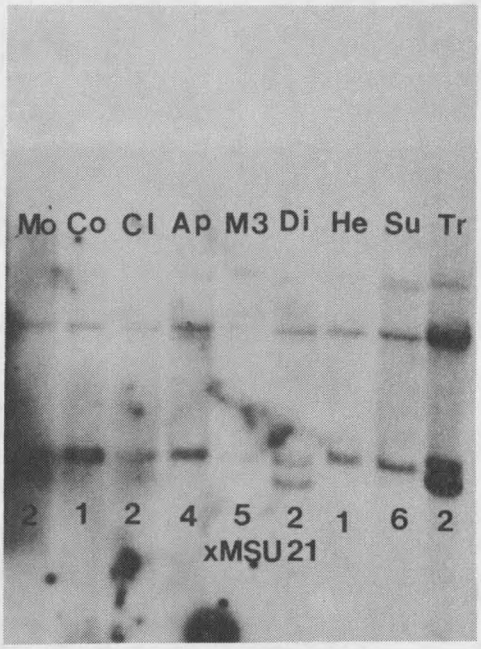


Figure 17. Polymorphic allelic patterns of pxMSU 21. DNA from 9 barley cultivars were digested with Hind III restriction endonuclease.

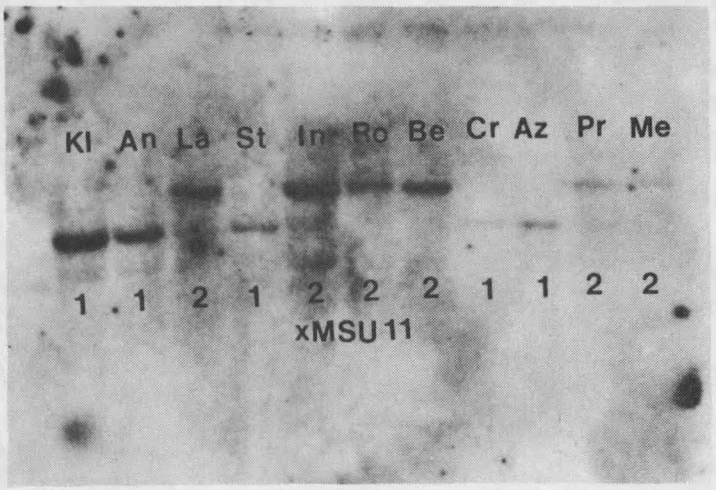


Figure 18. Polymorphic allelic patterns of pxMSU 11. DNA from 11 barley cultivars were digested with Hind III restriction endonuclease.

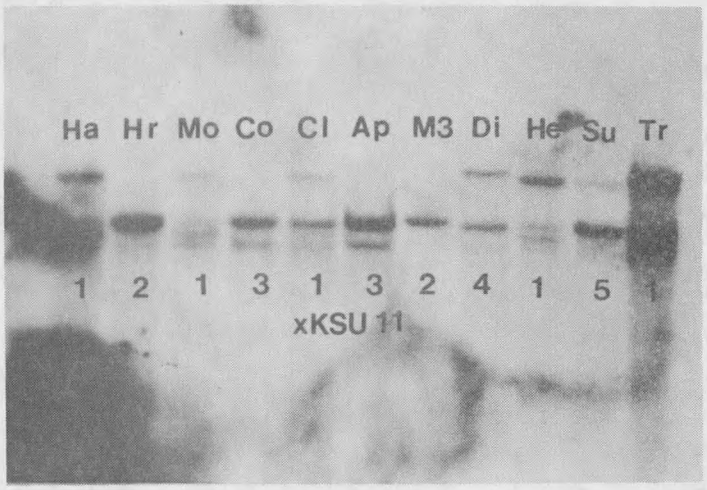


Figure 19. Polymorphic allelic patterns of pxKSU 11. DNA from 11 barley cultivars were digested with Hind III restriction endonuclease.

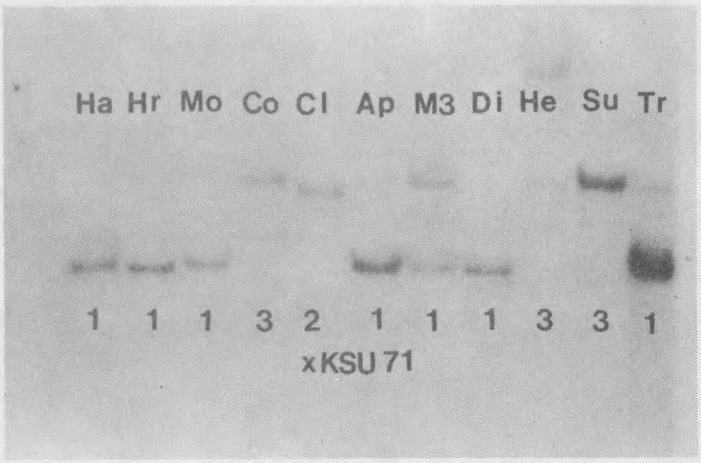


Figure 20. Polymorphic allelic patterns of pxKSU 71. DNA from 11 barley cultivars were digested with Bam HI restriction endonuclease.

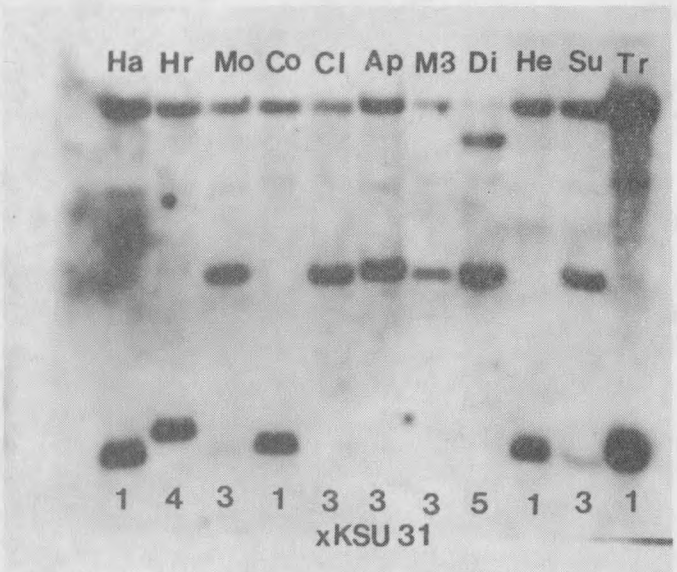


Figure 21. Polymorphic allelic patterns of pxKSU 31. DNA from 11 barley cultivars were digested with Hind III restriction endonuclease.

hordeins is the reason for their common use in varietal identification. The mean allele number of the tested DNA markers was about one-third of the hordeins. As the polymorphic DNA markers are unlimited in number and as the total allele number of the seven DNA markers (=25) was more than that of 2 hordeins (=18), this technology can be utilized to supplement problems in varietal identification insoluble through the sole use of the hordeins.

Table 8. Data matrix of polymorphic alleles among 21 barley cultivars.

Cultivar	Polymorphic loci								
	xKSU 21	xKSU 32	xMSU 21	xMSU 11	xKSU 11	xKSU 71	xKSU 31	Hor 2	Hor 1
Klages	1	1	1	1	1	1	1	1	1
Andre	1	1	1	1	2	1	1	4	1
Larker	1	1	2	2	1	1	1	5	6
Ingrid	1	1	1	2	2	3	2	3	2
Robust	1	1	2	2	1	1	3	5	6
Bellona	1	2	3	2	2	3	1	7	3
Clark	1	1	1	1	1	3	1	1	1
Azure	1	1	2	1	1	1	1	8	6
Piroline	1	1	1	2	3	3	3	9	4
Menuet	1	1	1	2	3	3	1	4	2
Hazen	1	1	2	2	1	1	1	5	6
Harrington	1	1	1	1	2	1	4	3	1
Morex	1	1	2	2	1	1	3	5	6
Compana	1	1	1	2	3	3	1	9	4
Columbia	1	1	2	1	1	2	3	1	8
Apex	1	2	4	2	3	1	3	4	2
Moravian III	1	1	5	2	2	1	3	4	2
Dicktoo	2	1	2	2	4	1	5	10	7
Hector	1	1	1	1	1	3	1	1	4
Summit	1	1	6	1	5	3	3	1	5
Traill	1	1	2	2	1	1	1	6	6

The probabilities of nonidentity of 9 genetic loci were given by the Equation 2 and described in Table 9. The probability of nonidentity, H, is a measure of genetic variation of a population and

usually called heterozygosity. The H value in this varietal identification population implies sufficient gene diversity to discriminate cultivars. The overall mean of gene diversity was 0.554. Average gene diversities from two different methodologies showed that of hordeins is greater than that of DNA markers (0.829 > 0.476). This estimate is well associated with the allele richness. However, the degree of gene differentiation of this population using DNA markers (0.476) was higher than the previously reported average gene diversity across 30 allozyme loci in Hordeum spontaneum populations (0.096) (Nevo et al., 1986).

Table 9. Analysis of allele frequency in a locus and gene diversity among 21 barley cultivars.

Locus	No. of alleles	No. of sample	Allele frequency at a locus	Gene diversity
pxKSU 21	2	21	0.95, 0.05	0.095
pxKSU 32	2	21	0.90, 0.10	0.180
pxMSU 21	6	21	0.43, 0.38, 0.05 x 4	0.660
pxMSU 11	2	21	0.38, 0.62	0.471
pxKSU 11	5	21	0.48, 0.24, 0.19, 0.05 x 2	0.785
pxKSU 71	3	21	0.57, 0.05, 0.38	0.528
pxKSU 31	5	21	0.52, 0.33, 0.05 x 3	0.613
Mean	3.571			0.476
Hor-1	8	21	0.19 x 2, 0.29, 0.14, 0.05 x 4	0.814
Hor-2	10	21	0.24, 0.19 x 2, 0.09 x 2, 0.05 x 4	0.843
Mean	9.000			0.829

Some cultivars undifferentiated by hordeins were well separated using subset of the DNA markers. In the first case, while 'Klages' and

'Clark' both had the same allele types of hordeins, they were differentiated ('Klages' = 1 and 'Clark' = 3) by pxKSU 71. Second, 'Larker', 'Robust', 'Hazen', and 'Morex', all had allele type 5 in Hor-2 and 6 in Hor-1. 'Larker' and 'Hazen' were different from 'Robust' and 'Morex' in pxKSU 31, but 'Larker' and 'Hazen', and 'Robust' and 'Morex' were not further separated from each other. To discriminate between those two, more polymorphic DNA markers should be tested. Third, 'Piroline' and 'Compana' were further discriminated by pxKSU 31. Finally, The polymorphic DNA markers pxKSU 32, pxMSU 21, pxKSU 11, and pxKSU 71, readily discriminated among three cultivars 'Menuet', 'Apex' and 'Moravian III' that showed the identical allelic patterns in hordeins.

The tree of genetic relationships (dendrograph) among these was constructed by genetic similarity and genetic distances using the computer program, PAUP (Figure 22). As in previous papers (Shewry et al., 1978; Gebre et al., 1986), cultivars related by common ancestry tended to cluster together. Varieties were divided into 6 major groups; (1) Western 2-rowed malting and feed barley cultivars derived from Betzes (Klages, Andre, Harrington, Hector, and Clark), (2) European 2-rowed malting and feed cultivars (Ingrid, Bellona, Menuet, Moravian III, Apex, Piroline, and Compana), (3) Western 6-rowed feed barley (Columbia), (4) blue aleurone colored 6-rowed midwestern malting barley (Azure), (5) white aleurone 6-rowed malting cultivars (Robust, Morex, Hazen, Traill, and Larker), (6) old 6-rowed winter barley cultivar (Dicktoo).

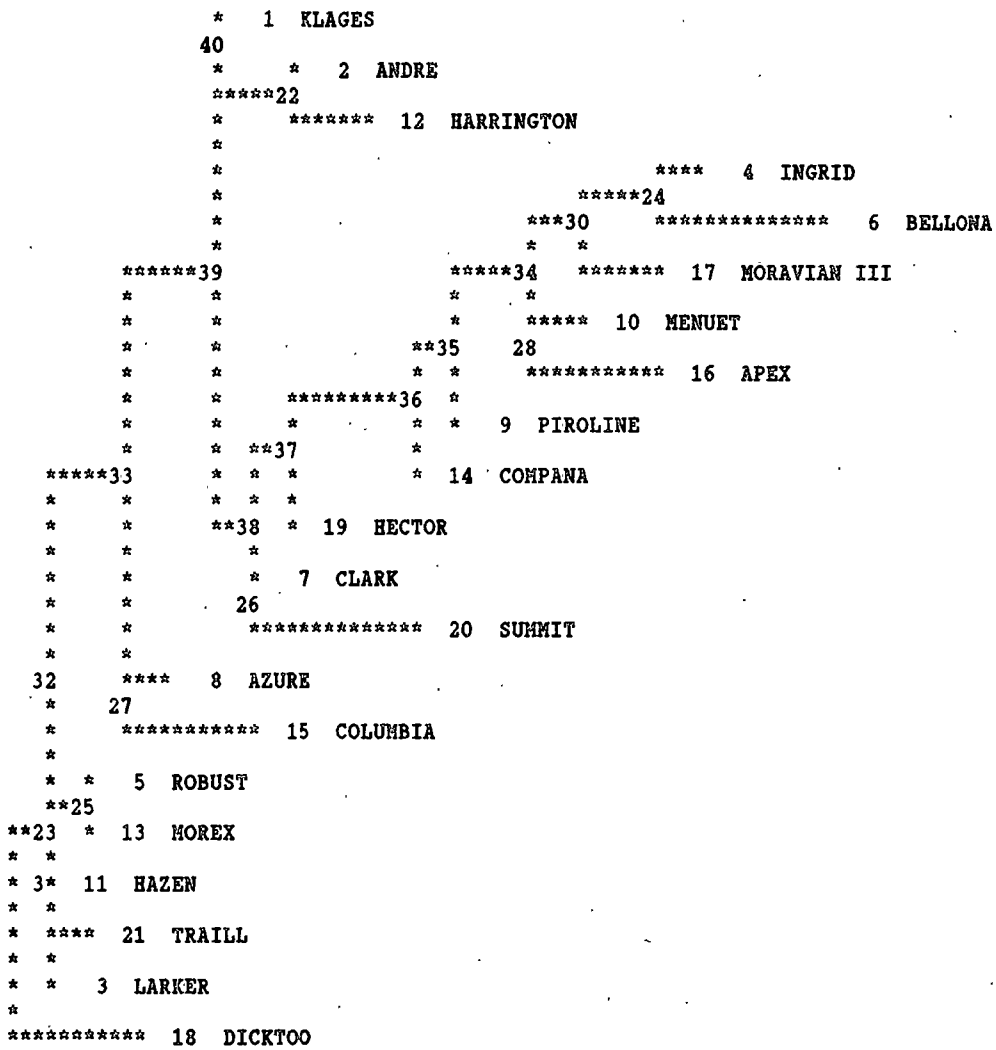


Figure 22. Dendrograph of 21 barley cultivars using the genetic distances given by computer program, PAUP.

Although barley varieties can be divided into groups on the basis of relatively discrete morphology, polymorphic DNA markers and biochemical markers identify variation within groups with similar morphologies and growth habit. DNA markers as well as biochemical markers could be utilized to differentiate barley cultivars in the case of seed mixture or varietal mislabeling.

CHAPTER 6

SUMMARY

This study focussed on the linkage analysis of molecular genetic markers based on restriction fragment length polymorphisms in the barley genome. For this purpose, barley random genomic DNA libraries were constructed using the plasmid vector pBR322 and the phage vector EMBL4. Repeat-free sequences from these libraries were screened against total genomic DNA using Southern blot analysis. Nine genomic clones and seven cDNA clones were identified which produced clear, consistent results in Southern blot analyses of segregating progeny. A multiple recessive marker stock as one parent provided seventeen 'benchmark' loci which had been previously mapped.

Seventeen RFLP markers, which utilized ten morphological markers, five isozyme loci and two hordeins as reference points in map construction, were located in barley linkage groups with the exceptions of chromosomes 4 and 6. The probes and markers utilized in this work span 680 recombination units of the barley genome, approximately 50 percent of its estimated recombinational length.

Physical maps of fifteen out of seventeen polymorphic DNA markers were constructed in detail using several restriction endonucleases. Twelve DNA clones were well differentiated using one or several restriction endonucleases. Three cDNA clones had no restriction site in 13 to 14 different restriction endonucleases, but had characteristic fragment sizes ranging from 500 to 650 base pairs. A total of 474 base

pairs of the polymorphic region of pxMSU 21 were also characterized by sequence analysis.

Seven polymorphic DNA markers and hordein traits were characterized for estimation of genetic diversity and allele frequency among 21 barley cultivars. Some cultivars undifferentiated by hordeins were well separated using a subset of the DNA markers. This technology could be utilized to supplement problems in varietal identification insoluble through the sole use of the hordeins.

The release of new informative RFLP markers with detailed restriction maps and the description of genotypes at thirty-four loci in 100 mapping lines provides two sets of tools which will simplify the mapping of additional RFLP loci in barley. We believe that this will provide the starting point for tracking agriculturally important quantitative trait loci in barley breeding programs.

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