



Characterization of diversity among members of multigene families in the barley genome
by Vladimir Kanazin

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Crop and Soil Science
Montana State University
© Copyright by Vladimir Kanazin (1993)

Abstract:

A substantial portion of the genome in higher plants consists of multigene families. Members of multigene families often exhibit great variability in structure and composition within as well as among genotypes and species. Analysis of their sequence and organization could illuminate the mechanisms involved in genome evolution.

Analysis of the structural organization of the B-hordein genes revealed the presence of a variable region within the structural portion of the Hor-2 gene. This domain appears to undergo frequent changes in the number of short tandem repeats due to unequal crossing over or DNA strand slippage during replication. The resulting differences in the length of the gene's repetitive domain could account for much of the variation observed on the protein level.

Nontranscribed spacers separating 5S rRNA genes were isolated and characterized from several species of the *Hordeum* genus. Cultivated barley (*H. vulgare*) had three clusters of 5S rRNA genes located on different chromosomes. Structural differences between spacers from different gene clusters allowed the isolation of gene-specific probes and served as convenient chromosome-specific markers. The distribution and copy number of 5S genes between clusters on chromosomes 2 and 3 varied widely between barley cultivars and in a population of doubled haploid lines derived from a cross between two varieties. However, this variation had small effect on plant phenotype. Sequence comparisons between rapidly diverging 5S spacers from different barley species provided new insight into evolutionary relationships within the genus *Hordeum*. New information was obtained concerning genomic constitution and the possible origin of polyploid barley species.

Histone H3 genes were shown to be present on at least four barley chromosomes. PCR amplification of the histones H3 with primers corresponding to conserved gene sequences permitted isolation of several histone H3 genes containing intervening sequences from wheat and barley genomes. The high level of sequence divergence between introns allowed us to use them as highly-specific probes for targeting individual gene copies.

Ability to characterize individual members of multigene families using sequence diversity between them could help in understanding the varied mechanisms responsible for heritable variation.

CHARACTERIZATION OF DIVERSITY AMONG MEMBERS OF MULTIGENE
FAMILIES IN THE BARLEY GENOME

by

Vladimir Kanazin

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Crop and Soil Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 1993

APPROVAL

of a thesis submitted by

Vladimir Kanazin

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.

Date 1/26/93

James K. Blake
Chairperson, Graduate Committee

Approved for Major Department

21/1/93
Date

Head, Major Department

Approved for College of Graduate Studies

Date 2/5/93

Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute copies of the dissertation in and from microfilm and the right to reproduce and distribute by abstract in any format."

Signature



Date

Jan. 28, 1993

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my major adviser, Dr. Tom Blake, for the opportunity to work in his lab, for his encouragement, guidance and help, without which this work would not have been possible. Special thanks to my advisors from N.I. Vavilov Institute of General Genetics in Moscow, Drs. Evgeny Ananiev and Alexander Kolchinsky, who encouraged and guided me throughout my study.

I would like to thank Drs. Tom McCoy, Luther Talbert, Robert Sharrock and Matt Lavin for serving as members of my graduate committee, and for their help and expert advice on many occasions.

I would like to express my gratitude to all my friends and colleagues from Montana State University and from Institute of General Genetics in Moscow. Special thanks to fellow graduate students Somvong Tragoonrung and Rosa Nieto, to Lynn Pederson and RaeAnn Magyar, and to all work study students in our lab.

I would also like to thank the Quaker Oats Company, Drs. Fran Webster, Gary Yarrow and Samuel Weaver in particular, for funding and support of this research.

TABLE OF CONTENTS

	Page
APPROVAL	ii
STATEMENT OF PERMISSION TO USE	iii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	x
INTRODUCTION	1
Origin and Evolution of the Multigene Families	2
Multigene Families and Phylogenetic Analysis	7
Gene-Specific Probes in Analysis of multigene families	9
VARIABILITY AMONG THE MEMBERS OF THE <i>Hor</i> -2 MULTIGENE FAMILY	11
Introduction	11
Materials and Methods	13
Plant Material	13
Biochemical Techniques	13
Results	15
Discussion	25
THE GENETICS OF 5S rRNA ENCODING MULTIGENE FAMILIES IN BARLEY	29
Introduction	29
Materials and Methods	31
Plant Material	31
Biochemical Techniques	31
Mapping and Analysis of Quantitative Traits	33
Results	33
Sequence and Structure of Two Classes of the 5S Nontranscribed Spacers	33
Mapping of the 5S rDNA Clusters	38
Variation in 5S rDNA copy number	44

TABLE OF CONTENTS - Continued

	Page
Phenotypic effect of the 5S rDNA	
copy number	45
Discussion	48
ANALYSIS OF EVOLUTIONARY RELATIONSHIPS AMONG <i>HORDEUM</i>	
SPECIES USING SEQUENCE POLYMORPHISMS	
IN 5S rDNA SPACERS	51
Introduction	51
Materials and Methods	53
Results	54
Discussion	63
CHARACTERISTICS OF THE HISTONE H3	
MULTIGENE FAMILY IN BARLEY	66
Introduction	66
Materials and Methods	67
Results	68
Cloning and Sequence Analysis of Barley	
and Wheat Histone H3 Genes	68
Mapping the Histone H3 Genes on	
Barley Chromosomes	73
Generation of Gene-Specific Markers	75
Discussion	81
CONCLUSIONS	84
REFERENCES CITED	87
APPENDIX	98

LIST OF TABLES

Table	Page
1. Amount of the 5S rDNA in different classes of doubled haploids	45
2. Description of the wild <i>Hordeum</i> species	60

LIST OF FIGURES

Figure		Page
1.	Schematic structure of the B-hordein gene	16
2.	Polymorphism among barley <i>Hor</i> -2 genes	17
3.	Segregation of the <i>Hor</i> -2 alleles in population of doubled haploid lines.....	19
4.	Sequence polymorphism among cloned B-hordein genes	20
5.	Amplification of the B-hordein gene variable domain from <i>Hordeum vulgare</i> cultivars	23
6.	Amplification of the B-hordein gene variable domain from different <i>Hordeum</i> species	24
7.	Sequence and organization of the 5S rDNA	36
8.	Length variation among 5S nontranscribed spacers	37
9.	Localization of 5S rDNA clusters using wheat- barley chromosome addition lines	39
10.	Mapping of the 5S rDNA clusters	41
11.	Genetic maps of barley chromosomes 2 and 3	42
12.	Amplification of the 5S NTS from wheat, barley, and wheat-barley addition lines	43
13.	5S rDNA copy number in different progeny classes	47
14.	Alignment of the consensus nucleotide sequences of the 5S NTS from <i>H. chilense</i> , <i>H. magellanicum</i>	55
15.	Cladistic analysis of evolutionary relationships in the genus <i>Hordeum</i>	58

LIST OF FIGURES - Continued

Figure	Page
16. Dot-matrix comparison of the 5S rDNA NTS sequences from <i>H. chilense</i> and <i>H. stenostachys</i>	58
17. Dot-blot hybridization of 5S NTS amplified from different <i>Hordeum</i> species	61
18. Alignment of the nucleotide sequences of isolated H3 histone genes	70
19. Nucleotide sequences of the introns from cloned wheat and barley histone H3 genes	72
20. Southern hybridization of barley and wheat genomic DNA with cloned H3 and H4 histone genes	74
21. Hybridization of the coding sequence of the clone B2, and the B2 introne with <i>EcoRI</i> digested DNA of Steptoe x Morex doubled haploid lines	76
22. Hybridization of the coding sequence of the clone B3, and the B3 introne with <i>EcoRI</i> digested DNA of Steptoe x Morex doubled haploid lines	77
23. PCR amplification of the introns from the histone H3 genes from several varieties of barley	79
24. PCR amplification of histone H3 introns in population of Steptoe x Morex DHL	80

ABSTRACT

A substantial portion of the genome in higher plants consists of multigene families. Members of multigene families often exhibit great variability in structure and composition within as well as among genotypes and species. Analysis of their sequence and organization could illuminate the mechanisms involved in genome evolution.

Analysis of the structural organization of the B-hordein genes revealed the presence of a variable region within the structural portion of the *Hor-2* gene. This domain appears to undergo frequent changes in the number of short tandem repeats due to unequal crossing over or DNA strand slippage during replication. The resulting differences in the length of the gene's repetitive domain could account for much of the variation observed on the protein level.

Nontranscribed spacers separating 5S rRNA genes were isolated and characterized from several species of the *Hordeum* genus. Cultivated barley (*H. vulgare*) had three clusters of 5S rRNA genes located on different chromosomes. Structural differences between spacers from different gene clusters allowed the isolation of gene-specific probes and served as convenient chromosome-specific markers. The distribution and copy number of 5S genes between clusters on chromosomes 2 and 3 varied widely between barley cultivars and in a population of doubled haploid lines derived from a cross between two varieties. However, this variation had small effect on plant phenotype. Sequence comparisons between rapidly diverging 5S spacers from different barley species provided new insight into evolutionary relationships within the genus *Hordeum*. New information was obtained concerning genomic constitution and the possible origin of polyploid barley species.

Histone H3 genes were shown to be present on at least four barley chromosomes. PCR amplification of the histones H3 with primers corresponding to conserved gene sequences permitted isolation of several histone H3 genes containing intervening sequences from wheat and barley genomes. The high level of sequence divergence between introns allowed us to use them as highly-specific probes for targeting individual gene copies.

Ability to characterize individual members of multigene families using sequence diversity between them could help in understanding the varied mechanisms responsible for heritable variation.

INTRODUCTION

Many of the cDNA and genomic clones isolated from cereals hybridize to multiple bands on Southern blots. This observation suggests that at the least, portions of many or most genic sequences are represented in plant genomes by more than a single copy. Some of these multigene families encode RNAs or proteins which require high levels of expression. Others encode proteins responsible for varied tasks during the life of the cell or organism. Some examples of the former group include the ribosomal RNAs and the seed storage proteins. A few examples of the latter group include the histones and many of the genes regulating plant response to environment. Individual members of the multigene families in eukaryotes may be dispersed throughout the genome, clustered at one locus, or organized into mixed arrays of clusterized and single-copy sequences. The wealth and versatility of information obtainable from analysis of multigene families makes their study an attractive tool for understanding the mechanisms of eukaryotic genome function and evolution. Experiments described in this thesis involve analysis of the B-hordein, 5S ribosomal RNA and histone H3 multigene families in barley (*Hordeum vulgare*, L.).

Origin and Evolution of the Multigene Families

Analysis of variation among members of gene families may provide insight into the mechanisms underlying variability in their origin. Patterns of sequence conservation may help identify the location of functionally or structurally important gene elements, while nucleotide sequence divergence could help identify those domains which provide variation in gene expression or function. The Neo-Darwinian synthesis of the late 1930's, coupled with mutagenesis research of the 1950's, provided a simple rationale for the generation of heritable variation through point mutation (Mayr, 1982). More recently, study of multigene families suggests that forces other than selection of favorable point mutations might be important in the evolution of structural gene organization and function.

The existence of multigene families suggests that sequence duplication may be important in genome evolution. Pichersky (1990) summarized several models of DNA duplication in plants, arguing that tandem duplication due to unequal crossing over, and DNA translocations are major mechanisms. He also proposed a model which provides a molecular mechanism for an initial duplication of DNA fragments in plant cells.

Duplication of sequences at genomic sites distal to the original location has been observed in several multigene families. In maize (*Zea mays*), histone H3 genes have been

mapped on most of the maize chromosomes (Chaubet et al., 1992). Several independent loci have also been identified in barley (Kanazin et al., *manuscript in preparation*).

Following an initial sequence duplication event, unequal crossing over can result in sequence loss or gain. This has occurred in several eukaryotic gene families, including the seed storage protein genes in barley, maize and wheat (*Triticum aestivum* L.), and the ribosomal RNA genes (rDNA) in barley and maize (Rivin et al., 1986; Kanazin et al., *in review*).

Unequal crossing over and gene conversion are major driving forces in the concerted evolution of multigene families. Unequal crossing over, besides changing copy number of the repetitive sequences, can accelerate rates of divergence or create sequence homogenization. Either outcome leads to fixation of a particular sequence with a probability based on the frequency of this allele (Hillis et al. 1991).

The actin genes provide an interesting example of the origin and evolution of an important multigene family. These small multigene families feature a high degree of sequence divergence between and within genotypes, as well as a conserved intron position. Many animal genomes contain as many as several hundred actin-derived sequences, most of which are intronless pseudogenes. Both angiosperms and gymnosperms carry actin genes with a single intron. This suggests that a single ancestral gene was duplicated before the separation of

gymnosperms and angiosperms (McElroy et al. 1990). Actin genes were isolated and characterized in several plant species. Soybeans (*Glycine max*), maize, rice (*Oryza sativa*), *Arabidopsis thaliana* and potato (*Solanum tuberosum*) contain 2-10 copies of actin genes (Baird and Meagher, 1987; McElroy et al. 1990). In contrast to the relatively simple organization of actin multigene families in these species, *Petunia* actin genes are organized into several subfamilies of 16-30 members, which form a complex superfamily consisting of over 200 genes. Duplication of these genes by unequal crossing over is the most likely mechanism of this family amplification (Baird and Meagher, 1987).

Although sequence conversion events might play an important role in the maintenance of sequence homogeneity, members of multigene families do not necessarily diverge within a species at rates comparable to that of single copy loci. Gene conversion is often viewed as an unbiased process, which can propagate or reverse a mutation with equal frequency (Hickey et al, 1991). However, there are known examples of selective propagation of the mutant phenotype. Hillis et al. (1991) demonstrated that in different lineages of parthenogenic lizards, biased gene conversion plays an important role in the evolution of ribosomal DNA. Biased gene conversion which favors one genotype supports the hypothesis of a "molecular drive" (Dover, 1982), which can rapidly spread mutations in repetitive gene families.

Although gene conversion promotes concerted evolution of a gene family within one locus, it does not necessarily produce homogenization of the noncoding gene sequences, nor can it promote homogenization of multigene family members at distant loci. Reddy and Appels (1989) demonstrated the presence of two independent lineages of 5S rDNA units located on different chromosomes in wheat. These two classes of 5S rDNA differed in levels of sequence polymorphism and fixation rate of nucleotide changes. This difference can be explained by a model of unequal crossing over and base pair change accumulation, leading to quantitative variation in cluster size and an homogenized array.

The major urinary protein (MUP) genes of the mouse show both duplication of intact and presumably active genes as well as pseudogenes. Ghazal et al. (1985) suggested that unequal crossing over and gene conversion could have resulted in the development of these two families of sequences while maintaining sequence similarity within families. The active copies and MUP pseudogenes are found in two separate subfamilies and organized into tandem pairs of sequence repeats. Substantial sequence variation differentiates the two subfamilies, while within each subfamily sequence similarity is very high. This suggests that either the two subfamilies derived from a single copy of the primary gene and a single copy of the pseudogene and underwent recent duplication, or that gene conversion resulted in the

maintenance of sequence similarity between members of the same gene cluster. Subfamilies of maize storage proteins, the zeins, show similar patterns of variation between different loci and conservation between closely linked genes (Heidecker et al., 1991).

The coding sequence of the small subunit of ribulose biphosphate carboxylase (*rubisco* or *rbcS*) is highly conserved throughout the plant kingdom. Analysis of adjacent and distant members of this multigene family in tomato (*Lycopersicum esculentum*) was performed by Sugita et al. (1987). The three tandem-repeated copies at the *rbcS3* locus shared approximately 98% sequence identity. Sequence similarity with the unlinked *rbcS1* and *rbcS2* loci was close to 90%. However, flanking regions surrounding all five copies showed no significant similarity among any of the five copies analyzed. This indicates that recent gene duplication is unlikely, since it would have had to involve only coding regions of the gene. Sequence similarity between linked gene copies may not be a good indicator of when the duplication event occurred, but sequence divergence among unlinked members may be more informative. The genes encoding the small subunit of *rubisco* contain introns, *Phaseolus vulgaris* and *Glycine max* (soybeans) genes contained two introns, wheat genes have one. Seven out of eight sequenced *Petunia* genes contained 2 introns, and one gene copy had three introns. In all species these introns were located at the same positions as in *petunia* (Dean et al.

1987). The length and sequence of these introns varied greatly between, as well as within genotypes. The conservation of the position of these introns in different species indicates their presence in an ancestral gene, which was duplicated and subsequently diverged. Their sequence diversity, however, indicates that while their position may have selective importance, their nucleotide composition does not.

Through comparison of noncoding portions of genes (e.g. introns and flanking sequences) one might be able to distinguish between recent gene duplications and ancient events. Comparison of two closely linked α -amylase genes in two species of *Drosophila* revealed conservation of gene sequences within a species and divergence between the species. Also, sequence divergence was much greater in flanking regions than in protein-coding regions. Thus, duplication of the sequence occurred early and was accompanied by the concerted evolution of the gene pairs (Hickey et al, 1991).

In barley and wheat, the coding regions of the 5S rRNA loci appear to be remarkably uniform. However, the non-transcribed spacers, separating individual coding sequences, differ between loci within genotypes. They also vary greatly between species. Copy number variation is also observed among genotypes, suggesting unequal crossing over.

Multigene Families and Phylogenetic Analysis

The accumulation of tandem, highly repetitive sequences

in the genomes of eukaryotes might reflect their role in the process of species differentiation. While heterochromatin has long been thought to be composed largely of repeated sequences, Fitch et al. (1990) provided several examples of high copy number, tandem repeats which were found within heterochromatic regions of chromosomes. These may comprise regions of high frequency recombination which may result in unequal crossing over and sequence amplification.

Phylogenetic relationships could be inferred from sequence analysis of multigene family members. These sequences are often located at multiple loci and mark specific chromosomes. Dvorak et al. (1988) used differences in repetitive sequence abundance to establish sources of A genomes in polyploid wheats. This approach might be flawed because of rapid changes in copy number among tandem repeats. (Jeffreys et al. 1988; Kanazin and Blake, *in review*).

Reddy and Appels (1989) constructed phenetic and cladistic trees in the genus *Secale* (rye) based on nucleotide sequence comparison between 5S rRNA genes in different species. Scoles et al. (1988) based their cladistic analysis on sequence data from a 75bp "conserved" region of the 5S rDNA spacer from 38 clones. Variation for locus number and spacer length of the spacers, along with restriction maps of the 5S rDNA in wheat were used in phylogenetic analysis of diploid *Triticum* species and in reconstruction of the evolutionary origin of polyploid wheats (Dvorak et al., 1989).

Estimation of the time of divergence between species should be done with caution. Gene flow and occasional recombination events between lineages and evolutionary constraints on different sequence types may give anomalous apparent rates of sequence divergence (Barbier et al, 1991).

Gene-Specific Probes in Analysis of Multigene Families.

Each type of multigene family presents unique problems and opportunities for structural and functional analysis. Establishing a relationship between the structure of a gene and its function demands that the gene be unambiguously distinguished from other related genes.

Analysis of the soybean *rbcS* gene family, using gene-specific probes from the most variable part of the sequence, demonstrated that expression of eight genes varied dramatically between leaves and other plant organs (Dean et al., 1985). Similar observations of differential expression of individual *RbcS* genes in various organs and stages of development and differentiation were made in tomatoes (Sugita et al. 1987) and in *Lemna gibba* (Silverthorne et al. 1990). Gene-specific probes were also used to show differential expression in soybean chalcon synthase (*CHS*) genes in response to environmental stimuli (Ryder et al. 1987, Windenger et al. 1989). Besides exhibiting common developmental and spatial mechanisms of gene regulation, these experiments show that polymorphism between multigene family members is responsible

for differential gene response within the same organ to different stimuli.

The above examples illustrate that the duplication of a gene and differentiation of its regulatory mechanisms allow plants to have a set of genes encoding closely related polypeptides with similar properties, but utilized in different regulatory networks.

The objective of this thesis was to characterize individual members of the B-hordein, histone H3 and 5S rRNA multigene families in barley. Additionally approaches were developed and tested to analyze individual members of multigene families.

VARIABILITY AMONG THE MEMBERS OF THE *Hor-2* MULTIGENE FAMILYIntroduction

The hordeins have been the subject of study since Osborne (1924) developed an experimental approach which permitted their isolation. Oram et al. (1975) provided initial evidence that the hordein proteins could be grouped into families which appeared to be encoded by specific genes. Shewry et al. (1978) and Doll et al. (1981) developed reliable analytical techniques for their evaluation. The *Hor* loci contain enormous allelic richness and have provided an excellent basis for varietal identification (Faulks et al. 1981), cladistic analysis and the study of population dynamics (Alexander et al. 1990).

The B-hordeins are a group of sulphur rich barley storage proteins which account for about 80% of the grain prolamins (Shewry et al. 1990). They are encoded by the complex *Hor-2* locus which is located on the short arm of chromosomes 5 in barley (homoeologous chromosome 1H) (Oram et al. 1975). It was estimated that this locus contains approximately 15-30 gene copies. The structural genes encode polypeptides ranging in size from 36 to 44 kDa (Bunce et al. 1986; Kreis et al. 1985; Shewry and Tatham 1990). The B-hordeins are enormously variable when analyzed by SDS-PAGE. Cyanogen bromide mapping of the polypeptides (Faulks et al. 1981) suggested that the B-

hordeins could be grouped into three distinctive subfamilies of proteins (BI, BII, BIII). *In vitro* translation experiments of poly A⁺ mRNA selected by cDNA-mRNA hybridization showed that this diversity is not due to posttranslational modification of polypeptides. At least two subfamilies of independently regulated genes produce mRNA encoding these proteins (Kreis et al. 1983a).

Several genes and cDNAs encoding B-hordeins have been cloned and sequenced (Brandt et al. 1985; Chernyshev et al. 1989; Forde et al. 1981, 1985a, 1985b; Vicente-Carbajosa et al. 1992). However, despite enormous protein variability, the structure of these genes is very similar. Like most other cereal storage protein genes, they are not interrupted by introns. The amino-terminal domain of the gene is proline-rich and consists of short (9-21 bp) imperfect tandem repeats organized into larger repetitive units. The carboxy-terminus is proline-poor, not repetitive and contains most of the charged amino-acids in the protein (Shewry and Tatham 1990).

In this report we utilized the polymerase chain reaction (PCR) to study the organization and variability among members of this multigene family. We attempted to explain how this variation resulted in the production of proteins which vary enormously in molecular weight but have very similar composition.

Materials and Methods

Plant Material

Several varieties and experimental lines of cultivated barley, several wild *Hordeum* species, and two populations of doubled haploids developed by the North American Barley Genome Mapping Project were used (Kleinhofs et al., *in press*). The parental pairs were Apex and MMS (Shin et al. 1990; Schon et al. 1990) and Morex and Steptoe (Kleinhofs et al., *in press*). Doubled haploids were produced using the *Hordeum bulbosum* technique (Kasha and Kao, 1970) by Dr. Ken Kasha, University of Guelph, and Dr. Patrick Hayes, Oregon State University.

Biochemical Techniques

Hordeins were extracted and separated by SDS-PAGE according to the method of Doll and Andersen (1981). DNA was extracted from foliar tissue by the method of Dellaporta et al. (1983).

The primer sequences utilized in this project were selected from sequence data published by Forde et al. (1985a) and Chernyshev et al. (1989). Sequence analysis was performed on an IBM PC using Genepro^R (Riverside Scientific) and the Genbank database. Synthesis of primers was performed on a PCR-Mate 391EP DNA synthesizer (Applied Biosystems) using phosphoroamidite chemistry.

Polymerase chain reaction sequence amplification from

genomic DNA was carried out in 100 μ l volumes utilizing PCR reaction buffer (Perkin-Elmer), 100mM each of dNTPs, 0.2 μ g of each primer, 50ng of barley genomic DNA as a template and 2.5 units of *Taq*-polymerase (Perkin-Elmer). After incubation for 4 minutes at 94°C, 30 cycles of amplification were performed (45 seconds at 94°C and 15 seconds at 60°C with primers KV1-KV2; 1 min at 94°C, 1 min at 55°C and 2 min at 72°C with primers TB38-TB39).

For Southern blot analysis PCR products were separated in 6% polyacrylamide gels and transferred onto Hybond-N+ membranes (Amersham). Hybridization was carried out in standard conditions (Maniatis et al. 1989) using ³²P labeled probe produced by random priming (Feinberg and Vogelstein, 1983). Filters were washed in a series of wash solutions at 62°C for fifteen minutes each, terminating in a final wash consisting of 0.05xSSC, 0.1%SDS.

For cloning amplified fragments were purified using the "Prep-A-Gene" DNA purification kit (BioRad). After phosphorylation and repair of the ends with Klenow fragment they were ligated into the *Sma*I site of pUC19 and used for transformation of host strain *Escherichia coli* XL-1 Blue (Stratagene). Inserts from recombinant plasmids were amplified using primers for the B-hordein gene. For analysis of restriction fragment size polymorphism, products of amplification were digested with *Taq*I and *Rsa*I restriction endonucleases (Promega) according to the manufacturers

instructions, and fractionated on 6% polyacrylamide gels. For partial restriction map construction size of the resulting fragments was compared with expected size determined from the known sequences (Forde et al. 1985a; Chernyshev et al. 1989).

Results

The entire coding sequence of the B-hordein genes as well as flanking sequences were amplified using primers TB38 (5'-CAGTCTAGTCTAGAAGAACAG-3') and TB39 (5'-CGGTTGGCAGAGTGGAGAC-3') (Fig. 1). A 2.3 kb PCR amplification product was obtained from all tested cultivars of *Hordeum vulgare*. The uniformity of the PCR products from barley cultivars suggested that, perhaps, one single gene from the B-hordein family was amplified. However, digestion of this product with frequently cutting restriction endonucleases resulted in a production of larger number of fragments than expected from amplification of a single gene. Comparison of their size with known restriction maps indicated that the 2.3 kb fragment was heterogenous, the result of amplification from several copies of the B-hordein gene.

Digestion of the 2.3 kb fragment amplified from several cultivars with frequently cutting endonucleases revealed substantial intervarietal polymorphism. Five distinctive patterns were seen after analysis of ten barley cultivars (Fig.2).

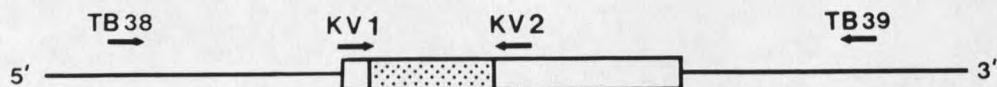


Figure 1. Schematic structure of the B-hordein gene showing location of the PCR primers. Lines represent 5' and 3' untranslated regions of the gene, the rectangle represents coding sequence, and the repetitive domain is indicated by dots.

According to Faulks et al. (1981), and Kreis et al. (1983a), the *Hor-2* locus consists of at least two gene subfamilies which produce proteins with different cyanogen bromide cleavage patterns. Although recombination within the locus is extremely rare, Shewry et al. (1990) isolated from a cross Bomi x P12/3 one recombinant line which was homozygous for a recombination event between the class I/II and class III genes. The recombinant line was shown to carry the class I/II subfamily allele from Bomi and the class III allele from line P12/3. Comparison of the *RsaI* digest of the 2.3 kb amplification product from this recombinant line (line 134/31 was a generous gift of Dr. Peter Shewry) and its parents (Bomi and P12/3) clearly indicates that the 2.3 kb fragment is identical in structure to the Bomi allele (Fig. 2). This suggests that the 2.3 kb amplification product derives from the class I/II gene subfamily.

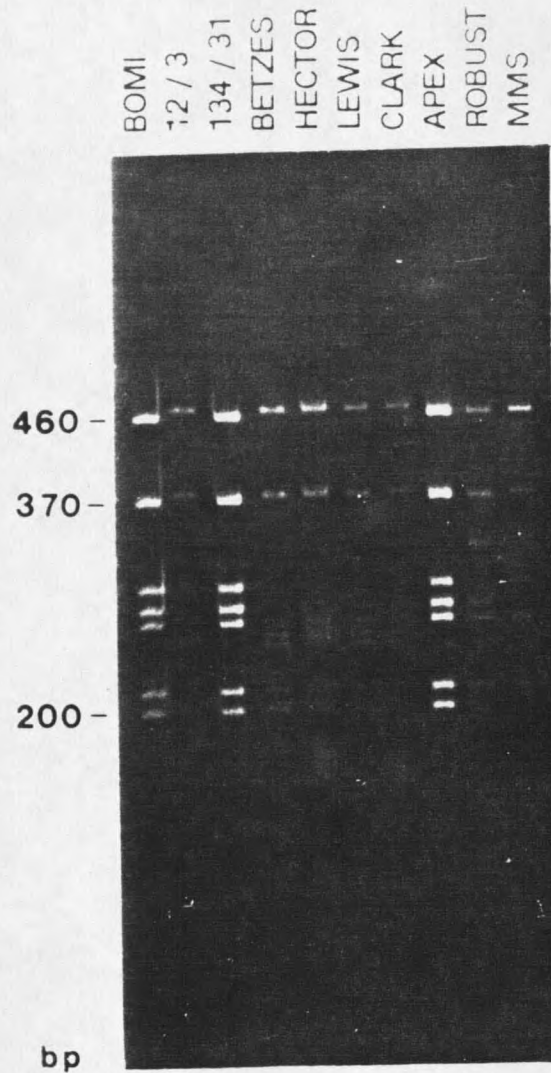


Figure 2. Polymorphism among barley *Hor-2* genes.
2.3 kb fragments amplified from different *H. vulgare* cultivars using primers TB38 and TB39, digested with *RsaI* and separated in polyacrylamide gel.

The banding patterns which were obtained after digestion showed high reproducibility from amplification to amplification. Segregation of RFLP patterns obtained by digestion of PCR products was compared with segregation of hordeins extracted from the grains and separated by SDS-PAGE. 42 doubled haploid lines (DHL) from cross Apex x MMS and 28 DHL from cross Steptoe x Morex were tested. Results of segregation analysis by both techniques were identical. An example of such analysis is shown on Fig. 3.

We cloned and characterized ten 2.3 kb fragments amplified from cv.Apex. Restriction mapping of the inserts from these ten independent clones with *TaqI* and *RsaI* endonucleases revealed several unique variants of the gene. Three out of ten analyzed clones had the same structure as "prototype" B-hordein sequence published by Forde (1985a), and four clones were missing a *TaqI* site within the coding region. Three more clones had restriction maps which differed from the prototype structure by one or two restriction sites (Fig. 4).

Following cloning, instability ("shrinking" in size) of some inserts was observed during their propagation in a *recA*⁻ strain of *E.coli* (XL-1 Blue). Restriction analysis of these variants identified deletions within the repetitive domain. This observation prompted us to analyze variability that might occur in this region of the gene.

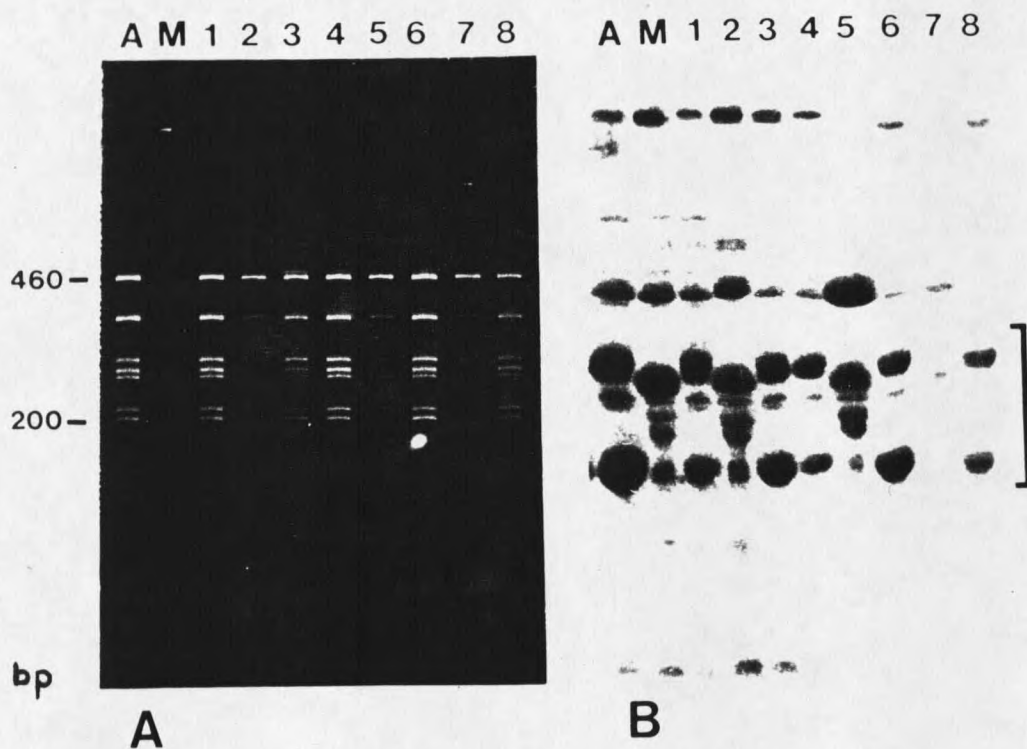


Figure 3. Segregation of the *Hor-2* alleles in population of doubled haploid lines

A: Polyacrylamide gel electrophoresis of *RsaI* digested B-hordein genes amplified from Apex (A), MMS (M) and eight doubled haploid lines from this cross (1-8).

B: SDS-PAGE of hordeins extracted from grains of Apex, MMS and doubled haploid lines from this cross. The bracket at right indicates B-hordeins. DHL 1, 3, 4, 6, 8 have the Apex allele, DHL 2, 5, 7 have the MMS type allele.

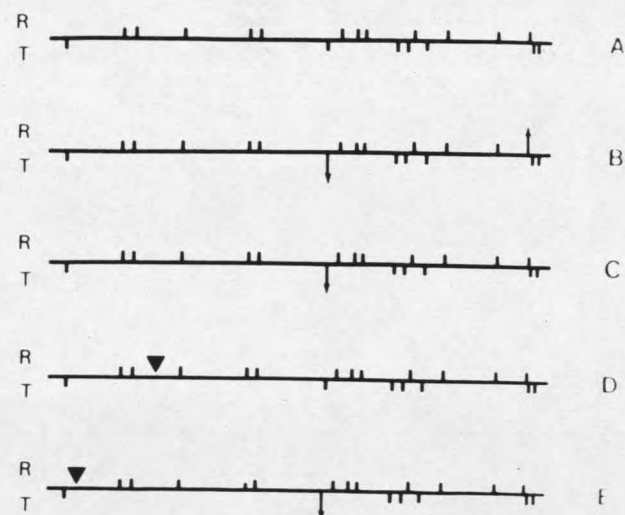
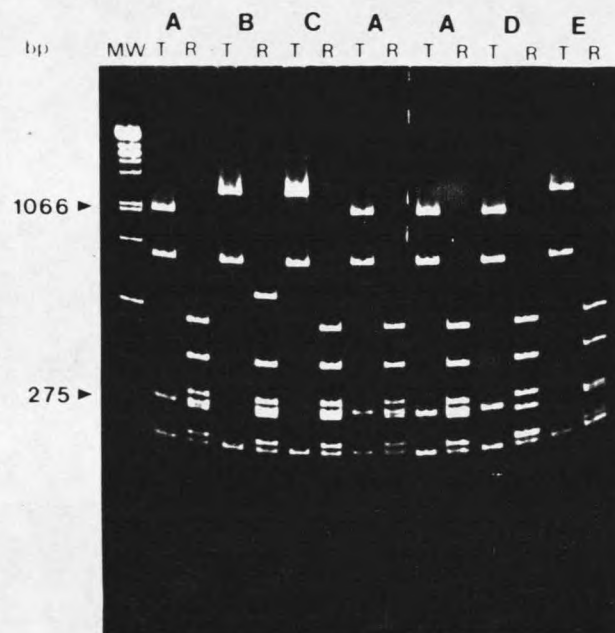


Figure 4. Left panel: *TaqI* (T) and *RsaI* (R) digests of different cloned variants of the B-hordein gene (A-E). Right panel: restriction maps of "prototype" B-hordein clone (A), and variants of it (B-E). Black triangles indicate new sites, arrows indicate lost sites.

Two primers were produced which flank the B hordein repetitive domain: KV1 (5'-CCACCATGAAGACCTTCCTC-3'), and KV2 (5'-ACCTTGCATGGGTTTAGCTG-3') (Fig. 1). Following amplification, the PCR products were resolved on polyacrylamide gels. This permitted the identification of multiple (up to 8 fragments) length variants in a single genotype ranging from 400 bp to 660 bp in size. Southern hybridization with ³²P labelled B-hordein probe showed homology between amplified fragments and the B-hordein gene (Fig. 5). These electrophoretic patterns were identical in cultivars of common origin (e.g. cv. Betzes, Hector, Bowman, Lewis, Clark), but distinctively different in unrelated genotypes.

The 540 bp long amplification product was found in amplifications from all tested *H. vulgare* cultivars. Other bands were specific for particular genotypes. The most extensive variability was observed within the 440-540 bp. range, though amplification products outside these limits were also observed (660 bp. in Cyclone, Dicktoo, Harrington and Kimberly). Attempts to clone and sequence the 540 bp fragment failed because of its instability in *E. coli*. Direct sequencing of PCR products also was not successful.

Comparison of amplification patterns in cv. Carlsberg II and isolated from this genotype cv. Riso 56 shows that latter lacks all but one of the bands presented in its parent. Also, the product produced in Riso 56 is one of the minor products from Carlsberg II. According to Kreis et al. (1983b) a major

deletion event occurred in the B-hordein locus in Riso 56, and as a consequence it fails to produce all major B-hordein polypeptides. Our observation supports Kreis' conclusion that Riso 56 is indeed a deletion mutant. If we accept this conclusion, then the products which are amplified with primers KV1 and KV2 from Carlsberg II and are missing from Riso 56 are produced from members of the B-hordein gene family.

Amplification of the repetitive domain from the B-hordein genes from wild *Hordeum* species resulted in unique banding patterns from each species (Fig. 6). Amplification patterns of four accessions of *Hordeum spontaneum*, the immediate ancestor of cultivated barley, were very similar to patterns obtained from *H. vulgare* DNA. The length of fragments amplified from more distant wild species with the exception of *Hordeum bulbosum* was significantly shorter than in *H. vulgare* (280-360 bp vs. 400-660 bp). The products of amplification from *H. bulbosum* spanned the ranges of sizes from both wild relatives and *H. vulgare*. Minor bands of high molecular weight were not reproducible and probably represent multimers formed during amplification. Attempts to amplify full-length B-hordein genes with primers TB38 and TB39 complementary to the noncoding gene flanking sequences from wild *Hordeum* species failed.

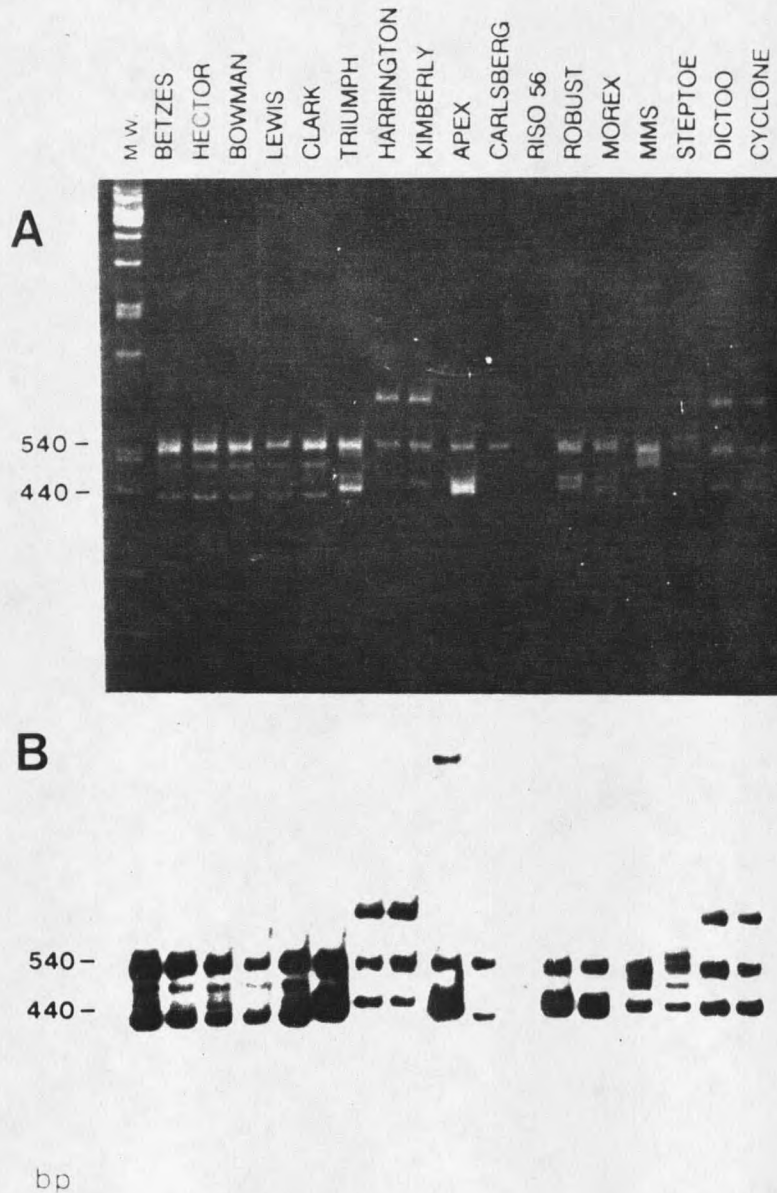


Figure 5. Amplification of the B-hordein gene variable domain from *H. vulgare* cultivars.

A: Polyacrylamide gel electrophoresis of PCR products amplified with primers KV1 & KV2 from different *H. vulgare* cultivars.

B: Autoradiograph of a Southern blot corresponding to the gel on the panel A. Filter was hybridized with ^{32}P -labelled B-hordein probe.

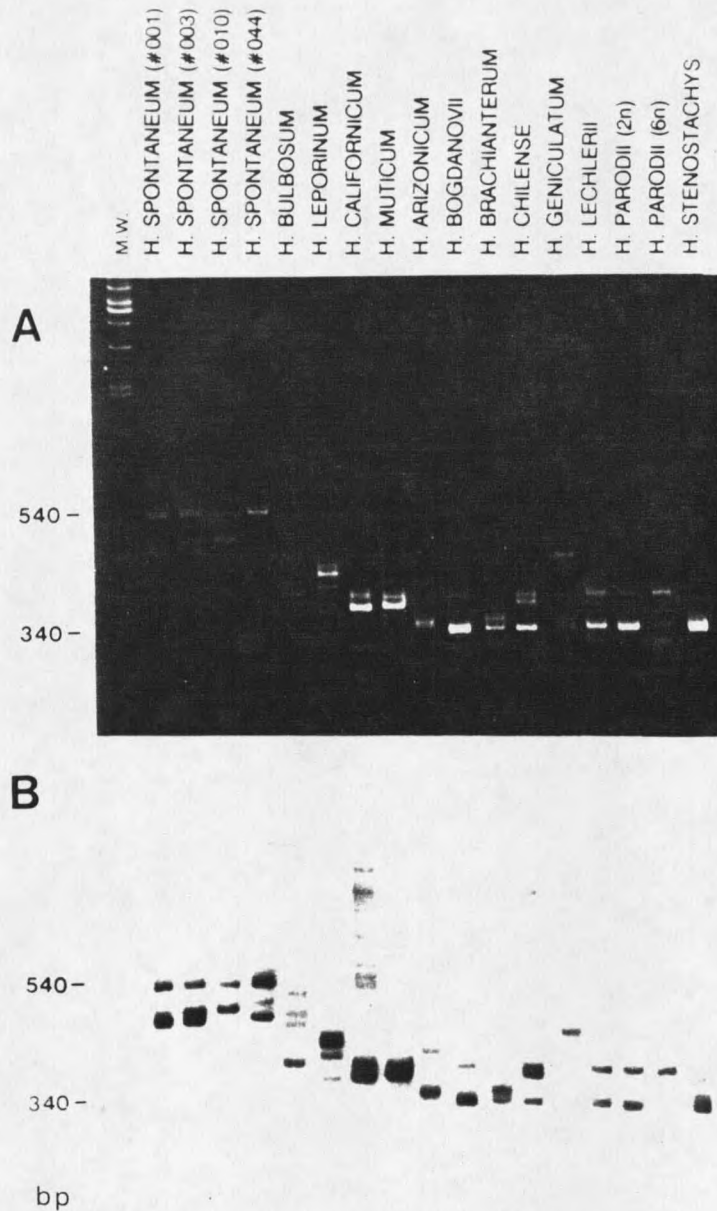


Figure 6. Amplification of the B-hordein gene variable domain from different *Hordeum* species.

A: Polyacrylamide gel electrophoresis of PCR products amplified with primers KV1 & KV2 from different *Hordeum* species.

B: Autoradiograph of a Southern blot corresponding to the gel on the panel A. Filter was hybridized with 32 P-labelled B-hordein probe.

Discussion

Cereal storage proteins are among the most commonly consumed proteins on earth. Their poor nutritional quality and their importance in human and animal diets have profound economic implications for both developing and developed nations. The prolamins, because of their tendency to accumulate variation which is readily identifiable, are also important as genetic and genotyping tools. While SDS-PAGE banding pattern variation among genotypes has been readily observed, little heterogeneity for amino-acid composition has been found (Kreis et al. 1985).

In this project, an attempt was made to determine how the variation observed at the protein level was generated at the level of DNA sequence. Digestion of PCR-amplified B-hordein genes using primers which were homologous to regions flanking the coding sequence uncovered multiple variants of a basic sequence within a single genotype. Polymorphisms were discovered which were due to acquired or lost restriction sites both within and between cultivars. Analysis of the 2.3 kb fragment amplified from a recombinant barley line 134/31 in which crossing over occurred within the *Hor-2* locus suggested that these polymorphic gene variants represented members of the BI subfamily.

In order to investigate the structure of other members of the B-hordein gene family we designed primers (KV1 and KV2)

homologous to regions within the coding sequence and which flanked a region containing multiple tandem repeats. Short (9-21 bp) imperfect repeats in this domain span over 200 bases. Amplification of this region revealed variation in the length of the repetitive domain of the B-hordein gene within cultivars and variation in banding patterns between cultivars. As many as eight length variants were observed within one genotype. The observed variation in length among products amplified using primers KV1-KV2 can most easily be attributed to changes in the number of tandem repeats within this repetitive domain. Amplification of only one minor band from barley line Riso 56 (a deletion derivative of the cultivar Carlsberg II) indicated that these products represent members of the B-hordein gene family. Wild barley species also contain this repetitive domain, although its length is generally much shorter.

The types of variability observed at this locus among different barley cultivars and species suggested that process of shrinking and expansion of the repetitive region occurs frequently in barley genome. The 540 bp long fragment obtained after amplification of barley genomic DNA with primers flanking the "repetitive box" may represent the oldest or most stable variant of the gene. Similar hypervariable blocks of simple tandem repeats have been found in genes from a wide range of organisms. These include the apolipoprotein B genes in humans, and the *period* and *glue* genes in *Drosophila* (Lyons

et al. 1988; Boerwinkle et al. 1989; Costa et al. 1991; Muskavitch and Hopness 1982).

Comparison of patterns obtained after amplification of the repetitive domain, and restriction digests of the 2.3 kb fragment, with SDS-PAGE of hordeins demonstrated perfect cosegregation in 70 doubled haploid lines from two crosses. This suggests that the variability observed represents real variation between genomes and is not a PCR artifact.

There are two major putative mechanisms for the generation of such variability - unequal crossing over and DNA strand slippage during replication. Intragenic unequal crossing over was indicated as a source of frequent insertion-deletion polymorphisms in a human proline-rich protein multigene family (Lyons et al. 1988). Rogers and Bendich (1983) proposed unequal DNA crossing over as source of generation of ribosomal RNA heterogeneity in plants. Other studies showed that not unequal crossing over, but DNA slipped strand mispairing is the major mechanism of variability generation in tandemly repetitive loci (Wolf et al. 1989; Levinson and Gutman 1987). In the case of hordeins either mechanism may be operating, although recombination events within *Hor-2* are extremely rare - only one such event has been reported in many thousands of analyses (Shewry et al. 1990).

PCR proved to be useful for characterizing the structure of members of the B-hordein multigene family. While clones containing multiple tandem repeats can undergo structural

modification during propagation in *E.coli* (our observations, data not shown; Mignotte et al. 1990; Studer et al. 1991; Vicente-Carbajosa et al. 1992), no modification of sequence size was attributed to artifacts of PCR.

These data suggest that the B-hordein genes are represented by multiple copies of a basic sequence which varies in nucleotide substitutions and length of the repetitive domain. Our data support the subdivision of the B-hordein genes into at least two gene subfamilies. A similar organization has been reported for the histone H3 and H4 multigene families in maize (Chaubet et al. 1989). Gene diversification due to expansion or contraction of the repetitive domain, and generation of variation in copy number due to gene conversion, could easily explain the large apparent differences in SDS-PAGE banding patterns of the B-hordeins as well as their similar amino acid composition.

THE GENETICS OF 5S rRNA ENCODING MULTIGENE FAMILIES IN BARLEY

Introduction

Genes encoding 5S ribosomal RNA (5S rDNA) have been cloned and characterized from many eukaryotes, including several members of the *Triticeae*. Usually they are located separately from the 18-26S rDNA clusters and are organized into one or two arrays of tandem repeats consisting of coding sequences separated by non-transcribed spacers (NTS). While the transcribed units of these large gene families are highly conserved among taxa, the non-transcribed spacers separating them show variation both within and between species. Wheat (*T. aestivum* L.) and many of the related *Triticeae* have two major 5S rDNA clusters per haploid genome. These two gene families can be differentiated on the basis of the NTS size, which can vary between species and among different gene families from less than 200 base pairs (bp) to over 400 bp in length. Within each gene family in a species the size of the NTS appears to be uniform. In wheat and rye, the 5S rDNA cluster containing the larger NTS is found on homoeologous group 5 chromosomes, while the shorter is found on homoeologous group 1 chromosomes (Dvorak et al. 1989, Reddy and Appels 1989; Mukai et al. 1990).

Khvyrleva et al. (1988) showed that two types of 5S rDNA sequences were present in barley, one class with a short,

approximately 180 bp long NTS, and a class with a larger NTS. Kolchinsky et al. (1990) identified chromosome 2 as the location of a 5S rRNA multigene family in barley. In a related report, Kolchinsky et al. (1991) showed that variation in the length of the NTS could be observed among copies within the same gene family.

In this report we demonstrate that the primary difference in structure of spacers is due to a 130 bp sequence which is absent in the shorter NTS variant. Length of the NTS varied within and between different *Hordeum vulgare* genotypes due to the presence of a (TAG)_n variable number tandem repeat (TAG VNTR) in both spacer classes. We confirmed the location of the 5S rDNA cluster containing the smaller NTS on barley chromosome 2, and located a major 5S rRNA multigene family containing the larger NTS on chromosome 3. The number of NTS copies at each locus varied greatly among genotypes. Doubled haploid progeny from a cross in which one parent (Morex) had a high number of 5S DNA sequences at the chromosome 2 locus and a low number at the chromosome 3 locus, and the other (Steptoe) had a high number of copies on chromosome 3 and a low number on chromosome 2, varied in total 5S DNA copy number approximately 4 fold. This variation had no significant effect on plant phenotype.

Materials and Methods

Plant Material

The doubled haploid lines used in this project were developed by Dr. Patrick Hayes, Oregon State University, using the *Hordeum bulbosum* technique (Hayes and Chen, 1988) and were distributed by the North American Barley Genome Mapping Project (NABGMP). The parents, Morex and Steptoe, are 6-rowed barley varieties developed by Dr. Don Rasmussen, University of Minnesota, and Dr. R.A. Nilan, Washington State University, respectively. The wheat-barley chromosome addition lines were developed by Islam et al. (1981). The wild species of barley were provided as seed by Dr. George Fedak, Ottawa Agricultural Research Center.

Biochemical Techniques

Plant DNA was extracted from fresh foliar tissue as described by Dellaporta et al. (1983). Digestion of DNA with restriction endonucleases and Southern blotting were performed as described in Shin et al. (1991).

Primers for PCR were synthesized using an Applied Biosystems model 391-EP DNA synthesizer using phosphoroamidite chemistry. Polymerase chain reaction sequence amplification (PCR) was performed using 2.5 units of *Taq* Polymerase and 10-20 nanograms DNA as template per 100 microliter reaction.

Twenty cycles of amplification were performed in a Perkin-Elmer thermocycler with a cycling regime of 40 seconds at 94°, 5 seconds at 55°. Longer extension times resulted in the production of large quantities of multimers (data not shown). The products of amplification were evaluated in either 1% agarose, 1% NuSieve gels or in 6% denaturing polyacrylamide gels (Maniatis et al. 1988).

Following amplification of the NTS from the barley cultivars Steptoe and Morex, ends of the amplification products were blunted using Klenow fragment and phosphorylated using polynucleotide kinase. Fragments were then ligated into the *Sma*I site of pGEM3. Plasmids containing NTS were isolated by alkaline lysis, purified using Prep-a-Gene DNA purification matrix (Bio-Rad), denatured in 0.2M NaOH, ethanol precipitated, and sequenced with Sequenase (USB) using the dideoxy approach of Sanger et al. (1977).

The relative copy number of the 5S rRNA genes was estimated in Morex, Steptoe and several doubled haploid lines of barley by measuring the amount of radioactive probe bound to a filter after Southern hybridization. Genomic DNA (1.5 micrograms) isolated from Steptoe, Morex and five representatives of each genotypic class was digested with *Bam*HI, electrophoresed in a 1.0% agarose gel in tris-borate buffer and transferred to a HyBond N+ membrane (Amersham) in 0.2N NaOH. 300 picograms of cloned 5S rDNA was applied to the gel prior to electrophoresis as a standard for copy number

estimation. One filter was hybridized with a P^{32} -labelled fragment specific for the long repeat, and another was hybridized with a complete 5S probe. After autoradiography the amount of radioactive probe bound to the membrane was estimated by Cherenkov counting in a Perkin-Elmer scintillation counter.

Mapping and Analysis of Quantitative Traits

Mapping of the 5S rDNA clusters and analysis of quantitative trait loci (QTL) were performed in a population of 150 DHL from Steptoe x Morex cross using MAPMAKER and MAPMAKER-QTL (Lander and Botstein 1989). Statistical analysis was performed using MSUSTAT v.3.20 (Lund 1986). Traits measured in dryland and irrigated conditions included flowering date, plant height at maturity, total tiller number, fertile tiller number, grain yield, kernel weight, grain protein content and plant lodging.

Results

Sequence and Structure of Two Classes of the 5S Nontranscribed Spacers

The location and orientation of each of the primers utilized for NTS amplification is shown in Figure 7(A). Primers S1 and S2 are complementary to the ends of 5S coding sequence and were designed to amplify spacers separating 5S rRNA genes from all species of cereals (Kolchinsky et al. 1991). After amplification we observed two classes of NTS, one

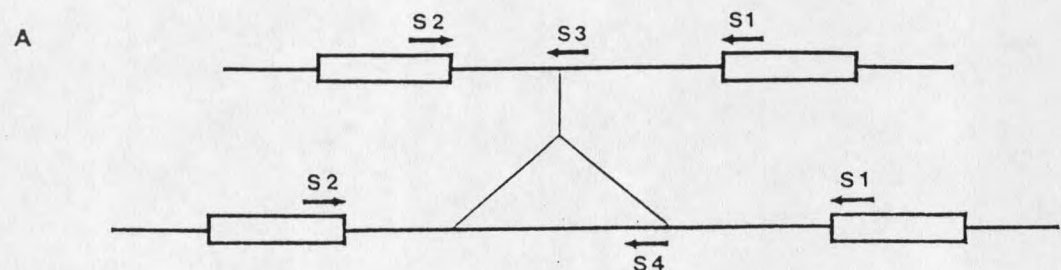
approximately 180 base pairs in size, the other approximately 330 bp. Several representatives of each class were cloned and sequenced. Examples of the sequences are shown in Figure 7(B).

The data derived from the short NTS group showed near identity of the sequences with the exception of a few point mutations and a variable number of internal TAG repeats. The longer class of NTS differed from the shorter class primarily by the inclusion of a 130 base pair unique sequence and by an 18 bp sequence duplication at the left border of the insert. The longer class also contained the VNTR region. The number of tandem TAG repeats in these clones varied from 6 to 34.

In order to determine how much variation could be observed at this VNTR region in the barley genome, nontranscribed spacers were amplified with ^{32}P labeled dCTP incorporated into the PCR reaction mix. Amplification was performed under standard conditions, but the products were denatured and the single stranded products separated in a sequencing gel (6% denaturing polyacrylamide gel). Following electrophoresis, the products were detected by autoradiography (Fig. 8). Products amplified from control plasmid produced a single band, indicating that PCR did not create apparent VNTRs by DNA strand slippage. Multiple bands similar to those reported by Kolchinsky et al. (1991) were observed for both the longer and shorter class of NTS. Different cultivars had very distinctive patterns of VNTRs and different amounts of short and long 5S spacers. It was possible to observe

variation in the distribution and number of TAG repeats, which varied from as low as 4 to almost 40 triplets within each class of spacers. In Morex and Betzes the majority of short NTS contained 7 TAG triplets, while in Harrington and Dicktoo the average number of repeats was 13. Several predominant classes of VNTRs were observed within the large spacer class as well. This VNTR was not observed in amplification products from oat, wheat and wild relatives of barley, which suggests that it may be a relatively recent addition to nontranscribed spacers of *Hordeum vulgare*.

The 5S NTS from barley showed several regions of near sequence identity with sequenced NTS units from rye and *Triticum tauschii* (Reddy and Appels 1989, Dvorak et al. 1989), two of which lie within the 130 base pair sequence unique to the long NTS class (Fig. 7). This suggests that the long NTS class is likely to be the older variant of the spacer, and that the shorter class may have derived from the longer by deletion.



B

TGGGAAGTCCTCGTGTTCATTCCTTTTAAATATATTTTTGCGCCACGTGAC
 TGGGAAGTCCTCGTGTTCATTCCTTTTAAATATATTTTTGCGCCACGTGAC

AAGGATGACGCGGGAGCGTGATCTATATGACCTCATTTTCTTATTTTTGACGATT
 AAGGATGACGCGGGACCGTGATCTATATGACCTCATTTTCTTATTTTTGACGTTT

ACTGTG.....
 ACTAGTTTTCTTATTTTTGACGTTTGTGATATGTTTGTAGCTTGCCCCTCCCGTGT

.....
 CCATTGCCACATTTGCCTCGACAACGGAGACGAGTTTAACACAAGAATTTACCG

.....TGACTTTTCCCA
 CTCCCTTTCTACCCCGACAACACGAGCACCGCCACGACCTCTCTGACTTTTCCCA

CCGCGCTTGACACCCAACGAC (TAG)₆₋₃₄ GGGCAAGCATAAGGAACAAAT
 CCGCGCTTGACACCCAACGAC (TAG)₆₋₃₄ GGGCAAGCATAAGGAACAAAT

AGATAGTTGCATGTGCGGATGCGATCATAACCAGCACTAAA
 AGATAGTTGCATGTGCGGATGCGATCATAACCAGCACTAAA

Figure 7. Sequence and organization of the 5S rDNA.

A). Organization of the short and long 5S rDNA classes. Rectangles represent 5S rRNA genes, lines represent nontranscribed spacers. Arrows represent location of primers S1 (5'-TTTAGTGCTGGTATGATCGC-3'), S2 (5'-TGGGAAGTCCTCGTGTTCGCA-3'), S3 (5'-CGCGGTGCGAAAAGTCACAC-3'), and S4 (5'-AGAGGTCGTGGCGGTGCTCG-3').

B). Alignment of the consensus sequences of the short (upper line) and long (lower line) nontranscribed spacers amplified with primers S1 and S2. Regions of homology with nontranscribed spacers from wheat and rye are underlined.

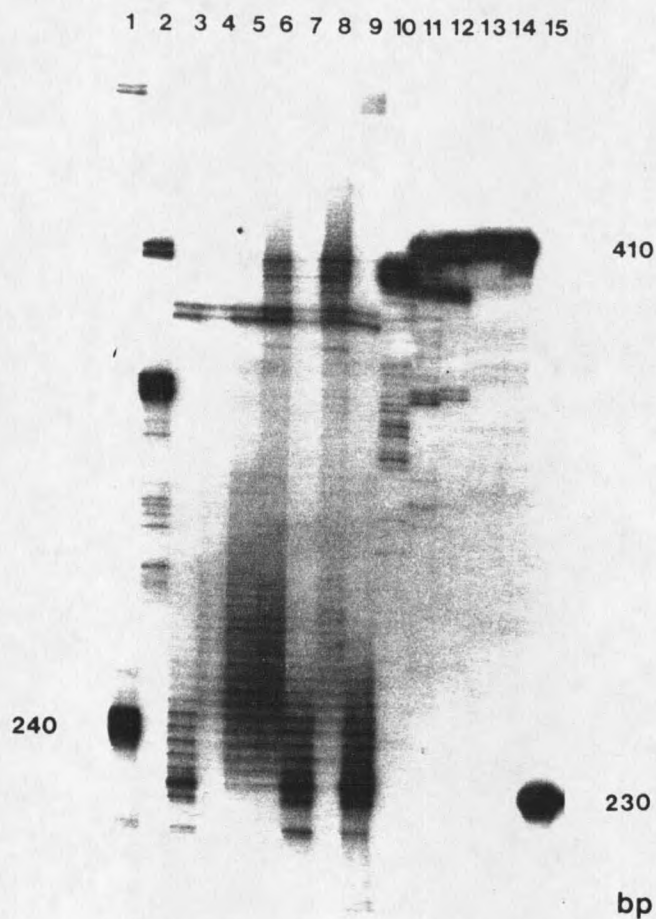


Figure 8. Length variation among 5S nontranscribed spacers. Spacers were amplified using primers S1 and S2 from different species of cereals in presence of P^{32} and separated in denaturing polyacrylamide gels. 1 - *Avena sativa*, 2 - *Triticum aestivum* cv. Chinese Spring, 3-8 - *Hordeum vulgare* cv. Betzes, TR306, Harrington, Dicktoo, Morex, Steptoe, 9 - *H. spontaneum*, 10 - *H. bulbosum*, 11 - *H. muticum*, 12 - *H. arizonicum*, 13 - *H. glaucum*, 14 - *H. leporinum*, 15 - control - amplification of a cloned spacer.

Mapping of the 5S rDNA Clusters

Both classes of NTS elements were amplified from genomic DNA using primers S1 and S2. In order to determine whether these two size classes of NTS were located within one multigene family or were organized into two distinct clusters, as is the case in many members of the *Triticeae*, (Reddy and Appels 1989; Dvorak et al. 1989), we utilized differences in sequence between the two spacer variants to synthesize PCR primers which would promote selective amplification of one type of the NTS. Amplification of short and long spacer classes from DNA of the wheat-barley addition lines permitted localization of the shorter NTS on barley chromosome 2, and the longer on chromosome 3, although chromosome 2 appeared to carry some long repeats as well (Fig. 9). We were unable to eliminate barley chromosome 5 (1H) from consideration as a possible location of additional 5S rRNA genes using this approach due to the unavailability of the appropriate line.

Pairs of primers S2-S3 and S2-S4 (see Fig. 7) failed to direct amplification from all wild barley species tested, with the exception of *Hordeum spontaneum* (data not shown). This suggested that substantial divergence may have occurred for NTS sequences among members of the genus *Hordeum*.

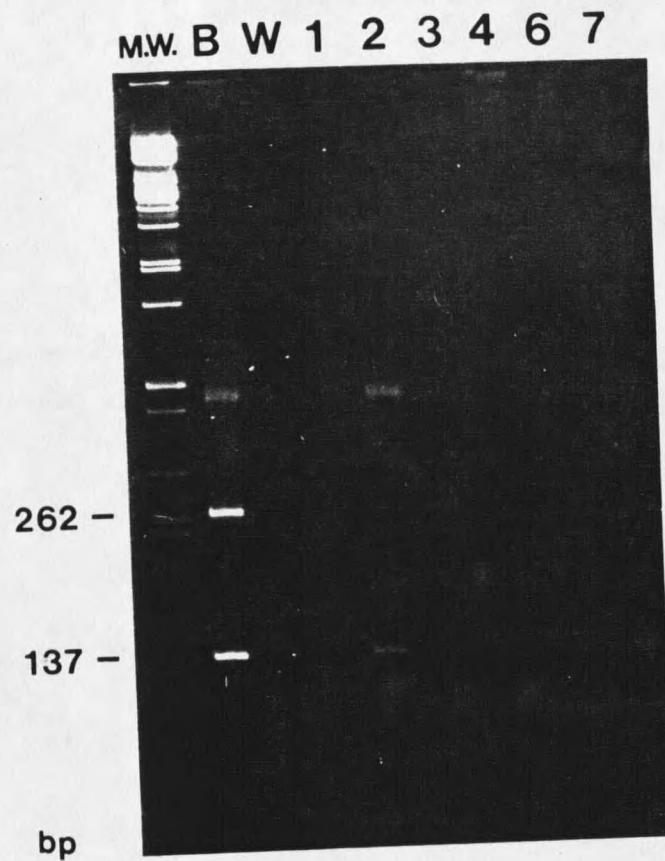


Figure 9. Localization of 5S rDNA clusters using wheat-barley chromosome addition lines. Amplification was performed using primer S2 and mixture of primers S3 and S4. B - Betzes barley, W - Chinese Spring wheat; 1, 2, 3, 4, 6, 7 - chromosome addition lines.

The two barley cultivars used by the NABGMP, Steptoe and Morex, showed dramatically different PCR amplification patterns when primers S1 and S2 were utilized. The lower band in Morex was substantially brighter than the upper, and in Steptoe the upper band was brighter than the lower (Fig. 10). Using relative intensity of the bands as a criteria, the upper band mapped to chromosome 3, 6 cM from locus PSR 156 and 2 cM from ABG 377. The shorter NTS mapped to the long arm of chromosome 2, 8 cM distal from ABC 152D and 4.9 cM proximal from KSU F15 (the map in Fig. 11 indicates relative locations of these loci, and is used with the permission of the NABGMP). A group of individuals proved difficult to classify because relative intensities of the upper and lower bands were essentially equivalent (for example: Fig. 10, lane 10). We utilized the 130 base pair fragment unique to the long NTS as a probe against *Bam*HI digested DNA from the 150 doubled haploid lines from the cross between Steptoe and Morex, and detected clear quantitative and qualitative polymorphisms which permitted us to unambiguously determine the identity of the long NTS allele for each of the doubled haploid lines. This additional data confirmed the location of the 5S rDNA locus containing the long NTS at the chromosome 3 locus identified by PCR.

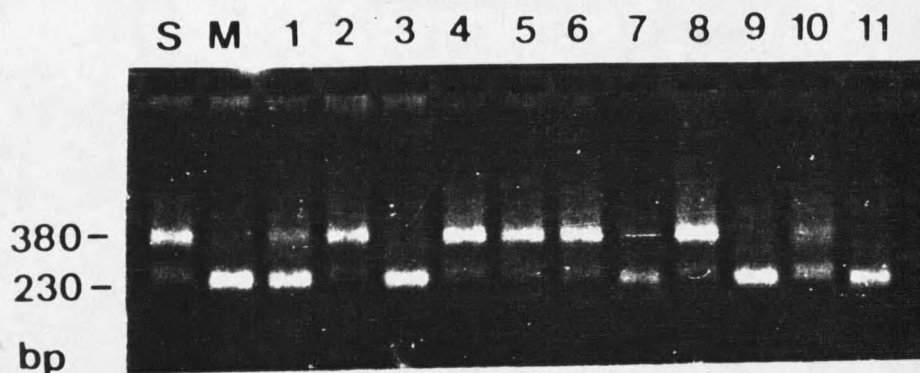


Figure 10. Mapping of the 5S rDNA clusters. NTS were amplified from Steptoe (S), Morex (M), and doubled haploid lines (1-11). Products of amplification were separated in 1% agarose/1% NuSieve gel.

We also can not exclude a possibility of other 5S loci present in barley genome, which we were unable to detect due to an absence of polymorphism between Steptoe and Morex. Indeed, amplification of 5S NTS from wheat-barley addition lines with primers S1 and S2, which correspond to conserved gene sequences, demonstrated the presence of an additional 5S rDNA cluster on chromosome 1 (Fig. 12).

Products of amplification from chromosome-addition line AD1 were cloned and several clones containing barley-specific inserts were isolated. Sequencing of these clones should determine structure of the non-transcribed locus on the chromosome 1.

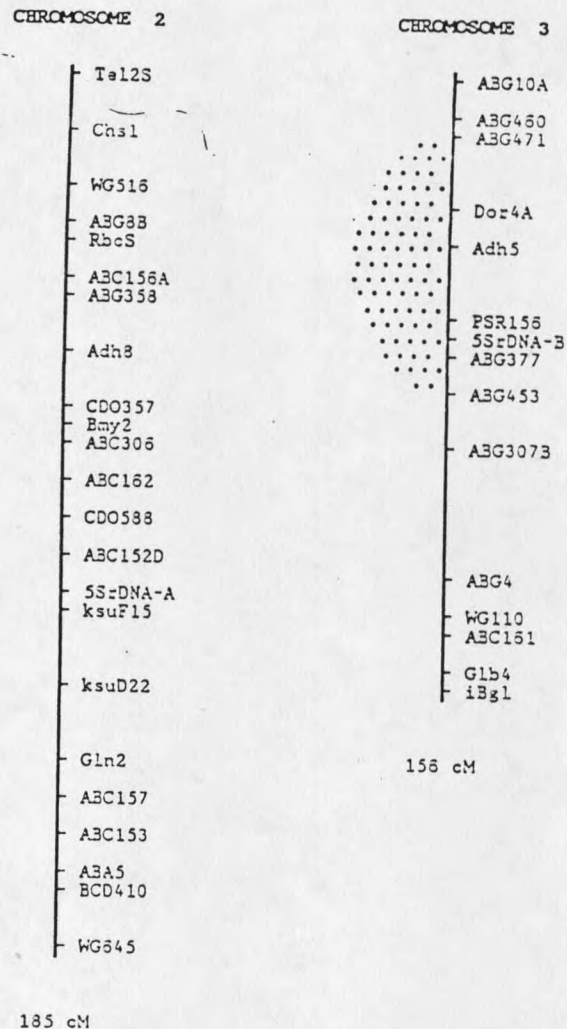


Figure 11. Genetic maps of barley chromosomes 2 and 3. LoD Score Estimates of the location of a quantitative trait locus having an effect on yield is indicated by shaded area.



Figure 12. Amplification of the 5S NTS from wheat Chinese Spring (W), barley Betzes (B), and wheat-barley addition lines (1-7). Top picture represents products of amplification separated in 1.5% agarose gel. These DNA fragments were transferred onto Hybond-N+ membrane and hybridized with ^{32}P labelled long variant of the barley 5S NTS (bottom panel).

Variation in 5S rDNA Copy Number

The preceding observations suggested that in Morex, which contains mostly the short class of the NTS, the preponderance of 5S rRNA sequences should be found at the chromosome 2 locus. In Steptoe, where 5S gene variants with long spacers are predominant, the preponderance of 5S ribosomal DNA sequences should be found on chromosome 3. This further suggested that among inbred progeny from a cross between these parents one progeny class would contain Morex chromosome 2 5S rDNA allele and the Steptoe chromosome 3 allele, while another class would carry the opposite allelic states. Four distinct progeny classes, the two parental and the two chromosomal recombinant classes, would then be expected to vary in total 5S rDNA copy number. We estimated 5S rDNA clusters size and copy number by counting radioactivity from Southern blot lanes containing carefully quantified *Bam*HI digested DNA from known genotypes. The results of these experiments are shown in Fig. 13 and Table 1. As expected, obvious differences in the amount of the 5S rDNA with short and long nontranscribed spacers and in total copy number of the 5S genes were observed between recombinant and parental genotypes. The two parental classes have similar total 5S rDNA copy numbers. The class bearing the chromosome 2 5S rDNA from Morex and the chromosome 3 5S rDNA from Steptoe (2^M3^S) showed about 1.3x parental levels, while the 2^S3^M class (chromosome 2 from Steptoe and

chromosome 3 from Morex) showed about 1/3 parental levels of the 5S rRNA genes amount. This experiment also confirmed the accuracy of the genotype determination performed by analysis of PCR data. Slight discrepancies in copy number estimation between parental and recombinant classes can be possibly attributed to presence of an additional locus on chromosome 1.

Table 1. Amount of the 5S rDNA in different classes of doubled haploids

Progeny class	Amount of bound probe, cpm	5S rDNA copy number	Amount of the 5S rDNA per haploid genome, b p
2^{S_3S}	2110	1050	472×10^3
2^{S_3M}	670	330	124×10^3
2^{M_3S}	2710	1310	491×10^3
2^{M_3M}	1840	890	268×10^3

Phenotypic Effect of the 5S rDNA Copy Number

This observation suggested that 4-fold variation in copy number for the 5S rRNA coding sequences could be observed among the Steptoe/Morex doubled haploid lines. Allelic variation at the nucleolar organizer regions in barley has been shown to be associated with variation in adaptation (Saghai Maroof et al. 1984, 1990; Allard et al. 1990). To determine whether either allelic variation at either 5S locus or copy number variation at both loci had an effect on plant phenotype, we performed quantitative trait locus analysis using data gathered at Bozeman, Montana in the 1991 field season. After analysis of eight characters (described in

"Materials and Methods") significant interactions were observed in several instances. The chromosome 2 5S locus was associated with variation for plant height, peduncle angle and length under dryland conditions. The chromosome 3 5S locus was associated with several traits (plant height, peduncle length and angle, lodging, number of fertile tillers, yield). However, after regression of values for these traits on 5S copy number, only a few traits (peduncle length, heading date and yield in dryland conditions) retained significance. Variation in 5S copy number contributed very little to these traits. After examination using the interval analysis approach implemented by MAPMAKER-QTL (Lander and Botstein 1989), linkage was observed between the 5S rDNA locus on chromosome 3 and a gene with major impact on yield (dotted area in Figure 12). The proximity of the chromosome 3 5S cluster to a locus with a large impact on yield and associated traits could explain the observed interactions.

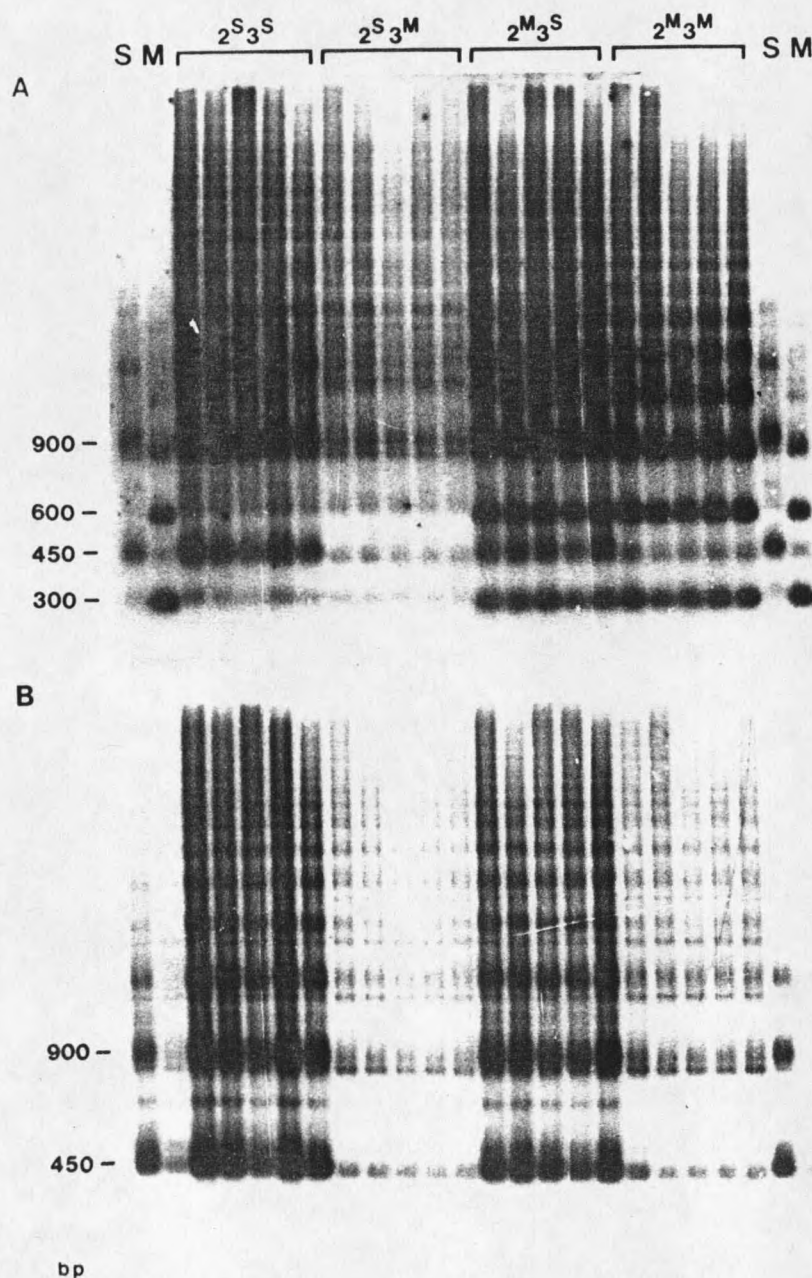


Figure 13. 5S rDNA copy number in different progeny classes: Blot hybridization of *Bam*HI digested genomic DNA from doubled haploid lines with: A) - full length 5S rDNA repetitive unit, B) - with fragment specific for a longer class of 5S NTS. S - Steptoe, M - Morex, 2^S3^S - DHLs with Steptoe alleles of 5S rDNA on chromosomes 2 and 3; 2^M3^M - DHLs with Morex alleles on both chromosomes; 2^S3^M , 2^M3^S - recombinant classes of DHLs.

Discussion

The barley 5S rDNA loci located in two large multigene families on barley chromosomes 2 and 3 were characterized. An additional 5S rDNA locus located on the chromosome 1.

The large NTS demarks the chromosome 3 family and the small NTS marks the chromosome 2 family. A TAG VNTR distinguishes both classes of barley NTS from all other characterized 5S spacers. The primary difference between long and short NTS classes consists of a 130 bp sequence absent in the smaller spacer variant. Frequent duplication and deletion effects in the 5S rDNA spacers were observed in many species of the *Triticeae* (Scoles et al. 1988). Three small sequences appear to be conserved among barley, rye and *T. tauschii*. Two of these three conserved regions are found within the 130 bp region unique to the large NTS. This observation suggested that the large NTS is the more ancient, and that the smaller was derived from the larger by deletion. This observation implies common evolutionary origin of the two 5S rDNA classes in barley, in contrast with independent lineages of different 5S rDNA classes in rye (Reddy and Appels, 1989).

These structural differences which distinguish spacers permit their use as convenient chromosome- and genome-specific markers for linkage analysis and evolutionary studies. Cordesse et al. (1992) used genome-specific ribosomal RNA gene spacer fragments from rice to analyze relationships among

different rice genomes. Cox et al. (1992) utilized differences in NTS length resulting from small deletions to locate 5S gene clusters on three different chromosomes of wheat.

The barley 5S rDNA loci are in chromosomal locations which are different from the locations of the 5S rDNA loci in wheat and rye. This is a case in which homoeology between wheat and barley appears not to have been maintained. Analysis of the genomic maps which have been produced in barley (Graner et al. 1989; Heun et al. 1991; Kleinhofs et al. 1992) and are in production in wheat will determine whether this is an isolated occurrence, or whether there are other large scale rearrangements which have gone unobserved to date. Mapping of the 5S rDNA loci also reveals discrepancies between genetical and physical maps. On the genetic map they are located close to the chromosome 2 and 3 centromers, while *in situ* hybridization with wheat and barley chromosomes shows a distal location of the 5S rDNA clusters. (Mukai et al. 1990; Leitch and Heslop-Harrison, in press). Variation in 5S rDNA copy number has been observed in many members of the *Triticeae*. Various inbred lines of maize widely utilized in breeding programs were found to contain 5,000 - 23,000 18-28S rRNA genes per nucleus (Phillips, 1978). Attempts were made to find a correlation between multiplicity of the ribosomal RNA genes and response of these inbred lines to selection for oil and protein content (Phillips, 1978).

In this report an attempt was made to determine whether

copy number variation at these loci had an impact on plant phenotype. Although many plant characters were evaluated, different alleles and 4-fold variation in copy number at these loci produced little phenotypic effect.

The TAG VNTR suggests that slippage during replication may be occurring. The variation among genotypes observed both in this report and by Kolchinsky et al. (1991) suggests that this may occur frequently within *Hordeum vulgare*. The variation in sequence copy number between chromosomes and between genotypes suggests that a mechanism such as unequal crossing over which could result in the large-scale reduction or generation of copies may also occur. Both observations may have an impact on the way in which we view the generation of variation in inbred species, such as barley.

ANALYSIS OF EVOLUTIONARY RELATIONSHIPS AMONG *HORDEUM*
SPECIES USING SEQUENCE POLYMORPHISMS IN 5S rDNA SPACERS

Introduction

The classification of species in the genus *Hordeum* is largely based on the classification proposed by Nevski (1941). He recognized 28 species separated into six sections. Bothmer et al. (1987) defined this genus as comprising of 30 species. Twelve tetraploid and seven hexaploid species were included in this classification. This classification was based on extensive taxonomic studies, incorporating data on classical characters (morphology, phenology, geographical distribution, habitat), cytogenetic characters (chromosome banding patterns and cytological behavior of interspecific hybrids), and biochemical and molecular traits. The results of this research are summarized in a series of papers detailing intra- and interspecific relationships in the genus *Hordeum* (Bothmer and Jacobsen, 1985; Doebley et al. 1992; Jacobsen and Bothmer, 1992; Jorgensen et al, 1986; Linde-Laursen et al. 1992; Pelger, 1991).

Based on these studies, *Hordeum* species are clustered into several major groups, with each group containing a unique genome. *H. vulgare* and *H. bulbosum* comprise the section *Critesion* and carry the I genome, *H. murinum* carries the Y genome, the *H. marinum* group contains the X genome and

remaining species, which include Asiatic and American diploids and polyploids, contain the *H* genome (Jacobsen and Bothmer, 1992).

Cluster of closely related American and Asiatic species proved to be difficult to resolve. Largely artificial subdivisions have been proposed for these species (Bothmer et al. 1991). Uncertainties remain concerning the origin of the polyploid species. Conventional cytogenetic methods are often ineffective in characterizing the donor species in allopolyploids. Preferential chromosome pairing can be influenced by genes which strongly regulate pairing (Pohler et al. 1986, Bothmer et al. 1989). Also, investigations of chloroplast genome structure typically fail to reveal more than the maternal parent in a hybrid species (Doebley et al., 1992).

The objective of this study is to investigate the structure, organization and evolution of nontranscribed spacers (NTS) from the 5S rRNA multigene family in wild barley species. These nuclear genetic markers appear to evolve rapidly and may provide insight into evolutionary relationships among closely related American species.

Materials and Methods

Seeds of wild species used in this research were kindly provided by the National Small Grains Collection (USDA-ARS, Aberdeen, ID) and by Dr. G. Fedak (Ottawa Agricultural Experimental Station, Canada).

DNA for amplification was extracted from green leaves or from embryos by crushing them in microcentrifuge tubes containing lysis buffer (100 mM Tris-Cl, pH 7.4; 100 mM EDTA, pH 8; 250 mM NaCl). After brief centrifugation, DNA was precipitated by adding an equal volume of isopropanol. After centrifugation, the DNA pellet was dissolved in 30 μ l of TE. Approximately 10 μ g of this DNA was used as a template for PCR.

Nontranscribed spacers were amplified from DNA of using primers S1 and S2 (Kolchinsky et al., 1991). After 25 cycles of amplification (94°C - 30 seconds, 55°C - 5 seconds) products were cloned into the SmaI site of plasmid pGEM4. Cloning, isolation of recombinant clones and sequencing were performed as described in Kanazin et al. (*in press*).

Dot-blot analysis was performed using approximately 10 ng of amplified 5S NTS DNA loaded onto Hybond-N+ membranes (Amersham). Probes for Southern hybridization were prepared by digesting cloned 5S NTS and extracting appropriate restriction fragments from polyacrylamide gels. Hybridization was performed as described in Kanazin et al. (*in press*).

Nucleotide sequence alignments and homology searches were performed on an IBM PC using GenePro^R (Riverside Scientific). Phenetic and cladistic analyses were performed with components of PHYLIP (Felsenstein, 1987).

Results

Nontranscribed spacers were amplified from ten diploid and three hexaploid *Hordeum* species using primers S1 and S2. These primers are complementary to the ends of the 5S coding sequence and flank the NTS. Several clones bearing 5S NTS were isolated and sequenced from each species. The complete nucleotide sequences of these clones are given in Appendix.

Different clones isolated from the same diploid species were very similar in structure, except for a few single base substitutions. There was much less similarity between clones from different species.

Based on nucleotide sequence similarity between 5S spacers isolated from the diploid barleys, these species were divided into several groups. *Hordeum vulgare*, *H. bulbosum*, *H. geniculatum*, and *H. stenostachys* 5S rRNA spacers were essentially unalignable with those of other species and were each considered to be unique. *H. glaucum* and *H. leporinum* formed a cluster, as did *H. muticum* and diploid *H. parodii*, and *H. magellanicum* and *H. chilense*. Clones from latter four species were very similar. Homology within each of those group was 93-95%, and between latter two groups approximately

85%. Figure 14 demonstrates alignment of the consensus nucleotide sequences from representatives of these three groups.

Figure 14. Alignment of the consensus nucleotide sequences of the 5S NTS from *H. chilense* (CHI), *H. magellanicum* (MAG), *H. parodii* (PAR), *H. muticum* (MUT), *H. leporinum* (LEP), and *H. glaucum* (GLA). Matching nucleotides represented by lines, gaps in the alignment represented by dots.

```

MUT  TGGGAAGTCCTCGTGTTCATTCTCTTTTAAATAAATTTTGCTCCGCGC
PAR  -----A-----A---
CHI  -----C-----T-----A-A-
MAG  -----C-----TC-----A-A-
LEP  -----C-----T-----T-G---
GLA  -----C-----T-----T-G---

```

```

MUT  GAGAAACGATGACGCACGTGCGCGGTA..TTTATTAAA.CAATGTTTCTT
PAR  -----TA-----
CHI  -----T--T--..T-T-C.GGA-T-TA-T--A--CG---C---
MAG  -----T--T--..T-T-C.GGAA-ATA-T--G--CG---C---
LEP  -G-G-T-.---G--T---T--AT--AA..---C-.--TG-----GA
GLA  -G-G--..---G-----A--TAAA-AT-AC-C-..TG-----AA

```

```

MUT  ATTTTTT.GGCGTTTGCGGTAAAGTTATAGCTTGC.GCG.CGGTACTCACGC
PAR  -----A-----
CHI  -----C.-A-----T-----T-----G-GT.-AT--TC-----
MAG  -----C.-A-----TT-----T-----TG-GT.-A.--TC-----
LEP  -A---T-A-----C-----T-----T.-GCT-AT--A-----
GLA  .....--C-----T-----T.-GTT-AT--A-----

```

```

MUT  GTCTAG...GGGCGGCGTTGCGGTGGCAAAGGTAGCGCGTTGTGAAAGGG
PAR  -----A-----A-----
CHI  --G--A...-----A---TGGT--G--AC-----
MAG  A-G---...-----A---TGGT--G-AAC-C-----
LEP  -----AGGAC--ATAA--A-T-T-----C-----CA-----
GLA  -----AGGCC--ATAA--A-T-T-----C-----CA-----

```

(continued on the next page)

(continued from the previous page)

MUT GGGTGAAACCGC.GGAAGAACTCGTGTTGGTGCGGTAGAGAGGGAAG.GG
 PAR -.-----T-----
 CHI -.-----AT-A-G-.A-----G-
 MAG -.CA-----T-A-G-CA-----G-
 LEP -.GT-----T.-TGA-----A---GT-.T-
 GLA -.GT-----T.-TGA-----A---GG-GT-

MUT TGGAAACGGTGGAAAGCCC..GTCTTTGTTGTTGA.GCGGGAGAGTACGT
 PAR -----A---.A-A-----T-----
 CHI ----G-C---TT-A-A-GT-G-GC-----C-----A--
 MAG ----G-C---TT-A-A-.G-GC-----A-----A--
 LEP -----A--T--A-TA..---CC-C---A-.---AC-----A--
 GLA -----A--T--A-TA..---CC-C---A-.---AC-----A--

MUT GGTACGGTGCGGTATCCGTTATT.AGG.AGCGGTGAAAAAGAA.TGTACG
 PAR -----T-----G-----
 CHI -----C-A---T-----G---C---TC-GC-----G.--CT--
 MAG T---T---A---T-----C---TC-GC-----G.--CT--
 LEP A-G-A---GA-----C-T-C---C-----G---G-
 GLA A-G-A---GA-----C-T-C---C-----AG---G-

MUT GAGGTGTTTATGGTGGAGCTGAGAGGGGCTAGAATAAGGGACGAAGGCGG
 PAR -----G-----A-----
 CHI ATC--A---G-----C-G---A-G-C---T-----
 MAG ATC--A---G-----C-G---G-C---T-----
 LEP -TC-G-----C-----G-----G-C-----TT--
 GLA -TC-G--G---A-----G-----G-C-----TT--

MUT GAG.TAACATGTCGGATGCGATCATACCAGCACTAAA
 PAR -----
 CHI -G-.G---G-----
 MAG -G-G-----
 LEP .G-.AT-----
 GLA .G-.A-----

5S NTS clones from the hexaploid barley species *H. arizonicum*, *H. lechlerii*, and a hexaploid accession of *H. parodii* were structurally similar to spacers cloned from the diploid species. Two sequenced clones from *H. arizonicum* and one clone from hexaploid *H. parodii* had 90-95% sequence homology with NTS from the diploid *H. parodii* - *H. muticum* group. Two clones from hexaploid *H. parodii* and two clones from *H. lechlerii* had equivalent homology to spacers from the *H. magellanicum* - *H. chilense* group. One clone from *H. lechlerii* was practically identical to the *H. stenostachys* spacer, with the exception of a small deletion.

Sequence conservation varied throughout the nontranscribed spacers. Approximately 50 nucleotides on both ends of the NTS, upstream and downstream of the coding sequence, are fairly highly conserved. Some degree of homology in these regions was observed among all species used in this study. Since these sequences could be aligned, a cladistic analysis was performed (courtesy of Dr. M. Lavin) and the results of this analysis are presented in Figure 15. The central portion of the NTS is substantially more diverse among species.

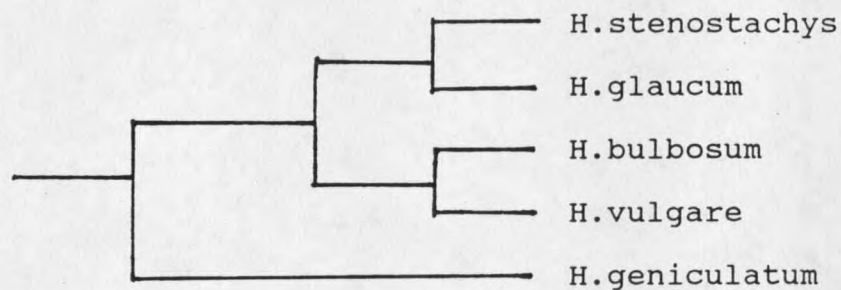


Figure 15. Cladistic analysis of evolutionary relationships in the genus *Hordeum*.

X-Axis: Clone #881, *H. stenostachys*
 Y-Axis: Clone #682, *H. chilense*
 Window 18, Matches 5, Ktup 5, Speed 1

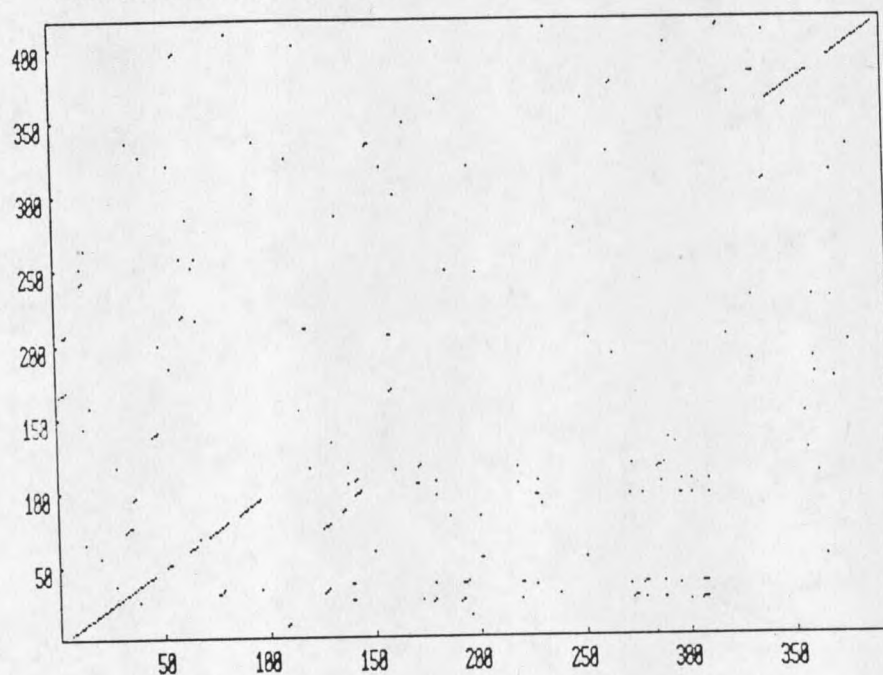


Figure 16. Dot-matrix comparison of the 5S rDNA NTS sequences from *H. chilense* and *H. stenostachys*.

Figure 16 illustrates the diversity among species observed in the central portion of the NTS. This sequence diversity permitted the isolation of 5S NTS spacer fragments unique to each group of species. Following restriction digestion, NTS fragments were extracted from polyacrylamide gels and used as probes against dot-blot containing 5S rDNA amplified from wild species of barley (Fig. 17). The nontranscribed spacers were amplified from genomic DNA of 41 different accessions belonging to 20 different *Hordeum* species (Table 2). Spacers amplified from the 5S rDNA NTS, cloned into plasmids, served as positive controls for hybridization.

Hybridization specificity in this experiment appeared to be quite high. The species which could be clustered based on homology to specific, unique 5S NTS probes fit well with prior classifications (Jacobsen and Bothmer, 1992) and with our expectations based on the sequence data. Several accessions fell into a "wrong" category, presumably due to misidentification or mislabelling of the samples. One "bulbosum" line was morphologically similar to *H. murinum*; one *H. chilense*, one *H. secalinum* and a *H. jubatum* line were morphologically similar to *H. bulbosum*.

Table 2. Description of the wild *Hordeum* species

# on blot	Species	Ploidy level	Accession number and origin
B2	<i>H. spontaneum</i>	2	PI400010 -
B3	<i>H. bulbosum</i>	2	-
B4	<i>H. bulbosum</i>	2	PI211040 Afghanistan
B5	<i>H. bulbosum</i>	2	PI304322 Israel
B6	<i>H. glaucum</i>	2	-
C1	<i>H. murinum</i>	2	PI223373 Iran
C2	<i>H. murinum</i>	2	PI304360 France
C3	<i>H. murinum</i>	2	PI255142 Chili
C4	<i>H. murinum</i>	2	PI247054 Canada
C5	<i>H. murinum</i>	2	PI304363 Argentina
C6	<i>H. murinum</i>	2	PI220100 Afghanistan
D1	<i>H. magellanicum</i>	2	-
D2	<i>H. chilense</i>	2	-
D3	<i>H. chilense</i>	2	PI255151 Chili
D4	<i>H. parodii</i>	2	-
D5	<i>H. stenostachys</i>	2	-
D6	<i>H. geniculatum</i>	2	-
E1	<i>H. bogdanii</i>	2	-
E2	<i>H. bogdanii</i>	2	PI531762 Tajikistan
E3	<i>H. bogdanii</i>	2	PI531761 China
E4	<i>H. brevisibulatum</i>	2	PI531771 Kirgizstan
E5	<i>H. brevisibulatum</i>	2	PI531768 Tajikistan
E6	<i>H. brevisibulatum</i>	2	PI531772 China
F1	<i>H. marinum</i>	2	PI200341 Israel
F2	<i>H. marinum</i>	2	PI185155 Iraq
F3	<i>H. marinum</i>	2	PI220522 Afghanistan
F4	<i>H. marinum</i>	2	PI247055 Argentina
F5	<i>H. marinum</i>	2	PI203462 Turkey
F6	<i>H. marinum</i>	2	PI304346 U.S.A.
G1	<i>H. californicum</i>	2	-
G2	<i>H. brachyanterum</i>	2	-
G3	<i>H. brachyanterum</i>	2	PI531763 U.S.A.
G4	<i>H. arizonicum</i>	6	-
G5	<i>H. parodii</i>	6	-
G6	<i>H. lechlerii</i>	6	-
H1	<i>H. secalinum</i>	4	PI278746 Spain
H2	<i>H. capense</i>	4	PI531780 South Africa
H3	<i>H. jubatum</i>	-	PI304350 Poland
H4	<i>H. jubatum</i>	-	PI304349 Belgium
H5	<i>H. jubatum</i>	-	PI304348 Germany
H6	<i>H. jubatum</i>	-	PI255155 Argentina

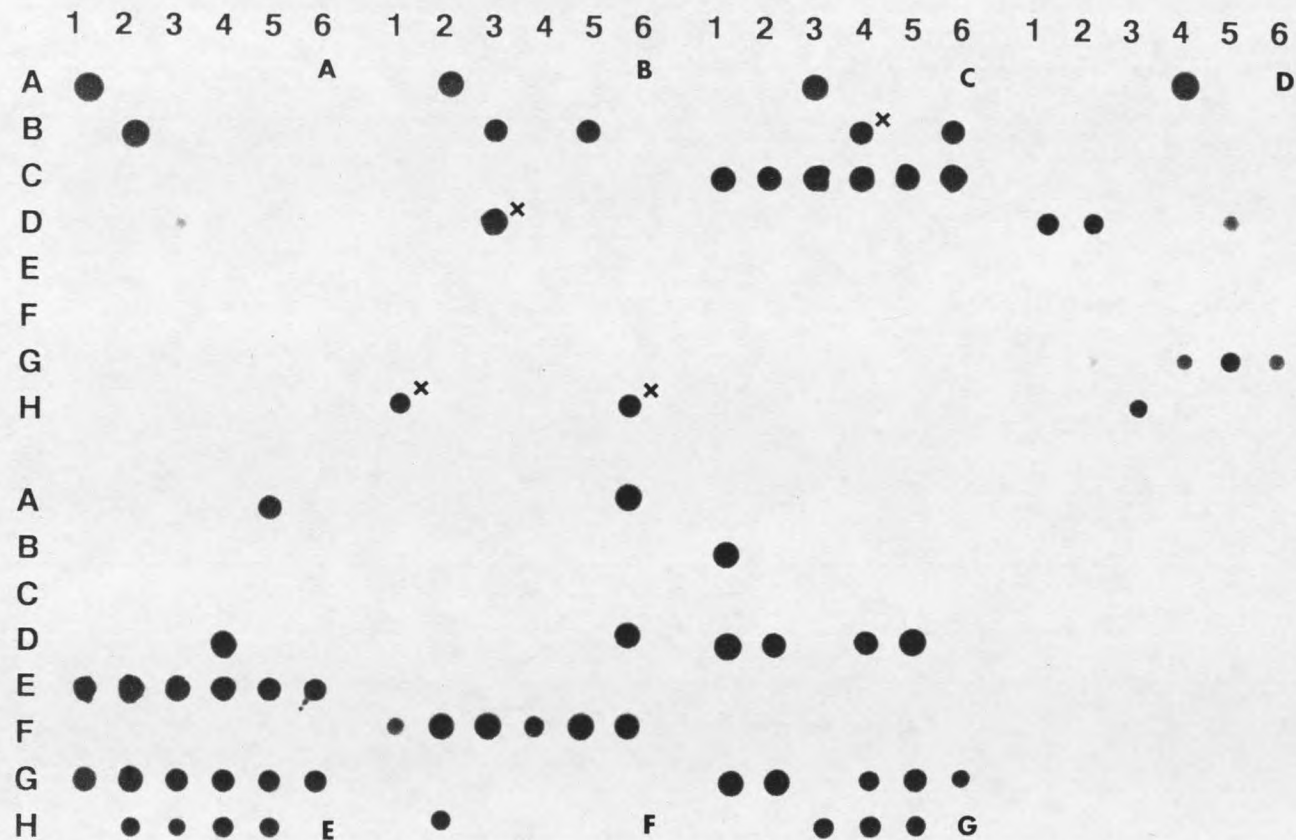


Figure 17. Dot-blot hybridization of 5S NTS amplified from different *Hordeum* species (#B2 - H6) with species-specific fragments isolated from cloned NTS. A - *H.vulgare*, B - *H.bulbosum*, C - *H.leporinum*, D - *H.magellanicum*, E - *H.muticum*, F - *H.geniculatum*, G - *H.stenostachys*. Dots #A1 - B1 served control and represent cloned NTS from species A - G, respectively.

Among the diploid species, the *H. vulgare* 5S spacer had homology only with DNA of *H. spontaneum*, which is its closest relative. *H. bulbosum* fell into a separate group. The unique spacer fragment isolated from *H. leporinum* hybridized to *H. leporinum*, *H. glaucum*, and *H. murinum* 5S rDNA. *H. geniculatum* and *H. marinum* formed another homology group. The unique portions of the *H. stenostachys* and *H. magellanicum* NTS clones failed to cross-hybridize. However, each hybridized to DNA from *H. magellanicum*, *H. chilense*, *H. parodii* (2x) and *H. stenostachys*. This suggests that each may carry two gene families, one with homology to each class of NTS sequence.

The polyploid species examined were *H. jubatum*, *H. capense*, *H. lechlerii*, *H. parodii* (6x) and *H. arizonicum*. *H. lechlerii*, *H. arizonicum* and *H. parodii* all shared homology with the unique fragments from *H. stenostachys*, *H. muticum* and *H. magellanicum*. One of the three *H. jubatum* accessions shared sequence similarity with unique 5S fragments from *H. stenostachys*, *H. muticum* and *H. magellanicum*. The other two *H. jubatum* accessions hybridized only to the unique sequences from *H. stenostachys* and *H. muticum*. This may indicate that two of the accessions were tetraploid and the third hexaploid. We were unable to test this hypothesis as the seed from these accessions failed to germinate and chromosome number could not be determined. *H. capense* DNA hybridized to unique NTS sequences cloned from *H. geniculatum* and *H. muticum*.

Discussion

The cloned 5S NTS showed enormous sequence divergence between species. The uniqueness of spacer fragments from different species permitted their use as probes to qualitatively evaluate homology among 5S rRNA encoding gene families across the genus *Hordeum*. This approach also allowed estimation of the degree of similarity between different accessions of the same species. This led to hypotheses on the genome formation of polyploid barleys.

Prior classifications and our current assessment of the phylogeny of the *Hordeum* genus are quite similar. The major difference between our classification and those of Baum and Bailey (1991), Doebley et al. (1992), Jacobsen and Bothmer (1992), Linde-Laursen et al. (1992) and Pelger (1991) concerns the origin of the polyploids. There is general agreement that *H. vulgare* and *H. bulbosum* form one monophyletic group, that *H. murinum* and sibling species form another, that *H. marinum* and *H. geniculatum* form a third. The remaining species form either a closely allied set of three sections of the genus or they share one common genome and can be considered as belonging to one section.

Presented data either supports, or fails to directly dispute these contentions. Our sequence data suggests that some revision within the American diploid groups may be

necessary. The NTS sequences cloned from *H. chilense* and *H. magellanicum* shared approximately 95% sequence homology. The NTS sequences from *H. muticum* and *H. parodii* (2x) also shared approximately 95% sequence homology. Between groups the homology was approximately 80-85%. According to Bothmer and Jacobsen (1985), *H. muticum* and *H. chilense* are members of the *Anisolepsis* group while *H. magellanicum* and *H. parodii* are members of the *Stenostachys* group. Our data suggests that the section divisions of Bothmer and Jacobsen (1985) are indeed artificial. This contention is supported by the work of Doebley et al. (1992). The cladistic analysis of chloroplast restriction sites suggests that *H. marinum* is distantly related to the other members of the *Hordeum* genus.

The NTS fragment unique to *H. geniculatum* hybridized to 5S rDNA amplified from *H. marinum* and *H. capense*. This is evidence that one of the genomes present in the tetraploid *H. capense* derived from the lineage leading to the *H. marinum*-*H. geniculatum* group, while the other was provided by *H. muticum* or an ancestral species. Doebley et al. (1992) provided evidence that the maternal lineage of *H. capense* included a close relative of tetraploid *H. brachyanterum*. The polyploids associated with polyploid *H. brachyanterum* accessions were found to include *H. jubatum*, *H. lechlerii* and *H. marinum*. Both *H. jubatum* and *H. lechlerii* appeared to share *H. muticum* as a common ancestor. Further testing of *H. brachyanterum* should determine whether *H. muticum* provided one of its genomes. It

would be interesting to determine whether the hexaploid *H. brachyanterum* accession #H2001, which carries a chloroplast genome similar to that of *H. marinum* accession #H800, also carries a *H. muticum* nuclear genome.

A critical interpretation of the data of Doebley et al. (1992) reveals several cases in which diploid and polyploid members of a species contain dramatically different chloroplast genomes. The simplest explanation for this observation is that the polyploids are indeed allopolyploids. If *H. muticum* provided the novel genome for many or most of these polyploids, then the genome of this species could be considered to be the pivotal genome and the other genome(s) in the polyploids could be considered to be the differential genomes (Kimber and Yen, 1988).

The reticulate nature of the evolution of polyploids in the *Triticeae* makes classical interpretation of the evolutionary relationships difficult. Molecular markers can be useful in dissecting the evolutionary paths along which species have evolved. Through analysis of the sequence homology between nontranscribed 5S rDNA spacers, we determined which *Hordeum* species shared similar sequences, and proposed a scheme for the genome origins of polyploid *Hordeum* species.

CHARACTERISTICS OF THE HISTONE H3 MULTIGENE FAMILY IN BARLEY

Introduction

Early studies of the histone genes led to several general conclusions about their structure. Histone genes were considered to be intronless, highly reiterated in the genome, and organized into tandemly repeated, highly conserved blocks. Their expression was thought to be developmentally controlled and their mRNA not polyadenylated (Old and Woodland, 1984). However, later research demonstrated that the organization and evolution of histone gene families is much more diverse and complex than previously thought. The organization of histone genes varies widely from highly reiterated, clustered quintets of genes encoding the major histone classes to randomly distributed single copy genes. Even in closely related organisms the pattern of gene organization is often different (Maxson et al. 1983). Clustered and scattered histone genes might be combined in a single genome. Such distribution may reflect differential regulation of these genes, or diverse mechanisms of gene duplication and gene family evolution (Tripputi et al. 1986).

Histone genes have been isolated and analyzed from surprisingly few plant species. Histone H3 and H4 genes have been cloned from *Medicago sativa* (alfalfa) (Wu et al. 1988), *Arabidopsis thaliana* (Chaboute et al. 1988), maize (Chaubet et

al. 1986, 1989; Gigot et al. 1987), rice (Thomas and Padayatty 1983, 1984) and wheat (Tabata et al, 1983, 1984). Only a portion of a histone H3 cDNA clone was isolated and sequenced from barley (GenBank^R).

This report describes the cloning, characterization, and mapping to chromosomal location of the histone H3 genes from barley and wheat. Several isolated H3 genes contained introns located in the same position, but of different length and sequence. Divergence among intervening sequences permitted to use them as gene-specific probes. Histone H3 genes were mapped on several chromosomes of barley.

Materials and Methods

Plant DNA was extracted from fresh foliar tissue as described by Dellaporta et al. (1983). Digestion of DNA with restriction endonucleases, gel electrophoresis and Southern blotting were performed as described in Kanazin et al. (in press).

Primers for PCR were synthesized with an Applied Biosystems model 391-EP DNA synthesizer. Polymerase chain reaction sequence amplification (PCR) was performed using 2.5 units of *Taq* Polymerase and 10-20 nanograms DNA as template per 100 microliter reaction. Thirty cycles of amplification were performed in a Perkin-Elmer thermocycler with a cycling regime of 40 seconds at 94°C, 5 seconds at 55°C. Amplified fragments were cloned into the *Sma*I site of plasmid pGEM4z.

Cloning, sequencing, sequence analysis procedures and other biochemical procedures were performed as described in chapter 2 of this thesis.

Gene-specific probes for Southern blotting were obtained by excising introns from cloned histone genes with appropriate endonucleases, separating and extracting them from a polyacrylamide gel.

Results

Cloning and Sequence Analysis of Barley and Wheat Histone H3 Genes.

PCR primers for amplification of the barley histone H3 genes were chosen from conserved regions of the gene based on nucleotide sequence comparisons with other cereal species. Primer KV12 (5'-ATGGCCCGCAC(C/G)AAGCAGAC -3') included the start codon and corresponded to the first 20 nucleotides of the coding sequence. Primer KV13 (5'-AGCTGGATGTCCTTGGGCAT-3') was located 28 bp upstream from the termination codon. PCR amplification using these primers produced a major band of the expected length (380 bp) and several minor fragments, approximately 500 bp long. Southern hybridization with a α -³²P labelled maize H3 gene demonstrated that these bands bore a high degree of sequence similarity to coding sequences of the histone H3 genes. They may represent variants of the histone H3 gene that contain intervening sequences. Introns has been shown to be present in H3.3 histone genes in chickens (Engel et al., 1982), in humans (Wells et al., 1985, 1987), and in H3

and H4 genes in *Neurospora crassa* (Woudt et al., 1983). Less efficient amplification of heavier fragments from plant genomic DNA could be reflection of their lower copy number, larger size or minor differences in primer annealing sites.

Histone H3 genes amplified from genomic DNA of wheat cv. Chinese Spring and barley cv. Steptoe were cloned into the *Sma*I site of plasmid pGEM4Z. Three wheat (W1, W2, W3) and two barley (B2, B3) putative intron-bearing clones and one intronless H3 histone clone were sequenced. Analysis of the nucleotide sequences showed that larger clones indeed contained intervening sequences (Fig. 18). Comparison of the coding sequences from the cloned genes showed multiple single nucleotide differences between sequences. All analyzed clones differed in their nucleotide sequence. In most cases, nucleotide changes occurred at the third codon position, with no subsequent changes in the resulting amino-acid sequence, except for a few conservative amino-acid substitutions.

The most striking feature of these cloned histone genes was their strong bias in codon usage. In three of the sequenced clones (W1, W2, B2) tendency of nonrandom codon usage is maintained. Clone W1 had only one, clone W2 had three, and clone B2 had five codons out of 127 which did not end with an C or G nucleotide. All of these gene variants contained introns. Clone B1 did not contain an intron and did not exhibit nonrandom codon usage, since substantially more (27) codons in this clone end with an A or T nucleotide.

Figure 18. Alignment of the nucleotide sequences of isolated H3 histone genes. Top line represents nucleotide sequence of an H3 gene isolated from wheat (Tabata et al. 1983) and used as a reference. Matching with it nucleotides in clones W1, W2, W3, B1, B2, B3 are indicated by lines. Clone W3 sequenced partially. Clone B1 represents part of the H3 gene. It contains first exon, intron, and 5' fragment of the second exon. Due to absence of homology between central parts of the introns they could not be aligned and their sequence is presented separately

	Exon 1		Intron		Exon 2
ref	ATGGCCCGCACCAAGCAGACGGCGAGGAAGTCGACCGGCGGCAAGGCGCC				GCCGCTCG
W1	-----G-----C--CC-C-----C-----				--G--CA-
W2	-----C-----C--CC-C-----C-----				--G--GA-
W3	-----G-----C--CC-C-----C-----C--				--T-GCT-
B1	-----C-----C--C-AG---C---A-----				--T--C--
B2	-----G-----C--CC-C-----C-----				--G--GA-
B3	-----N--NN-N-----C-----NNN-----C--				--G--GA-
ref	GAGGAAGCAGCTGGCGACCAAG				
W1	CC-C-----C--C-----		gtccg(.....)ttgcag		
W2	CC-C-----C--C-----		gtccg(.....)ttgcag		
W3	CC-C-----C--C---CC		atcgt(.....)tttcag		
B1	T-----C--T-----				
B2	CCAC-----C--C---CC		gtccg(.....)tttcag		
B3	CGCT-----C--C---CC		atcgt(.....)ttgcag		
ref	CAAGTCCGCCCCGGCCACCGGCGGCGTGAAGAAGCCCCACCGCTTCCGCC				
W1	G-----G--G---A-----A-----A-A-G-				
W2	G-----G--G---A---G---A-----A-A-G-				
W3	-----GG				
B1	-----A--TN-C-----T--T-A--A--				
B2	G-----G--N--N---N--N--NN-----TT-----A-A-G-				
B3	G-----*				

(continued on the next page)

(continued from the previous page)

```

ref  CCGGCACCGTGGCGCTCCGCGAGATCCGCAAGTACCAGAAGAGCACGGAG
W1   ---T-----G---C-----
W2   -G-----C---G-----T
W3                                     -----N-A---
B1   ---T---C---T---T-----TCGT-----T---
B2   ---G-----C-----

```

```

ref  CTGCTCATCCGCAAGCTCCCCTTCCAGCGCCTAGTGCGCGAGATCGCCCA
W1   ----G-----G---G-----G---A-G-----G---
W2   -----G---G-----GC---G-----
W3   ----G---G---N---G---G-----G---N-----T-G---
B1   --T---G---A-G-----T---T---A---T---G---
B2   ----G-----G---G-----G---A-G-----T---

```

```

ref  GGACTTCAAGACCGACCTCCGCTTCCAGTCCTCCGCCGTCTCCGCCCTGC
W1   -----G-----CA-----GCTG-----C-
W2   -----G---T---G-----AG-CA-----GCTG-----C-
W3   -----G-----CA-----GCTG-----C-
B1   -----A-----TTCGA-A-----A---AG-----ATGG-----
B2   -----A-G-----CA-----GCTG-----C-

```

```

ref  AGGAGGCCGCGGAGGCCTACCTCGTGGGCCTCTTCGAGGACACCAACCTC
W1   -----G-----G---G---G---G-----G-
W2   -----G-----C---G-----C-
W3   -----G-----G---C---G---G-----G-
B1   -----GT-----G---T---T---T-----T-G-
B2   -----T-----NA-----T---C---G---G-----G-

```

```

ref  TCGGCCATCCACGCCAAGCGCGTCACCATCATGCCCAAGGACATCCAGCT
W1   -----
W2   -----T-----
W3   -----GC-----
B1   -----GC-----
B2   -----

```

Three wheat (W1, W2, W3) and two barley (B2, B3) clones contained one intron located at an identical position, between nucleotides 72 and 73 of the coding region. Intron length was similar (98 - 120 bp.), but sequence similarity between them was practically absent. Nucleotide sequences of the introns presented on Figure 19.

NUCLEOTIDE SEQUENCE OF THE INTRONS

W1	GTCCGTCCCCTCCCGTCTTCTGAATTCGATCTGTTTCATCCTTGTTGTTTC GAGATTGGTTGGCTGACGTTTTGGATTGAATCGTTGTGTGTTTGCAG
W2	GTCCGCCTCATCCCCTCCCTTCTCCCCATCATCTGTTCCCCTTCCTCCGT TCCGATCTGTTTCGATGTGGTTTTGTTTGCTGACGGTGATTCCGCGCGTGT GATTGCAG
W3	ATCGTACGTCTCGCCGCCTCCCTTGTCTCCTTCTTCCCCTGTTCCCATCG GTTGCTATTTTCGCTGATGATCGACGGTTTCGATTGTGGCGTTTCCTTTTC AG
B1	GTCCGTCCCCTCTCCTCTCCCCTCCCTTATCCTTCCCCTCTTTTGATTTC GATACGTTTCGTCGTCGGGAGTTGTTGACTGACGCTTCGGATTTCGTTTCAT CGTTGTGTTTTTCAG
B2	ATCGTCGGTCCCGCCTCCCCCCCCCTTTTCCCGTCCGTTTCATGAGGATTT CGTTGACGATCGTTTCTTTTCCCTTTTCCCGTCCGTTTCATGAGGATTTTCG CTGACGATCGTGTCGTTGCAG

Figure 19. Nucleotide sequences of the introns from cloned wheat and barley histone H3 genes.

However, short nucleotide stretches on the 5' and 3' ends of each of the introns were well-conserved. Introns from clones W1, W2 and B2 had a GTCCG sequence at the 5' end, while clones W3 and B3 had a different motif - ATCGT at the 5' end. All introns had a TT(T/G)CAG sequence at their 3' end. These

two groups differed in the structure of the exon-intron junction as well: clones W1, W2 and B2 had "classical" exon-intron junction sequences (agGT...AGgc) (Senapathy et al. 1990), while clones W3 and B3 had slightly different boundary sequences at the 5' splicing site (ccAT...AGgc). Despite the absence of internal homology among the intervening sequences of cloned H3 genes, one commonly observed among all introns characteristic was long interspersed stretches of C and T nucleotides.

Mapping the H3 Histone Genes on Barley Chromosomes

Southern hybridization of cloned histone H3 and H4 genes with digested with several restriction endonucleases genomic DNA of barley and wheat revealed over 20 hybridizing bands (Fig. 20). The majority of bands represent single copies of the genes. Only a few bands hybridize stronger than others. Several bands on these blots hybridize to both H3 and H4 probes. This suggests an association between H3 and H4 genes. Analysis of three barley cultivars, Nutans, Odessky, and Betzes, and wheat cv. Chinese Spring reveal a high level of intervarietal polymorphism.

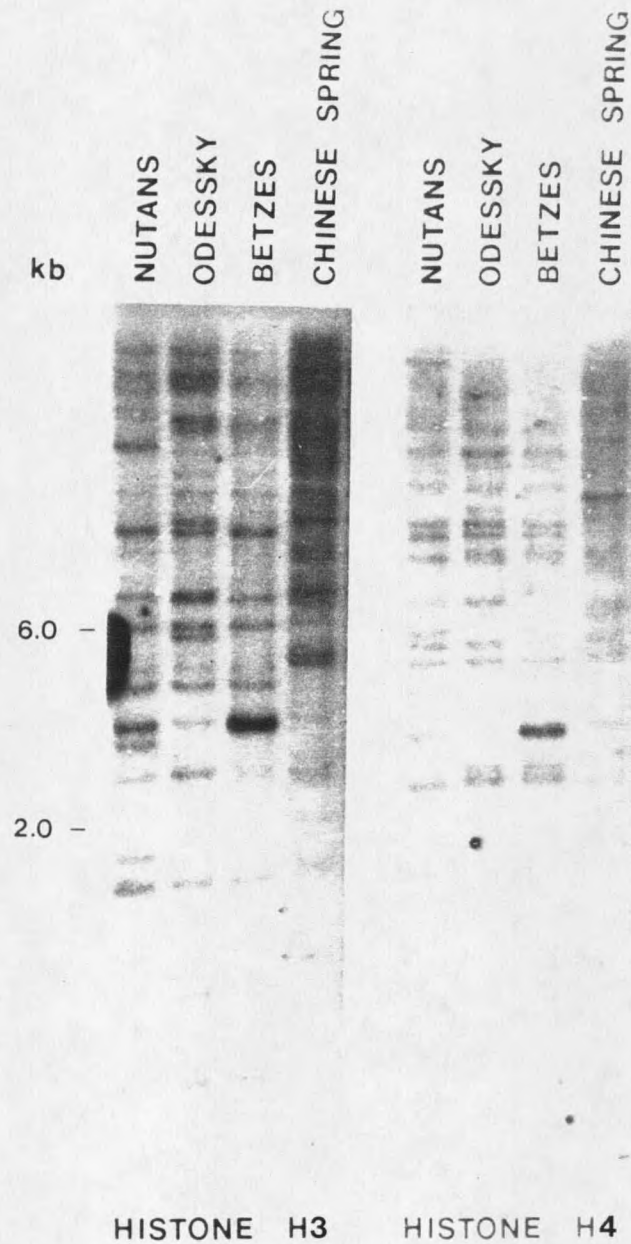


Figure 20. Southern hybridization of barley and wheat genomic DNA with cloned H3 and H4 histone genes. Genomic DNA was digested with *Bam*HI and separated in 1% agarose gel.

To localize histone genes on barley chromosomes, cloned H3 genes were used as probes against Southern blots containing genomic DNA from 150 doubled haploid lines (derived from a Steptoe x Morex cross and used in the North American Barley Genome Mapping Project; Kleinhofs et al. *in press*). Each tested enzyme (*EcoRI*, *EcoRV*, *DraI*, *HindIII*) revealed several polymorphic bands. This allowed mapping of these genes at several loci located on at least four barley chromosomes. It appears likely that histone genes are present on all barley chromosomes. However, this data is difficult to adapt for genetic analysis in barley. High copy number and substantial levels of apparent polymorphism at each locus makes it difficult to critically compare allelic variants over several genotypes. Generation of allele-specific probes would help to resolve this problem.

Generation of Gene-Specific Markers

In order to distinguish individual copies of the H3 gene and to individually map cloned barley H3 genes, introns from clones B2 and B3 were isolated, labelled with ^{32}P , and used as probes in Southern blot analysis. Two blots which contained DNA of doubled haploid lines, digested with *EcoRI*, were first hybridized with the coding region of the barley H3 gene. After autoradiography this probe was removed and the blots were rehybridized with introns isolated from clones B2 and B3 (Fig. 21) and B3 (Fig. 22).

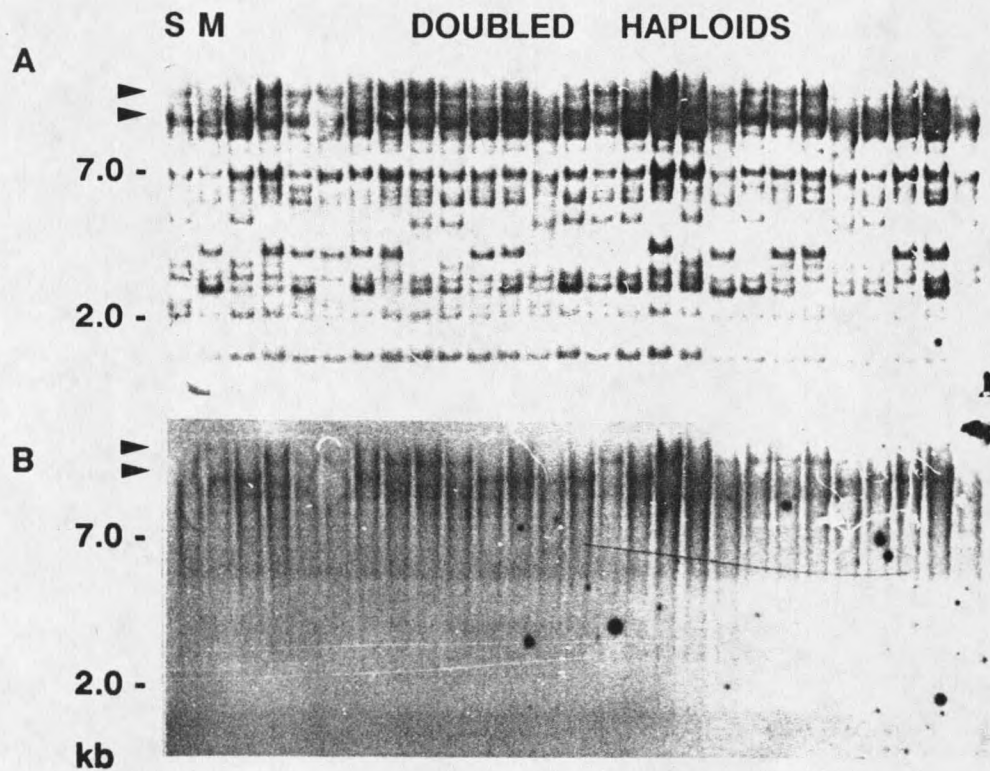


Figure 21. Hybridization of the coding sequence of the clone B2 (top), and the B2 intron (bottom) with *EcoRI* digested DNA of Steptoe x Morex doubled haploid lines. Arrows indicate bands hybridizing with the sequence of the intron.

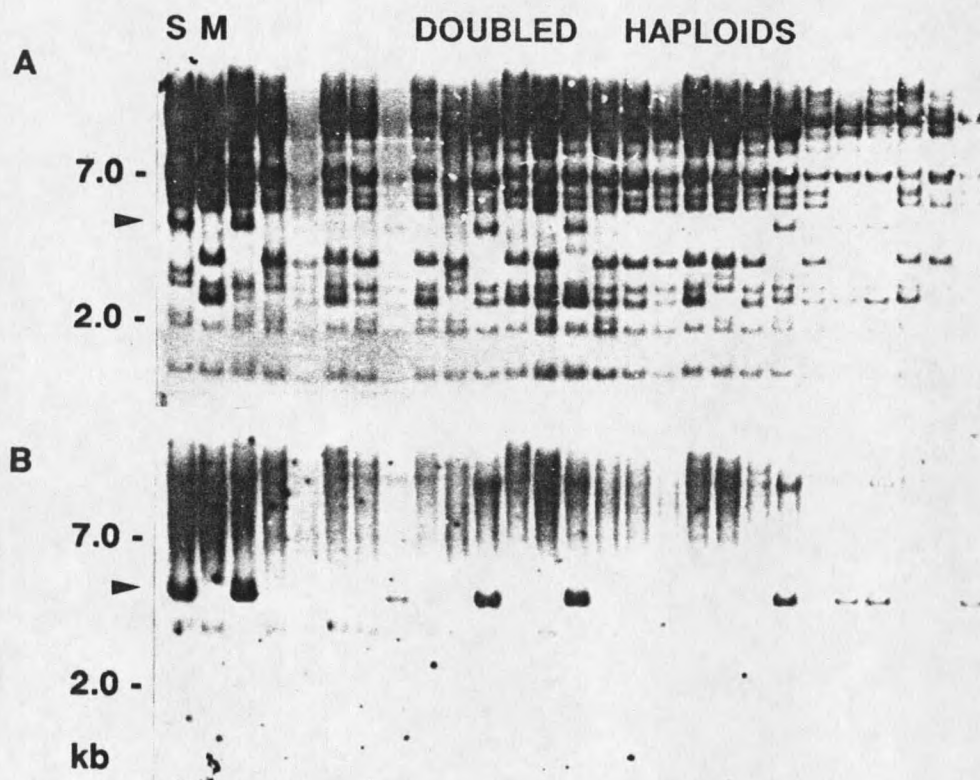


Figure 22. Hybridization of the coding sequence of the clone B3 (top), and the B3 intron (bottom) with *Eco*RI digested DNA of Steptoe x Morex doubled haploid lines. Arrows indicate bands hybridizing with the sequence of the intron.

Introns hybridized to single bands which correspond to one of the dozen bands visible on autoradiographs after initial hybridization with the coding sequence. Intron B2 hybridized to an approximately 11 kb band in Steptoe, and an 10 kb band in Morex. Intron B3 identified a presence\absence of a single hybridizing band type polymorphism between Steptoe and Morex. That indicates that gene variants bearing these introns probably are present in the barley genome in a single copy. The B2 intron was mapped to barley chromosome 1, and the B3 intron B3 mapped to chromosome 4.

The presence of a conserved sequence TT(T/G)CAG on the 3' end of all sequenced introns permitted the synthesis of an intron-specific primer KV24 (5'-GACTTCCTC/GGCCGCCTGCAA-3'). This primer, used with KV12, directed amplification only from histone H3 genes which contain intervening sequences, and eliminated the problem of being "outcompeted" by the intronless variants. Amplification of the first exon and an intron from genomic DNA of several cultivars of wheat and barley resulted in the production of several bands ranging in size from 160 to 210 bp. The degree of polymorphism in PCR product number and size between cultivars was very high (Fig. 23).

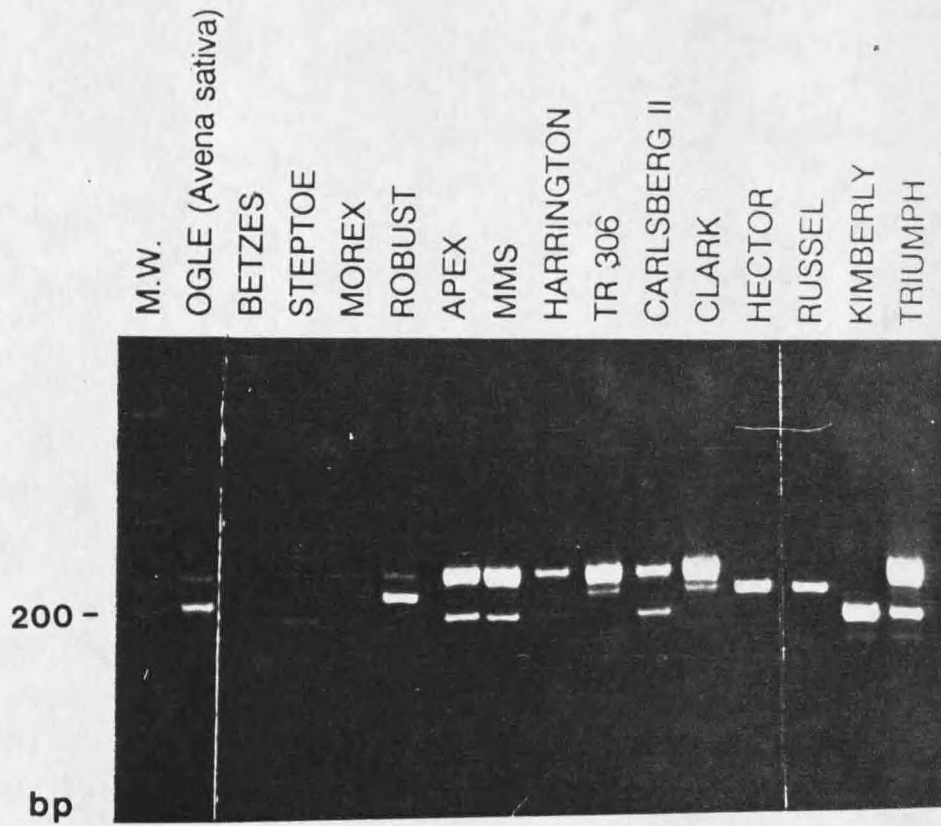


Figure 23. PCR amplification of the introns from the histone H3 genes from several varieties of barley. Products of amplification were separated in a 7% polyacrylamide gel.

Steptoe and Morex had two prominent bands; one was common to both cultivars while the other showed a difference. Segregation of the PCR amplification pattern was tested in the doubled haploid population (Fig. 24). The results were identical to results seen by Southern blot analysis with B2 intron as a probe, previously mapped to chromosome 1.

When primers KV12 - KV24 were tested on a set of wheat nullitetrasonic lines (Sears, 1954; DNA kindly provided by Dr. L. Talbert), one prominent band mapped to wheat chromosome 7A. Wheat chromosome 7 is the homeologue of barley chromosome 1 (data not shown).

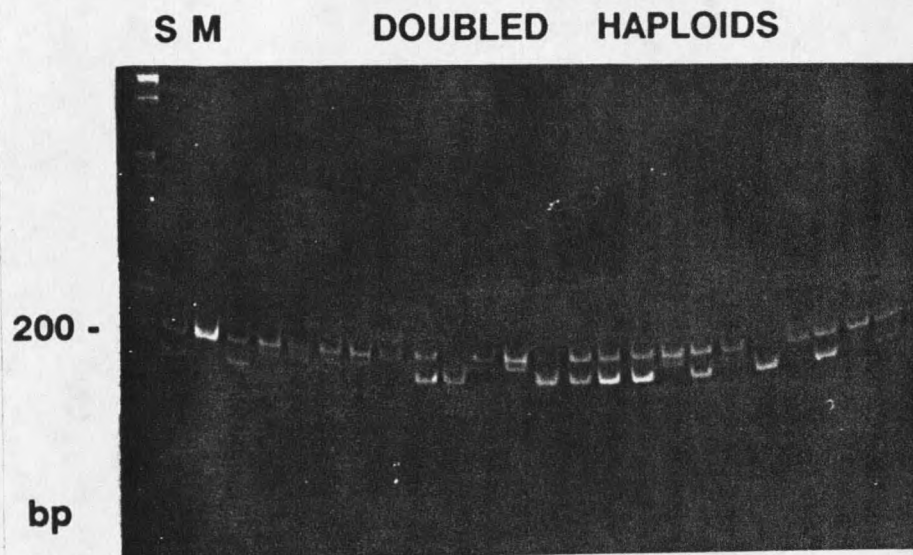


Figure 24. PCR amplification of histone H3 introns in population of Steptoe x Morex DHL. Products of amplification were separated in 7% polyacrylamide gel.

Discussion

Organization and copy number of the histone genes in higher eukaryotes is very diverse and different from the organization seen in lower eukaryotes. In sea urchin, whose histone organization is extensively studied, H1 and each of the core (H2A, H2B, H3 and H4) histone genes are arranged into tandemly repetitive units (Hentschel and Birnstiel, 1981). In chicken, mouse and humans, histone genes are clustered into random arrays with differences in degree of homology, order and spacing between genes from cluster to cluster. Solitary and clustered histone genes may coexist within genome. Preferential pairing of H3-H4 and H2A-H2B genes is observed (Maxson et al. 1983; Sierra et al. 1982).

Little is known about the organization of the histone genes in plants. The copy number of histone genes is estimated to be 80-100 for H3 and 100-125 for H4 in hexaploid wheat (Tabata et al 1983, 1984), and 60-80 for H3 and 100-120 for H4 in maize (Chaubet et al. 1986). Rogers and Bendich (1992) estimated the number of H3 and H4 genes in *Vicia faba* to be from 2 to 55 copies. Of monocotyledonous species, H3 and H4 genes have been cloned only from wheat, rice and maize (Chaubet et al. 1986; Gigot et al. 1987; Tabata et al. 1983, 1984). Chaubet et al. (1989) demonstrated, using 5'-noncoding regions of the genes as probes, that maize H3 and H4 genes are organized into eight to ten subfamilies containing four to

sixteen copies. Similar clusters were found in teosinte, sorghum, and sugar cane. No association was observed between H3 and H4 subfamilies. The same group of researchers later demonstrated that H3 and H4 gene subfamilies are located on many, possibly on all chromosomes of maize (Chaubet et al. 1992).

Using PCR primers chosen from the conserved regions of the histone H3 genes several copies of the H3 genes were amplified and cloned from wheat and barley genomes. Three clones derived from wheat and two barley clones contained a single intron. Intron was located in the same position, but its length and sequence varied among different cloned genes. Comparison of the coding sequences among cloned genes showed multiple nucleotide changes, although derived amino-acid sequences were essentially the same.

Several cloned histone H3 genes exhibited nonrandom codon usage. Non-random codon usage in eukaryotes has been well documented (Grantham et al. 1981, Lycett et al. 1983). In maize and wheat H3 and H4 histones, codons ending with A and T are underrepresented and codons ending with AA or TA completely absent (Chaubet et al. 1986, Tabata et al., 1983, 1984). A similar, though less pronounced pattern was observed in H3 genes cloned from chicken, man, and *Xenopus* (Engel et al. 1983, Zhong et al. 1982). However, sequence analysis of H3 and H4 genes cloned from dicotyledonous plants including alfalfa and *Arabidopsis thaliana* did not show such a pattern

(Chaubot et al. 1987, Wu et al. 1988). Grantham et al. (1981) have proposed that nonrandom codon usage reflects differential gene expression rather than evolutionary differences.

Introns isolated from barley and wheat H3 histone genes demonstrated to be useful for targeting individual genes in this complex multigene family. Using them as probes for Southern blots permitted the detection of single bands on genomic blots, instead of a complex picture of over a dozen bands when the coding sequence was used as a probe. The generation of intron-specific primers for PCR also allowed us to isolate and track individual copies of the gene in a segregating population. Design of primers specific for individual sequenced introns might permit generation of convenient STS-PCR markers for analysis of cereal genomes.

CONCLUSIONS

A substantial portion of the genome in higher plants consists of multigene families. Members of multigene families often exhibit great variability in structure and composition within genotypes, among genotypes, within species and among species. Analysis of their sequence and organization could illuminate the mechanisms involved in genome evolution. This report demonstrates the presence of multiple sequence and length variants among members of the B-hordein, 5S rRNA, and histone H3 multigene families.

Analysis of the structural organization of the B-hordein genes revealed the presence of a variable region within the structural portion of the *Hor-2* gene. This domain appears to undergo frequent changes in the number of short tandem repeats due to unequal crossing over or DNA strand slippage during replication. The resulting differences in the length of the gene's repetitive domain could account for much of the variation observed on the protein level.

PCR served as a convenient tool for structural analysis of the variable region of B-hordein genes. Simple electrophoretic techniques coupled with PCR amplification of the variable number tandem repeat region of the B-hordein genes, revealed levels of diversity similar to that seen with SDS-PAGE of the B-hordeins.

PCR also proved useful in the analysis of variation among

5S rRNA encoding loci. Primers, complementary to the conserved coding sequences of the 5S genes, were used to amplify, clone, and sequence 5S nontranscribed spacers from these multigene families in cultivated barley and several wild species of barley. *H. vulgare* appeared to have three clusters of 5S rRNA genes located on different chromosomes. Structural differences between spacers from different gene clusters allowed the isolation of gene-specific probes and convenient chromosome-specific markers. Sequence comparisons between rapidly diverging 5S spacers from different barley species provided new insight into evolutionary relationships within the genus *Hordeum*. New information was obtained concerning genomic constitution and the possible origin of polyploid barley species.

Southern blot analysis showed the histone H3 genes to be located on several chromosomes. Primers complementary to the 5' and 3' regions of the histone H3 coding sequence amplified products, most of which corresponded to the previously described in plants intronless gene structure. In addition, products somewhat larger than expected were observed. After cloning and sequencing, they were identified as histone H3 genes with intervening sequences. The coding sequences were very similar in all the histone H3 genes cloned. Conversely, nucleotide sequences of the introns varied dramatically from clone to clone. Primer sequences which incorporated these intron sequences and isolated introns were utilized as markers

in the North American Barley Genome Mapping Project.

Sequence diversity among members of multigene families permitted the evaluation of individual genes within several barley gene families. These approaches should elaborate the varied mechanisms of heritable variation and elucidate the role of individual gene family members in development and evolution of species.

REFERENCES CITED

- Alexander, W.L., Hockett, E.A., Blake, T.K., and Hoffman, D.L. 1990. Esterase 1 and C hordein allelic and genotypic frequencies in CC XXXI, a male sterile barley population. *Euphytica* 46:165-168.
- Allard, R.W., Saghai Maroof, M.A., Zhang, Q., and Jorgensen, R.A. 1990. Genetic and molecular organization of ribosomal DNA (rDNA) variants in wild and cultivated barley. *Genetics* 126:743-751.
- Baird, W.V., and Meagher, R.B. 1987. A complex gene superfamily encodes actin in petunia. *EMBO J.* 6:3223-3231.
- Barbier, P., Morishima, H., and Ishihama, A. 1991. Phylogenetic relationships of annual and perennial wild rice: probing by direct DNA sequencing. *Theor. Appl. Genet.* 81:693-702.
- Baum, B.R., and Bailey, L.G. 1991. Taxonomy of the North and South American species of *Hordeum* section *Hordeastrum*. *Can. J. Bot.* 64:1745-1759
- Blake, T., Lybeck, N., and Hayes, P. 1991. Good, bad and untested ideas in RFLP and QTL analysis. *Plant Breeding Abstr.* 16:1-7.
- Boerwinkle, E., Xiong, W., Fourest, E., and Chan, L. 1989. Rapid typing of tandemly repeated hypervariable loci by the polymerase chain reaction: Application to the apolipoprotein B 3' hypervariable region. *Proc. Natl. Acad. Sci. USA* 86:212-216.
- Bothmer, R., Flink, J., and Jacobsen, N. 1989. Meiosis in interspecific *Hordeum* hybrids. *Evol. Tr. in Plants.* 3:53-58.
- Bothmer, R., and Jacobsen, N. 1985. Origin, taxonomy, and related species. In *Barley*, 19-56. Agronomy Society of America, Madison, WI.
- Bothmer, R., Jacobsen, N., Baden, C., Jorgensen, R.B., and Linde-Laursen, I. 1991. An ecogeographical study of the genus *Hordeum*. Systematic and ecogeographic studies on crop genepools 7. IBPGR. Rome.
- Bothmer, R., Jorgensen, B., and Linde-Laursen, I. 1987.

- Natural variation, phylogeny and genetic resources in *Hordeum*. *Barley Genet.* 5:22-33.
- Brandt, A., Montembault, A., Cameron-Mills, V., and Rasmussen, S. 1985. Primary structure of a B1 hordein gene from barley. *Carlsberg Res. Commun.* 50:333-345.
- Bunce, N.A.C., Forde, B.G., Kreis, M., and Shewry, P.R. 1986. DNA restriction fragment length polymorphism at hordein loci: application to identifying and fingerprinting barley cultivars. *Seed Sci. Tech.* 14:419-429.
- Chaboute, M.-E., Chaubet, N., Philipps, G., Ehling, M., and Gigot, C. 1987. Genomic organization and nucleotide sequence of two histone H3 and two histone H4 genes in *Arabidopsis thaliana*. *Plant. Mol. Biol.* 8:179-191.
- Chaubet, N., Philipps, G., Chaboute, M.-E., Ehling, M., and Gigot, C. 1986. Nucleotide sequences of two corn histone H3 genes. Genomic organization of the corn histone H3 and H4 genes. *Plant Mol. Biol.* 6:253-263.
- Chaubet, N., Philipps, G., and Gigot, C. 1989. Organization of the histone H3 and H4 multigenic families in maize and in related genomes. *Mol. Gen. Genet.* 219:404-412.
- Chaubet, N., Philipps, G., Gigot, C., Guitton, C., Bouvet, N., Freyssinet, G., Schneerman, M., and Weber, D.F. 1992. Subfamilies of histone H3 and H4 genes are located on most, possibly all of the chromosomes in maize. *Theor. Appl. Genet.* 84:555-559.
- Chernyshev, A.I., Khadirzhanova, D.K., Pomortsev, A.A., Ananiev, E.V., and Sozinov, A.A. 1989. Nucleotide sequence of a B1 hordein gene of barley *Hordeum vulgare* *Genetika (USSR)* 8:1349-1355.
- Childs, G., Maxson, R., Cohn, R.H., and Kedes, L. 1981. Orphans: dispersed genetic elements derived from tandem repetitive genes of eucaryotes. *Cell* 23:651-663.
- Cordesse, F., Grellet, F., Reddy, A.S., and Delseny, M. 1992. Genome specificity of rDNA spacer fragments from *Oryza sativa* L. *Theor. Appl. Genet.* 83: 864-870.
- Costa, R., Peixoto, A., Thackeray, J.R., Dalglish, R., and Kyriacou, C.P. 1991. Length polymorphism in the threonine-glycine-encoding repeat region of the period gene in *Drosophila*. *J. Mol. Evol.* 32:238-246.
- Cox, A.V., Bennett, M.D., and Dyer, T.A. 1992. Use of

- the polymerase chain reaction to detect spacer size heterogeneity in plant 5S-rRNA gene clusters and to locate such clusters in wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* 83: 684-690.
- De Kochko, A., Kiefer, M.C., Cordesse, F., Reddy, A.S., and Delseny, M. 1991. Distribution and organization of a tandemly repeated 352-bp sequence in the *Oryzae* family. *Theor. Appl. Genet.* 82:57-64.
- Dean, C., Elzen, P., Tamaki, S., Black, M., Dunsmuir, P., and Bedbrook, J. 1987. Molecular characterization of the rbcS multi-gene family of *Petunia* (Mitchell). *Mol. Gen. Genet.* 206:465-474.
- Dean, C., Elzen, P., Tamaki, S., Dunsmuir, P., and Bedbrook, J. 1985. Differential expression of the eight genes of the petunia ribulose biphosphate carboxylase small subunit multi-gene family. *EMBO J.* 4:3055-3061.
- Dellaporta, S.L., Wood, J., and Hicks, J.B. 1983. A rapid method for DNA extraction from plant tissue. *Plant Mol. Biol. Rep.* 1:19-21.
- Doebley, J., Bothmer, R., and Larson, S. 1992. Chloroplast DNA variation and the phylogeny of *Hordeum* (Poaceae). *Amer. J. Bot.* 79:576-584.
- Doll, H., and Andersen, B. 1981. Preparation of barley storage proteins, hordein, for analytical sodium dodecyl sulfate - polyacrylamide gel electrophoresis. *Anal. Biochem.* 115:61-66.
- Dover, G. 1982. Molecular drive: a cohesive mode of species evolution. *Nature.* 299:111-117.
- Dvorak, J., McGuire, P.E., and Cassidy, B. 1988. Apparent sources of the A genomes of wheats inferred from polymorphism in abundance and restriction fragment length of repeated nucleotide sequences. *Genome.* 30:680-689.
- Dvorak, J., and Zhang, H.-B. 1992. Reconstruction of the phylogeny of the genus *Triticum* from variation in repeated nucleotide sequences. *Theor. Appl. Genet.* 84:419-429.
- Dvorak, J., Zang, H.-B., Kota, R.S., and Lassner, M. 1989. Organization and evolution of the 5S ribosomal RNA gene family in wheat and related species. *Genome* 32: 1003-1016.
- Engel, J.D., Sugarman, B.J., and Dodgson J.B. 1982. A chicken

- histone H3 gene contains intervening sequences. *Nature* 297:434-436.
- Faulks, A.J., Shewry, P.R., and Mifflin, B.J. 1981. The polymorphism and structural homology of storage polypeptides (hordein) coded by the *Hor-2* locus in barley (*Hordeum vulgare*, L.). *Biochem. Genet.* 19:841-858.
- Feinberg, A.P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Felsenstein, J. 1987. PHYLIP (Phylogenetic inference package) version 3.0 manual. University of Washington, Seattle.
- Fitch, D.H.A., Strausbaugh, L.D., Barret, V. 1990. On the origin of tandemly repeated genes: does histone gene copy number in *Drosophila* reflect chromosomal location? *Chromosoma*. 99:118-124.
- Forde, B.G., Heyworth, A., Pywell, J., and Mifflin, B.J. 1985a. Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. *Nuc. Acids Res.* 13:7327-7339.
- Forde, B.G., Kreis, M., Bahramian, M.B., Matthews, J.A., Mifflin, B.J., Thompson, R.D., Bartels, D., and Flawell, R.B. 1981. Molecular cloning and analysis of cDNA sequences derived from poly A⁺ RNA from barley endosperm: identification of B hordein related clones. *Nuc. Acids Res.* 9:6689-6707.
- Forde, B.G., Kreis, M., Williamson, M.S., Fry, R.P., Pywell, J., Shewry, P.R., Bunce, N., and Mifflin, B.J. 1985b. Short tandem repeats shared by B- and C-hordein DNAs suggest a common evolutionary origin for two groups of cereal storage protein genes. *EMBO J.* 4:9-15.
- Ghazal, P., Clark A.J., and Bishop J.O. 1985. Evolutionary amplification of a pseudogene. *Proc. Natl. Acad. Sci. USA* 82:4182-4185.
- Gigot, C., Chaubet, N., Chaubote, M.E., Ehling, M., and Philipps, G. 1987. Nucleotide sequence of two histone H3 and H4 genes of corn. Further insight into the structure and organization of the histone genes in higher plants. *Plant Physiol. Biochem.* 25:235-247.
- Graner, A., Jahoor, A., Schondelmaier, J., Siedler, H., Pillen, K., Fishbeck, G., Wenzel, G., and Herrmann, R.G. 1991. Construction of an RFLP map of barley. *Theor. Appl.*

Genet. 83:250-256.

- Grantham, R., Gauthier, C., Gouy, M., Jacobzone, M., and Mercier, R. 1981. Codon catalog usage is a genomic strategy modulated for gene expressivity. Nucl. Acids Res. 9: r43-r74.
- Hayes, P., and Chen, M. 1989. Genotypic variation for *Hordeum bulbosum* -mediated haploid production in winter and facultative barley. Crop Sci. 29:1184-1188.
- Heidecker, G., Chaudhuri, S., and Messing, J. 1991. Highly clustered zein gene sequences reveal evolutionary history of the multigene family. Genomics 10:719-732.
- Hentschel, C.C., and Birnstiel, M.L. 1981. The organization and expression of histone gene families. Cell 25:301-313.
- Heun, M., Kennedy, A.M., Anderson, J.A., Lapitan N.L.V., Sorrells, M., and Tanksley, S.D. 1991. Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34:437-447.
- Hickey, D.A., Bally-Cuif, L., Abukashawa, S., Payant, V., and Benkel, B.F. 1991. Concerted evolution of duplicated protein-coding genes in *Drosophila*. Proc. Natl. Acad. Sci. USA 88:1611-1615.
- Hillis, D.M., Moritz, C., Porter, C.A., and Baker, R.J. 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science. 251:308-310.
- Islam, A.K.R.M., Sheperd, K., and Sparrow, D.H.B. 1981. Isolation and characterization of the wheat-barley chromosome addition lines. Heredity 46:161-174.
- Jacobsen, N., and Bothmer, R. 1992. Supraspecific groups in genus *Hordeum*. Hereditas 116:21-24.
- Jeffreys, A., Royle, N.J., Wilson, V., and Wong, Z. 1988. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature. 332:278-281.
- Jorgensen, R.B. 1986. Relationships in the barley genus (*Hordeum*): An electrophoretic examination of proteins. Hereditas 104:273-291.
- Kanazin, V., Ananiev, E., and Blake, T. 1993. Variability among the members of the *Hor*-2 multigenefamily. Genome (in press).

- Kanazin, V., Ananiev, E., and Blake, T. The genetics of 5S rRNA encoding multigene families in barley. *Genome* (in review).
- Kasha, K.G., and Kao, K.N. 1970. High frequency haploid productio in barley (*Hordeum vulgar*, L) *Nature* 225:874-876.
- Khvyrleva, C.D., Gazumyan, A.K., and Ananiev, E.V. 1988. Organization and primary nucleotide sequence of the 5S rRNA genes in barley. *Genetica* (Moscow) 24: 1830-1840.
- Kimber, G., and Yen, Y. 1988. Analysis of pivotal-differential evolutionary patterns. *Proc. Natl. Acad. Sci. USA* 85:9106-9108.
- Kleinhofs, A., Kilian, A., Saghai-Marooof, M.A., Biyashev, R.M., Hayes, P., Chen, F.Q., Lapitan, N., Fenwick, A., Blake, T.K., Kanazin, V., Ananiev, V., Dahleen, L., Kudrna, D., Bollinger, J., Knapp, S.J., Liu, B., Sorrels, M., Heun, M., Franckowiak, J.D., Hoffman, D., Skadsen, R., and Steffenson, B.J. 1993. A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. *Theor. Appl. Genet.* (in press).
- Koes, R.E., Spelt, C.E., Mol, J.N.M., and Gerats, A.G.M. 1987. The chalcone synthase multigene family of *Petunia hybrida* (V30): sequence homology, chromosomal localization and evolutionary aspects. *Plant Mol. Biol.* 10:375-385.
- Kolchinsky, A., Kanazin, V., Yakovleva, E., Gazumyan, A., Koe, C., and Ananiev, E. 1990. 5S-RNA genes of barley are located on the second chromosome. *Theor. Appl. Genet.* 80:333-336.
- Kolchinsky, A., Kolesnikova, M., and Ananiev, E. 1991. "Portraying" of plant genomes using polymerase chain reaction amplification of ribosomal 5S genes. *Genome* 34: 1028-1030.
- Kreis, M., Rahman, S., Forde, B.G., Pywell, J., Shewry, P.R., and Miflin, B.J. 1983a. Sub-families of hordein mRNA encoded at the *Hor-2* locus of barley. *Mol. Gen. Genet.* 191:194-200.
- Kreis, M., Shewry, P.R., Forde, B.G., Forde, J., and Miflin, B.J. 1985. Structure and evolution of seed storage proteins and their genes with particular reference to those of wheat, barley and rye. *Oxford Surv. Plant. Mol. Cell Biol.* 2:253-317.
- Kreis, M., Shewry, P.R., Forde, B.G., Rahman, S., and Miflin,

- B.J. 1983b. Molecular analysis of a mutation conferring the high-lysine phenotype of the grain of barley (*Hordeum vulgare*). Cell 34:161-167.
- Lander, E.S., and Botstein, D. 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185-199.
- Lagudah, E.S., Clarke, B.C., and Appels, R. 1989. Phylogenetic relationships of *Triticum tauschii*, the D-genome donor to hexaploid wheat. 4. Variation and chromosomal location of 5S DNA. Genome 32: 1017-1025.
- Leitch, I.J., and Heslop-Harrison, J.S. 1993. Physical mapping of four sites of 5S ribosomal DNA sequences and one site of the α -amylase 2 gene in barley (*Hordeum vulgare*). Genome (in press).
- Levinson, G., and Gutman, G. 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol. Biol. Evol. 4:203-221.
- Linde-Layrsen, I.B., Bothmer, R., and Jacobsen, N. 1992. Relationships in the genus *Hordeum*: Giemsa C-banded karyotypes. Hereditas 116:111-116.
- Lund, R.E. 1986. MSUSTAT Statistical Analysis Package. Montana State University.
- Lycett, G.W., Delauney, A.J., and Croy, R.R.D. 1983. Are plant genes different? FEBS Lett. 153:143-146.
- Lyons, K.M., Stein, J.H., and Smithies, O. 1988. Length polymorphisms in human proline-rich protein genes generated by intragenic unequal crossing over. Genetics, 120:267-278.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Maxson, R., Kohn, R., and Kedes, L. 1983. Expression and organization of histone genes. Ann Rev. Genet. 17:239-277.
- Mayr, E. 1982. The growth of biological thought. Bellknap. Cambridge.
- McElroy, D., Rothenberg, M., Reece, K.S., and Wu, R. 1990. Characterization of the rice (*Oryza sativa*) actin gene family. Plant Mol. Biol. 15:257-268.

- Mignotte, F., Gueride, M., Champagne, A., and Mounolou, J. 1990. Direct repeats in the non-coding region of rabbit mitochondrial DNA. *Eur. J. Biochem.* 194:561-571.
- Mukai, Y., Endo, T.R., and Gill, B.S. 1990. Physical mapping of the 5S rRNA multigene family in common wheat. *J. Hered.* 81:290-295.
- Muskavitch, M.A.T., and Hogness, D.S. 1982. An expandable gene that encodes a *Drosophila* glue protein is not expressed in variants lacking remote upstream sequences. *Cell* 29:1041-1051.
- Nevski, S.A. 1941. Materialien zur der kenntnis der wildwachsenden gersten im zusammenhang mit der frage uber den ursprung von *Hordeum vulgare* L. und *Hordeum distichum* L. *Acta Inst. Bot. Nomine V.L.Komarovii, Acad.Sci. U.S.S.R.*
- Old, R.W., and Woodland, H.R. 1984. Histone genes: not so simple after all. *Cell* 38:624-626.
- Oram, R.N., Doll, H., and Koie, B. 1975. Genetics of two storage protein variants in barley. *Hereditas*, 80:53-58.
- Osborne, T.B. 1924. The vegetable proteins. Longmans, Greene & Co, London.
- Phillips, R.L. 1978. Molecular cytogenetics of the nucleolus organizer region. In: *Maize Breeding and Genetics*, Edited by D.Walden. New York. 711-741.
- Pichersky, E. 1990. Nomad DNA - a model for movement and duplication of DNA sequences in plant genomes. *Plant Mol. Biol.* 15:437-448.
- Pohler, W., Clauss, E., and Staat, B. 1986. Confirmation of genetic diploidization in *Hordeum geniculatum* 4x by meiotic investigation of hybrid plants. *Arc. Zuchtungforschung.* 16:91-94.
- Reddy, P., and Appels, R. 1989. A second locus for the 5S multigene family in *Secale* L.: sequence divergence in two lineages of the family. *Genome* 32:456-467.
- Rivin, C.J., Cullis, C.A., and Walbot, V. 1986. Evaluating quantitative variation in the genome of *Zea mays*. *Genetics.* 113:1009-1019.
- Rogers, S.O., and Bendich, A.J. 1988. Recombination in *Escherichia coli* between cloned ribosomal RNA intergenic spacers from *Vicia faba*: a model for the

- generation of ribosomal RNA gene heterogeneity in plants. *Plant Sci.* 55:27-31.
- Rogers, S.O., and Bendich, A.J. 1992. Variability and inheritance of histone genes H3 and H4 in *Vicia faba*. *Theor. Appl. Genet.* 84:617-623.
- Ryder, T.B., Hedrick, S.A., Bell, J.N., Liang, X., Clouse, S.D., and Lamb, C.J. 1987. Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. *Mol. Gen. Genet.* 210:219-233.
- Saghai Maroof, M.A., Allard, R.W., and Zhang, Q. 1990. Genetic diversity and ecogeographical differentiation among ribosomal DNA alleles in wild and cultivated barley. *Proc. Natl. Acad. Sci. USA* 87:8486-8490.
- Saghai Maroof, M.A., Solliman, K.M., Jorgensen, R.A., and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian distance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* 81:8014-8018.
- Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Scoles, G.J., Gill, B.S., Xin, Z.-Y., Clarke, B.C., McIntyre, C.L., Chapman, C., and Appels, R. 1988. Frequent duplication and deletion events in the 5S RNA genes and the associated spacer regions of the *Triticeae*. *Pl. Syst. Evol.* 160:105-122.
- Schon, C., Sanchez, M., Blake, T., and Hayes, P.M. 1990. Segregation of Mendelian markers in doubled haploid and F_2 progeny of a barley cross. *Hereditas* 113:69-72.
- Sears, E. 1954. The aneuploids of common wheat. *Res. Bull. Mo. Agric. Exp. Sta.* No.472.
- Senapathy, P., Shapiro, M.B., and Harris, N.L. 1990. Splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. *Methods Enzymol.* 183:252-278.
- Shewry, P.R., Ellis, J.R.S., Pratt, H.M., and Mifflin, B.J. 1978. A comparison of methods for the extraction and separation of hordein fractions from 29 barley varieties. *J. Sci. Food Agric.* 29:433-441.
- Shewry, P.R., Parmar, S., Franklin, J., and Burgess, S.R.

1990. Analysis of a rare recombination event within the multigenic *Hor-2* locus of barley (*Hordeum vulgare*). Genet. Res. Cambr. 55:171-176.
- Shewry, P.R., and Tatham, A.S. 1990. The prolamine storage proteins of cereal seeds: structure and evolution. Biochem. J. 267:1-12.
- Shin, J.S., Chao, S., Corpuz, L., and Blake, T. 1990. A partial map of the barley genome incorporating restriction fragment length polymorphism, polymerase chain reaction, isozyme, and morphological marker loci. Genome 33:803-809.
- Sierra, F., Lichter, A., Marashi, F., Rickles, R., Van Dyke, T., Clark, S., Wells, J., Stein, G., and Stein, J. 1982. Organization of human histone genes. Proc. Natl. Acad. Sci. USA 79:1795-1799.
- Silverthorne, J., Wimpee, C.F., Yamada, T., Rolfe, S.A., and Tobin, E.M. 1990. Differential expression of individual genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in *Lemna gibba*. Plant Mol. Biol. 15:49-58.
- Studer, R., Kammerbauer, C., Zischler, H., and Hinkkanen, A. 1991. Highly instable (GATA)_n-containing sequences of the mouse during cloning process. Electrophoresis, 12:153-158.
- Sugita, M., Manzara, T., Pichersky E., Cashmore, A., and Gruissem, W. 1987. Genomic organization, sequence analysis and expression of all five genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from tomato. Mol. Gen. Genet. 209:247-256.
- Tabata, T., Fukasawa, M., and Iwabuchi, M. 1984. Nucleotide sequence and genomic organization of a wheat histone H3 gene. Mol. Gen. Genet. 196:397-400.
- Tabata, T., Sasaki, K., and Iwabuchi, M. 1983. The structural organization and DNA sequence of a wheat histone H4 gene. Nucl. Acids Res. 11:5865-5874.
- Thomas, G., and Radayatty, J.D. 1983. Organization and bidirectional transcription of H2A, H2B and H4 histone genes in rice embryos. Nature 306:82-84.
- Thomas, G., and Radayatty, J.D. 1984. Restriction map and partial sequence of a rice DNA fragment carrying histone

genes H2A, H2B and H4. Indian J. Biochem. Biophys. 21:1-6.

Tripputi, P., Emanuel, B.S., Croce, C.M., Green, L.G., Stein, G.S. and Stein, J.L. 1986. Human histone genes map to multiple chromosomes. Proc. Natl. Acad. Sci. USA 83:3185-3188.

Vicente-Carbajosa, J., Betashvili, D.R., Kraev, A.S., and Skryabin, K.G. 1992. Conserved structure and organization of B-hordein genes in the *Hor-2* locus of barley. Plant Mol. Biol. 18:453-458.

Wells, D., Hoffman, D., and Kedes, L. 1987. Unusual structure, evolutionary conservation of non-coding sequences and numerous pseudogenes characterize the human H3.3 histone multigene family. Nucl. Acids Res. 15:2871-2889.

Wells, D., and Kedes, L. 1985. Structure of a human histone cDNA: evidence that basally expressed histone genes have intervening sequences and encode polyadenylylated mRNAs. Proc. Natl. Acad. Sci. USA. 82:2834-2838.

Windenger, J., Rohrig, H., Horicke, C., Wing, D., and Schell, J. 1989. Differential regulation of soybean chalcone synthase genes in plant defence, symbiosis and upon environmental stimuli. Mol. Gen. Genet. 218:315-322.

Wolff, R., Plaetke, R., Jeffreys, A., and White, R. 1989. Unequal crossingover between homologous chromosomes is not the major mechanism involved in the generation of new alleles at VNTR loci. Genomics 5:382-384.

Woudt, L.P., Pastink, A., Kempers-Veenstra, A., Jansen, A.E.M., Mager, W.H., and Planta, R.J. 1983. The genes coding for histone H3 and H4 in *Neurospora crassa* are unique and contain intervening sequences. Nucl. Acids Res. 11:5347-5360.

Wu, S.-C., Bogre, L., Vincze, E., Kiss, G.B., and Dudits, D. 1988. Isolation of an alfalfa histone H3 gene: structure and expression. Plant Mol. Biol. 11:641-649.

APPENDIX

NUCLEOTIDE SEQUENCE OF THE CLONED 5s rDNA NONTRANSCRIBED SPACERS FROM WILD HORDEUM SPECIES

H. bulbosum

Clone #1

TGGGAAGTCC	TCGTGTTGCA	TTCCTTTTTG	AATATGTTTT	TACGCCACGT
GGCAGCGATG	ACGCATGTGA	GTGCTATATA	TGAAGCCCAT	TTTATTATTT
TTGACGTTTT	GTGAAGTGTT	TTAGCTCACG	ACTCATTGTT	CAGGCGTATA
TGTGCGGCAA	GGGCGTTCAG	AAAGTGGTCG	GGGATGTGTT	AGCGCTCGTG
TTGTGAGAGT	AGAGGGGGCG	GGGCGAAGTC	TGTGGTAACC	TCGTGTCCGT
TGTTGAGCGG	AGGGTGAGTA	GTGTGGTGCG	GTATCCATTA	CTAGGCAATG
GTGGCGAGAA	TGCACGGCCC	GTCTTTTTAG	TGAAACGTGG	GAGGGACTGC
ATAGGAAACA	AATAGTTATG	AACATGTCGG	ATGCGATCAT	ACCAGCACTA
AA				

Clone #2

TGGGAAGATC	CTCGTGTTGC	ATTCCTTTTT	GAATATATTT	TTACGCCACG
TGGCAGCGAT	GACGCACGTG	AGCGTTATAT	ATGAAGCCCT	TTTATTATTT
TTGACGTTTT	GTGAAGTGTT	TTAGCTCACG	GCTCATTGTT	CAGGCGTATA
TGTGCGGCAA	GGGCGTTCGG	AAAGTGGTCG	GGGACGTGTT	AGCGCTCGTG
TTGTGAGAGT	AGAGGGGGTG	GGACGAAATC	TGTGGTAACC	TCGTGTCCGT
TGTTGAGCGG	AGGGTGAGTA	GTGTGGTGCG	GTATCCATTA	CTAGGCAATG
GTGGTGAGAA	TGCACGGCCC	GTCTTTTTAG	TGAACGTGGG	AGGGACTGCA
TAAGGAAACA	AATAGTTATG	AACATGTCGG	ATGCGATCAT	ACCAGCACTA
AA				

H. glaucum

Clones #101, 102

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATATTTT	TTCTGCGCGC
GGGGAACATG	GCGCACGTGC	CAGTTAAATA	TTACACATGG	TTTCAACCGG
TAAGTTTTAG	CTTGTTGGTT	ATTAATCACG	CGTCTAAGGC	CGGATAAGTA
GTGTTGGCAA	AGGTACCGCG	TTGAGAAAGG	GGTGTAAGCC	GTGGTGAAAC
TCGTGTTGGT	GCGGTAGAAA	GGGGGGGTGT	GGAAACGATG	GTAAACTAGT
CTCCGTCGTT	AAGCGACAGA	GTAAGTAGTG	CAGTGGAGTA	TCCGTTATTA
GGCAGTGCTG	ACAAAGAAAG	GTAGGGTCGG	GTGTATAGTG	GAGCTGGGAG
GGGCGAGCAT	AAGGGACGAA	GTTGGGGAAA	CATGTCGGAT	GCGATCATAC
CAGCACTAAA				

H. leporinum

Clone #206

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATATTTT	TTCTGCGCGC
GGGGATCATG	GCGCTCGTGT	GCATTAAATA	TTACACATGG	TTTCGAAATT
TTTGACGTTT	CCGGTAAGTT	TTAGCTTGTG	GCTCATTAAT	CACGCGTCTA
AGGACGGATA	AGTAGTGTTG	GCAAAGGTAC	CGCGTTCAGA	AAGGGGTGTA
AACCGTGGTG	AAACTCGTGT	TGGTGCGGTA	GAAAGGGGGT	GTGTGGAAAC
GATGGTAAAC	TAGTCTCCGT	CGTTAAGCGA	CAGAGTAAGT	AGTGCAGTGG
AGTATCCGTT	ATTAGGCAGT	GCTGACAAAG	AAGGTAGGGT	CGGGTTTATC
GTGGAGCTGG	GAGGGGCGAG	CATAAGGGAC	GAAGTTGGGG	ATACATGTCC
GATGCGATCA	TACCAGCACT	AAA		

H. geniculatum

Clone #301

TGGGAAGTCC	TCGTGTTGCA	ATTCTTTTTT	CATTATTATT	TTTGCTGCAC
GCGAGAAACA	TGAGGCCCGT	GCGCGATACA	TATTCAATGC	ATGTGCGGGG
TAAATAGTAA	AGATGGTTTT	TTCTTTCTAA	CACTTGAAGT	GAGTTGCAGC
TTGCGATCGA	TGTTGACGCG	TGTAGGGGCA	GCGTTATGGT	GGGAGAGGCA
CCGCTCTCTG	AAATCGTCGA	CGGCATATGA	AAACTCGTGC	AACCT.....
GGAGGGGTGA	AACCGTGGTA	AATGTGCGAA	CGGCTGTTGA	GGTGGAGAGA
AAGTAGTATG	GTGAAGTATC	GAGCGGTGGC	AAGGAATGTA	TGGTAGAGTT
TGTAGTGGAT	CTAGGAGGGG	CGAGCATAAG	GGACGAAGGG	ATATGAAACA
TGTCGGATGC	GATCATACCA	GCACTAAA		

Clone #302

TGGGAAGTCC	TCGTGTTGCA	TTCGTTTTTT	CATTATTTTA	TTTGCTGCAC
GCCGAGAAAC	ATGAGGCACG	TGCGCGATAC	GTATTGAACG	CACGTGCGGG
GTAATAGTAA	AAGATGGTTT	TTTCTTTCTG	ACATTTCAG	TGAGTTGCAG
CTTGCGATCG	ATGTTGACGC	GTGTAGGGGC	GCGTTATGG	T.....
AGAAGGAGGG	TGAAAACCGT	GGTAAACGTG	CGAACGGCTG	TTGAGGTGGA
GAGAAAGTAG	TATGGTGAAG	TATCGAGCGG	TGGCAAGGAA	TGGATGGTAG
AGTTTGTAGT	GGAGCTAGGA	GGGCCGAGCA	TAAGGGACGA	AGGGATATGA
AACAAGTCGG	ATGCGATCAT	ACCAGCACTA	AA	

H. magellanicum

Clone #401

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATCTTTT	TGCTCCACAC
GAGAAACATT	CGTACGTGTG	CGGAATATAT	TAAGCACGGT	TCCTTATTTT
CGACGTTTTT	GGTAAGTTTT	AGCTTGTGGG	TCATATCCAC	GCATGTAGGG
GCGGCGTTGC	GGTGGAAAAG	TGGTCGGGAA	CTCAAAGGGG	CAGAAACCGC
GGTAAAAGTC	CAGTTGTGCG	GTAGAGAGGG	AGGGGTGGAG	ACCGTGTTAA
ACACGTGTGC	GTTGTTGAGC	GAGAGAGTAA	GTTGTATGGT	GCAGTATTCG
TTATTAGGCA	GCGTCGGCAA	AGAGTGCTCG	ATCGTATTTG	TGGTGGAGCC
GGGAGGGGCG	AGCATAATGG	ACGAAGGCGG	GGGGTAACAT	GTCGGATGCG
ATCATACCAG	CACTAAA			

Clone #402

TGGGAAGTCC TCGTGTGCA TTCCCTTTTT AATATATTTT TCTCCACGCA
 AGAAACATGC GTACGTGCGC GGAACATATT AAGCACGGTT CCTTATTTTC
 GACGTTTTCT ATAAGTTTTA GCTTGCGGGT CATTATCCAC GCGTGTAGGG
 GCGGCGTTGC GGTGAAAAAG TGGTCGGGTA CTGAAAGGGG TCGGTACCGC
 GTAAAAGTCC AGTTGGTGCG ATAGAGAGAG AGGGATGGAG ACGGTGTTAA
 ACAGGTGTGC GTTGTGAGC GGGGGAGTAA GTGGTATGGT GCGGTATTTCG
 TTTTATAGCA GCGTCGGCAA AGAGTGCTCG ATCGTATTTG TGGTGGAGCC
 GGGAGGGGCG AGCATAATGG ACGAAGGCGG GGGTAACATG TCGGATGCGA
 TCATACCAGC ACTAAA

H. muticum

Clone #501

TGGGAAGTCC TCGTGTGCA TTCTCTTTTT AATAAATTTT TGCTCCGCGC
 GAGAAACGAT GACGCACGTG CGCGGTATTT ATTAACAAT GTTTCTTATT
 TTTGGCGTTT GCGGTAAGTT ATAGCTTGCG CGCGGTACTC ACGGTCTAG
 GGGCGGCGTT GCGGTGGCAA AGGTAGCGCG TTGTGAAAGG GGGTCGAAAC
 CGCGGAAGAA CTCGTGTTGG TCGGTAGAG AGGGAAGGGT GGAAACGGTG
 GAAAGCCCGT CTTTGTGTT GAGCGGAGA GTACGTGGTA CCGTGCGGTA
 TCCGTTATTA GGAGCGGTGA AAAAGAATGT ACGGAGGTGT TTATGGTGGA
 GCTGAGAGGG GCTAGAATAA GGGACGAAGG CGGGAGTAAC ATGTCCGATG
 CGATCATACC AGCACTAAA

Clone #502

TGGGAAGTCC TCGTGTGCA TTCTCTTTTT AATAAATTTT TGCTCCGCGC
 GAGAAACGAT CACGCACGTG CGTGGTATTT ATTAACAAT GTTTCTTATT
 TCTGACGTTT GCGGTAAGTT ATAGCTTGCG CGCGGTACTC ACGGTCTAG
 GGGCGGCGTT GCGGTGGCAA AGGTAGCGCG TTGTGAAAGG GGGTCGAAAC
 CGCGGTAGAA CTCGTGTTGG TCGGTAGAG AGGGAAGGGT GGAAACGGTG
 GAAAGCCCGT CTTTGTGTT GAGCGGAGA GTACGTGGTA CCGTGCGGTA
 TCCGTTATTG GGAGCGGTGA AAAAGAATGT ACGGAGGTGT TTATGGTGGA
 GCTGGGATGG GCTAGAATAA GGGACGAAGG CGGGAGTAAC ATGTCCGATG
 CGATCATACC AGCACTAAA

H. chilense

Clone #601

TGGGAAGTCC TCGTGTGCA TTCCCTTTTT AATATAATTT TTCTCCACGC
 GAGAAACATG CGTACGTGTG CGGAATATAT TAAGCTCGGT TCCTTATTTT
 TGACGTTTTT GGTAAGTTTT AGCTTGCGGG TCATTATCCA CGCGTGTAGG
 GGCGGCGTTG CCGGTGAAAA GTGGTCGGGT ACTGAAAGGG GTGGAACGTT
 GGAAATGTCC AGTTGGTGC. GTGTTCAACA CATGTGCGTT
 GTTGAGCGGG GGAGTAAGTG GTACGGTGCA GTATTCGTTA TTAGGCAGCG
 TCGGCAAAGA GTGCTCGATC GTATTTGTGA TGGAGCCGGG AGGGGCGAGC
 ATAGTGGACG AAGACGGGGG TAACATGTCT GATGCGATCA TACCAGCACT
 AAA

Clone #602

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATATTTT	TGCTCCACAC
GGGAAACATT	CGTACGTGTG	CGGAATATAT	TAAACACGGT	TCCTTATTTT
CGACGTTTTT	GGTAAGTTTT	AGCTTGCGGG	TCATTATCCA	CGCGTGTAAG
GGCGGCGTTG	CGGTGGAAAA	GTGGTCGGGT	ACTGAAAGGG	GTCGAAACCG
CGATAAAAGT	CAGTTGGTGC	GTAGAGAGGA	GGGGTGGAGA	CCGTGTTAAA
CACGTGTGTG	CGTTGTTGAC	CGGGAGAGTA	AGTGGTACGG	CGCAGTATTC
GTTATTGAGG	CAGCGTCGGC	AAAGAGTGCT	CGATCGTATT	TGTGGTGGAG
CCGGGAGGGA	CGAGCATAAT	GGACGAAGGC	GGGGGTGACA	GGTCCGATGC
GATCATACCA	GCATAAA			

Clone #603

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATATTTT	TACTCCACAC
GAGAAACATG	CGTACGTGCG	CGGAATATAT	TAAGCACGGT	TCCTTATTTT
CGACGTTTTT	GGTAAGTTTT	AGCTTGCGGA	CATACGG...	
TGGTCGGTCA	TCAAAGGAGG	TTGGAGACTG	TGTTAAACAC	GTGTGCGTTG
TTAAGCGGGG	GAGTAAGTGG	TACGGTGCAG	TATTCGTTAT	TAGGCAGCTT
CGGCAAAGAG	TGCTCGATCG	TATTTGTGGT	GGAGCCGGGA	GGGGCGAGCA
TAATGGACGA	AGGCGGGTGC	AACTTGTGGG	ATGCGATCAT	ACCAGCACTA
AA				

Clone #604

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATAATTT	TTCTCCACGC
GAGAAACATG	CGTACGTGTG	CGGAATATAT	TAAGCTCGGT	TCCTTATTTT
TGACGTTTTT	GGTAAGTTTT	AGCTTGCGGG	TCATTATCCA	CGCGTGTAAG
GGCGGCGTTG	CGGTGGAAAA	GTGGTCGGGT	ACTGAAAGGG	GTGGAAACGT
GGAAATGTCC	AGTTGGTGC.		GTGTTCAACA	CATGTGCGTT
GTTGAGCGGG	GGAGTAAGTG	GTACGGTGCA	GTATTCGTTA	TTAGGCAGCG
TCGGCAAAGA	GTGCTCGATC	GTATTTGTGA	TGGAGCCGGG	AGGGGCGAGC
ATAGTGGACG	AAGACGGGGG	TAACATGTGC	GATGCGATCA	TACCAGCACT
AAA				

H. parodii

Clone #701

TGGGAAGTCC	TCGTGTTGCA	TTCTATTTTT	AATAAATTTT	TGCTCCACGC
GAGAAACATG	ACGCACGTGC	GCGGTATATT	TATTAAACAA	TGTTTCTTAT
TTTTGACGTT	TGCGGTAAAGT	TATAGCTTGC	GGCGCGGTAC	TCACGCGTCT
AGGGGCGGCA	TTGCGGTGGC	AAAGGTAGCG	CGATGTGAAA	GGGGTCGAAA
CCGCGGTAGA	ACTCGTGTTG	GTGCGGTAGA	GAGGGAAGGG	TGGAAACGGT
GGAAAACCCG	TCATTGTTGT	TGATGCGGGA	GAGTACGTGG	TACGTTGCGG
TATCCGTTAT	TAGGAGCGGT	GGAAAAGAAT	GTACGGAGGT	GTTTATGGTG
GAGCTGGGAG	GGGCAAGAAT	AAGGGACGAA	GGCGGGAGTA	ACATGTCGGA
TGCGATCATA	CCAGCACTAA	A		

Clone #702

TGGGAAGTCC	TCGTGTTGCA	TTCTATTTTT	AATAAATTTT	TGCTCCACGC
GAGAAACATG	ACGCACGTGC	GCGGTATATT	TATTAAACAA	TGTTTCTTAT
TTTTGACGTT	TGCGGTAAAGT	TATAGCTTGC	GGCGCGGTAC	TCACGCGTCT
AGGGGCGGCA	TTGCGGTGGC	AAACGTAGCG	CGATGTGAAA	GGGGTCGAAA
CCGCGGTAGA	ACTCGTGTTG	GTGCGGTAGA	GAGGGAAGGG	TGGAAACGGT

GGAAAACCCG TCATTGTTGT TGATGCGGGA GAGTACGTGG TACGTTGCGG
 TATCCGTTAT TAGGAGCGGT GGAAAAGAAT GTACGGAGGT GTTTATGGTG
 GAGCTGGGAG GGGCGAGAAT AAGGGACGAA GCGGGGAGTA ACATGTCCGA
 TCGGATCATA CCAGCACTAA A

H. stenostachys

Clone #801

TGGGAAGTCC TCGTGTTGCA TTCCCTTTTT AATATATTTT TGCTCCACGC
 GAGAAAGATG CGTACGTGCG CGGAATATAT TAAGCACGGT TCCTTATTTT
 CGACTTCTCG TGCTGATCAG TTTGATATAT TATACGGTTT TTNCGACATC
 GTATGCAAAA GTTATAGCCG TTTTACTTTT TCATAACACT TTTTGC AAA
 ACATGTCAAA ATTTATGTTT TTGAATTTT CTAAC TAATA GATGTAGTAA
 CATAAATACA TCTCGAAGGA TTTTAATTTT TGAAGTTTTT ATCATTTTCT
 TTTCTTTTTT TCAAACTAA AATGCGNATA GGGGGGCGAG CATAATGGAC
 GAAGGCGGGG GTAACATGTC GGATGCGATC ATACCAGCAC TAAA

H. spontaneum

Clone #901

TGGGAAGTCC TCGTGTTGCA TTCCCCTTTT TAAATATATT TTTGCGCCAC
 GTGACAAGGA TGACGCGGGA CCGTGATCTA TATGACCTCA TTTTCTTATT
 TTTGACGATT ACTGTGTGAC TTTTCCCACC GCGCTTGACA CCCAACCCT
 AGTAGTAGTA GTAGTAGGGG CCAGCATAAG GAACAAATAG ATAGTTGCAT
 GTCGGATGCG ATCATACCAG CACTAAA

H. arizonicum

Clone #1001

TGGGAAGTCC TCGTGTTGCA TTCTCTTTTT AATTAATTTT TGCTCCACGC
 GAGAAACATG ACGCACGTGC ACGGTATATT TATTAAACAA TGTTTCTTAT
 TTTTACGTT TGCGGTAAGT TATAGCTTGC GCGCGGTACT CACGCGTCTA
 GGGGCGGCAT TGCGGTGGCA AAGGTAGCGC GATGTGAAAG GGGTCGAAAC
 CGCGGTAGAA CTCGTGTTGG TGCGGTAGAG AGGGAAGGGT GGAAACGGTG
 GAAAACCCGT CATTGTTGTT GAGGCGGGAG AGTACGTGGT ACGTTGCGGT
 ATCCGTTATT AGGAGCGGTG GAAAAGAATG TACGGAGGTG TTTATGGTGG
 AGCTGGGAGG GCGGAGAATA AGGGACGAAG GCGGGAGTAA CATGTCGGAT
 GCGATCATAC CAGCACTAAA

Clone #1002

TGGGAAGTCC TCGTGTTGCA TTCTCTTTTT AATTAATTTT TGCTCCACGC
 GAGAAACATG ACGCACGTGG ACGGTATATT TATTAAACAA TGTTTCTTAT
 TTTTGACGTT TGCGGTAAGT TATAGCTTGC GCGCGGTAAT CACGCGTCTA
 GGGGCGGCAT TGCGGTGGCA AAGGTAGCGC GATGTGAAAG GGGTCGAAAC
 CGCGGTAGAA CTCGTGTTGG TGCGGTAGAG AGGGAAGGGC GGAAACGGTG
 GAAAACCCGT CATTGTTGTT GAGGCGGGAG AGTACGTGGT ACGTTGCGGT
 ATCCGTTATT AGGAGCGGTG GAAAAGAATG TACGGAGGTG TTTATGGTGG
 AGTTGGGAGG GGCAAGAATA AGGGACGAAG GCGGGAGTAA CATGTCGGAT
 GCGATCATAC CAGCACTAAA

H. parodii, 6x

Clone #1101

TGGGAAGTCC	TCGTGTTGCA	TTCCCCTTTT	TAATAAATTT	TTGCTCCACG
CGAGAAACAT	GACGCACGTG	CGCTGTATTT	GTTAAACAAT	GTTTCTCATT
CTTGACGTTT	GCGGTAAGTT	ATAGCTTGCG	CGCAGTACTC	ACGCGTCTAT
GGCGGCGTTG	CGGTGGAAT	GGTACCGCGT	TGTGAAAGGG	GTCGAAACCG
CGGTAGAACT	CGTGTGTTG	CGGTACAGAG	GGAAGGGTGG	AAACGGTGGA
AAACCCGTCT	TTGTTGTTGA	GCAGGGAGAG	TACGTGGTAC	GGTGCGGTAT
CCGTTATTAG	GAGCGGTGGA	AAAGAATGTA	CGAAGGTGTT	TATGGTGGAG
CTGGGAGGGG	CGAGAATAAG	GGACGAAGGC	GGGAGTAACA	TGTCGGATGC
GATCATACCA	GCACTAAA			

Clone #1102

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATATCTT	TGCTCCACCG
GAGAAACATG	CGTACGTGCG	CGGAATATAT	TAAGCACGGT	TCCTTATTTT
CGACGTTTTT	GGTAAGTTTT	AGCTTGCGGG	TCATTATCCA	CGCATGTAGG
GGCGGCGTTG	CGGCGGAAAA	GTGGTCCGGT	ACTGAAAGGG	GTCGAAACCG
CCGGTAAAAC	TCTAGTTGGT	GCGGTAGAGA	GGGAGGGGTG	GAGACCGTGG
TAAACACGTG	TACGTTGTTG	AAAGGGAGAG	TAAGTGGTAT	GGTGCCGTAT
TCGCTATGAG	GCAGCGTTGG	CAAAGAGTGC	TCGATCGTGT	TTGTGGTGGA
GCCGAGAGGG	GCGAGCATAA	TGGACGAAGG	CGGGGGTAAC	ACGTCCGATG
CGATCATACC	AGCACTAAA			

Clone #1110

TGGGAAGTCC	TCGTGTTGCA	TTCCCTTTTT	AATATATTTT	TGCTCCACCG
GAGAAACATG	CGTACGTCCG	CAGAATATAT	TAAGCATGGT	TCCTTAATTT
CGACGTTTTT	GGTGAGTTTT	AGCTTGCGGG	TCATTATCCA	CGCGTGTAGG
GGTGCGGTTG	CGGTGGAATA	GTGGTCATGT	TCTGGAAGGG	GCCGAAACCG
CGGTAAAACT	CTAATTGGTT	TGGTAGAGAG	GGAGGGGTGG	AGACTGTGGT
AAACATGTGT	ACATTGTTGA	GCGGGAGAGT	AAGTGGTATG	GTGCGGTATT
CGTTATTAGG	AAGCATTGGT	AAAGAGTGCT	CGATCGTGTT	AGTGGTGGAG
CCAGGAGGGG	CGAGCATAAT	GGATGAAGGC	AGGGGTAACA	TCTCGGATGC
GATCATACAG	CACTAAA			

H. lechlerii

Clone #1201

TGGGAAGTCC	TCGTGTTGCA	TTTTCTTTTT	TAATATATTT	TTGCTCCACG
CGAGAAACAT	GCGTACGTGC	GCGGAATATA	TTAAGCGCGG	TTCCTTATTT
TTGACGTTTT	CGGCAAGTTT	GAGCTTGCGG	TCATTATCCA	CGTGTGTAGG
GGCGTTCGTT	CGGTGGAATA	GTGGTCCGGT	ACTGAAAGGG	GTCGAAGCCG
CGGTAAACCT	CTAGTTGGTG	TGGTAGAGAG	GGAGGGGTGG	AGACCGTGGT
AAACACGAGA	CGTGTACGTT	GTTGAGCGGG	AGAGTAAGTG	GTACGGTGCA
GTATTTCGTTA	TTAGGAAGCG	TTGGCAAAGA	GTGCTCGGTC	GTGTTTGTGG
TGGAGCCGGG	AGGCGGGAGC	ATAATGGACG	AAGGCGGGGG	AAACATGTCG
GATGCGATCA	TACCAGCACT	AAA		

Clone #1207

TGGGAAGTCC TCGTGTTGCA TTCCCTTTTT AATATATTTT TGCTCCACGC
GAGAAACATG CGTACGTGCG CGGAATATAT TAAGCGCGGT TCCTTATTTT
TGACGTTTTT GGCTAGTTTT AGCTTGCGGT GCATTATCCA CAAGTGTAGG
GGCGTCGTTG CGGTGGAAAA GTGGTCGGGT ACTGAAAGGG GTCGAAACCG
TGGTAAACCT CTAGTTGGTG CGTAGAGAGG GAGGGTGAGA CCATGGTAAA
CACACACGTG TACGTTGTTG AGCGGGAGAG TAAGTGGTAC GGTGCAGTAT
TCGTTATTTG GAATCGTAGG CCAAGAGTGC TCGGTCGTGT TTGTGGTGGA
GTCGGGAGGC GGGAGCATAA TGGACGAAGG CGGGGGAAAC ATGTCGGATG
CGATCATACC AGCACTAAA

Clone #1208

TGGGAAGTCC TCGTGTTGCA TTCCCTTTTT AATATAATAT ATTAAGCACG
GTTCGTTATT TCCGACTTCT CGTGCTGATC AGTTTGATAT ATTATACGTT
TTTTTCGACA TCGTATGCAA AAGTTATAGC CGTTTTACTT TTTCATAACA
CTTTTTTGCA AAACATGTCA AAATTTATGT TTTTGAATTT TCCTAACTAA
TAGATGTAGT AACATAAAAT ACATCTCGAA GGATTTTAAT TTTTGAAGTT
TTTATCATT TTTTCTTTT TTTCAAACT AAAATGGCGA TACCGGGGGG
GGGGGCAGCA TAATGGACGA AGGCGGGGGT AACATGTCGG ATGCGATCAT
ACCAGCACTA AA

MONTANA STATE UNIVERSITY LIBRARIES



3 1762 10062417 8