Inferences of relationships in the genus Hordeum by molecular analysis of repetitive DNA
by Maria del Mar Sanchez Garcia

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Crop and Soil Science
Montana State University
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Abstract:
Five dispersed middle-repetitive elements were selected from a barley (Hordeum vulaare L.) random genomic library. Their genus specificity, nuclear origin, and chromosomal distribution was determined. Elements were characterized by restriction analyses, and sequence data was obtained. The objective of this project was to compare five different families of repeated sequences and to assess the extent of variation in organization of these repetitive DNA families across twenty-three Hordeum species during the evolution of the genus.

The distribution of five repetitive elements in the barley genome and variation in their structure across species of the genus Hordeum was evaluated.

Selected clones were used to probe Hordeum species genomes for the presence of interspecies polymorphic hybridization patterns. Variability in repeated sequence organization was found to be . considerably higher between different sections (according to Bothmer and Jacobsen 1985) than among species of the same section.

Southern blot hybridization data were included in a cladistic analysis that revealed relationships among species that were compatible with the four sections of the genus described by Bothmer and Jacobsen in 1985: section Hordeum. section Anisolepis, section Critesion, and section Stenostachys. A further clustering of H. vulgare and H. bulbosum in a separated subsection within the Hordeum section may be suggested by our results.

Partial sequence data was used to design five different sets of primers that amplified the corresponding sequences in samples of DNA from twenty-three Hordeum species via the polymerase chain reaction. Polymorphic amplification patterns were observed at interspecific and intraspecific levels for different species/primer combinations. Cladograms produced from total PCR data analyses were compared to those produced when only fragments that were monomorphic within species were considered or when alloploid taxa were excluded from the analysis. Monomorphisms proved useful for delineating taxonomic sections and species of alloploid origin were found to increase homoplasy in the analyses.

INFERENCES OF RELATIONSHIPS IN THE GENUS *HORDEUM* BY MOLECULAR ANALYSIS OF REPETITIVE DNA

by

María del Mar Sánchez García

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

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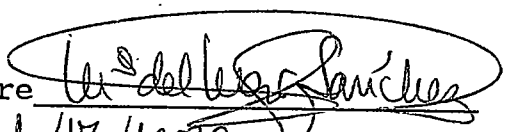
Signature 

Date 11/17/1989

To my husband Antonio Tomás

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

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The distribution of five repetitive elements in the barley genome and variation in their structure across species of the genus Hordeum was evaluated.

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Southern blot hybridization data were included in a cladistic analysis that revealed relationships among species that were compatible with the four sections of the genus described by Bothmer and Jacobsen in 1985: section Hordeum, section Anisolepis, section Critesion, and section Stenostachys. A further clustering of H. vulgare and H. bulbosum in a separated subsection within the Hordeum section may be suggested by our results.

Partial sequence data was used to design five different sets of primers that amplified the corresponding sequences in samples of DNA from twenty-three Hordeum species via the polymerase chain reaction. Polymorphic amplification patterns were observed at interspecific and intraspecific levels for different species/primer combinations. Cladograms produced from total PCR data analyses were compared to those produced when only fragments that were monomorphic within species were considered or when alloploid taxa were excluded from the analysis. Monomorphisms proved useful for delineating taxonomic sections and species of alloploid origin were found to increase homoplasy in the analyses.

CHAPTER 1

INTRODUCTION

Approximately 75% of the barley genome consists of families of repeated DNA sequences. A small proportion of these sequences may include essential coding sequences, but the functions, if any, of most of them remain enigmatic.

The classification of species in the genus Hordeum is still a matter of debate. The purpose of these studies was to elucidate whether or not the reported classification of species in the genus is supported by molecular analyses of dispersed middle repetitive DNA and to provide evidence for the mechanisms by which repetitive DNA becomes reorganized during Hordeum genome evolution.

The study began with the selection of five barley specific repetitive sequences and their characterization by restriction mapping and nucleotide sequencing. Subsequently the study considers the organization and evolution of families of repeated sequences across twenty Hordeum species and the utilization of Southern blot information to construct cladograms of relationships among species. The final part of this study focused on DNA amplification via the polymerase chain reaction.

CHAPTER 2

SELECTION AND CHARACTERIZATION OF REPETITIVE ELEMENTS

Introduction

Repeated sequences constitute much of the chromosomal DNA of higher eukaryotes. A large body of sequence information about repetitive fractions of DNA found in animal genomes is available at present (Jelinek 1982). No single unifying description of their arrangement has been proposed. However, repetitive sequences are found organized either in clusters, for instance satellites, or in a dispersed fashion. A number of possible functions has been suggested for these sequences, including involvement in recombination or replication processes. However, no direct evidence concerning their biological significance has been presented.

Higher plant genomes have been traditionally analyzed by DNA-reassociation kinetics (Sorenson 1984). In all plant species studied so far, reassociation experiments have demonstrated the presence of repetitive fractions which may comprise as much as 70 % of the plant cell DNA.

Amplification of repetitive DNA followed by sequence rearrangements occurs during evolution and enables closely related species to be distinguished on the basis of their repeated sequence DNA complements (Flavell et al. 1977).

The objectives of this part of the study were to select barley-specific nuclear repetitive DNA clones from a barley random genomic DNA library and to characterize them by restriction mapping analysis and DNA sequencing.

Materials and Methods

Selection of Clones

A barley genomic library previously constructed by Dr. Chao in the phage vector EMBL4 (Frischauf et al. 1983) using total DNA from the barley cultivar "Betzes" was utilized. Phage clones were randomly selected and amplified using methods described by Maniatis et al. (1982). DNA from each clone was digested with EcoRI according to manufacturer's instructions and fragments separated by electrophoresis in 0.8% agarose gels at 2V/cm overnight.

Gels were stained with ethidium bromide and photographed under UV light. The DNA was transferred to Zeta-Probe membranes (Reed 1985). Filters were hybridized with total DNA from barley, wheat and rye which had been radioactively labeled by nick-translation (Rigby et al. 1977). Repetitive and mid-repetitive barley DNA inserts were identified as those which bound large amounts of the total barley DNA probe and low or undetectable amounts of the wheat and rye probes (Fig. 1).

Elucidation of the Nature of Cloned Elements

Selected fragments were subcloned into the plasmid vector pBR325, radioactively labeled by nick-translation (Rigby et al. 1977) and utilized as probes against filters containing barley, rye and wheat DNAs digested with BamHI, HindIII, DraI and EcoRV, to verify their barley specificity.

Barley chloroplast and mitochondrial DNAs were isolated (Vedel et al. 1980) and digested with several restriction endonucleases. DNA was separated by gel electrophoresis, Southern transferred and probed with pBR325 clones to determine whether the repetitive elements were nuclear or cytoplasmic in origin.

Restriction Mapping

Repetitive elements were characterized by restriction mapping using about 500 ng of plasmid DNA and 12 different restriction endonucleases either singly or in combination. DNA fragments were separated in 0.8% agarose gels, stained by ethidium bromide, and photographed. The size of each fragment was determined using the HindIII digested fragments of the phage Lambda as standards.

Sequence Analysis

Subfragments of the clones were digested according to their restriction maps and cloned into the sequencing vector pIBI30. Partial nucleotide sequence was obtained using the chain termination method of Sanger et al. (1977).

Results and Discussion

Selection of Repetitive Clones

Approximately 200 genomic clones were screened from the EMBL4 library. Ten were selected as containing barley specific repetitive elements. The selection strategy is illustrated in Fig. 1. Inserts that bound a high amount of barley genomic DNA but low or undetectable amounts of the rye and wheat probes were considered to contain barley-specific repetitive DNA. The goal of this study was to determine whether it was possible to relate taxa at the species level using dispersed repetitive DNA elements. Also, comparative analysis of rapidly evolving repetitive sequences might provide useful information on mechanisms by which repetitive DNA elements have diverged in the course of evolution of the genus Hordeum. For this reason barley-specific repetitive clones were selected for this study.

Selected inserts were subcloned into pBR325 and hybridized to filters containing digested barley, wheat and rye DNA to verify the barley-specificity of the clones. No or very little signal was detected for each of the selected clones against either wheat or rye DNA (Fig. 2). Five subclones were identified as containing nuclear repetitive elements when chloroplast, mitochondrial, and total DNA were extracted from barley and probed with the selected clones. Sizes of inserts ranged from 2.26 kbp to 5.50 kbp.

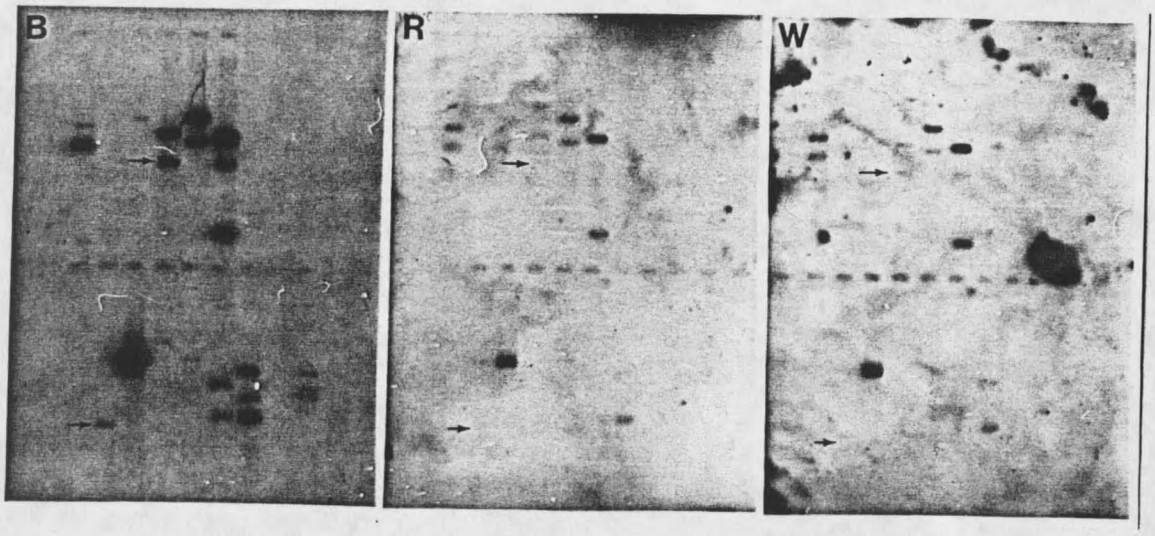

Figure 1. Repetitive element selection strategy. EMBL4 clones containing sequences that bound high amounts of barley total DNA (B) but low or undetectable amounts of either rye (R) or wheat (W) total DNA (arrows) were selected for further subcloning and use as probes.

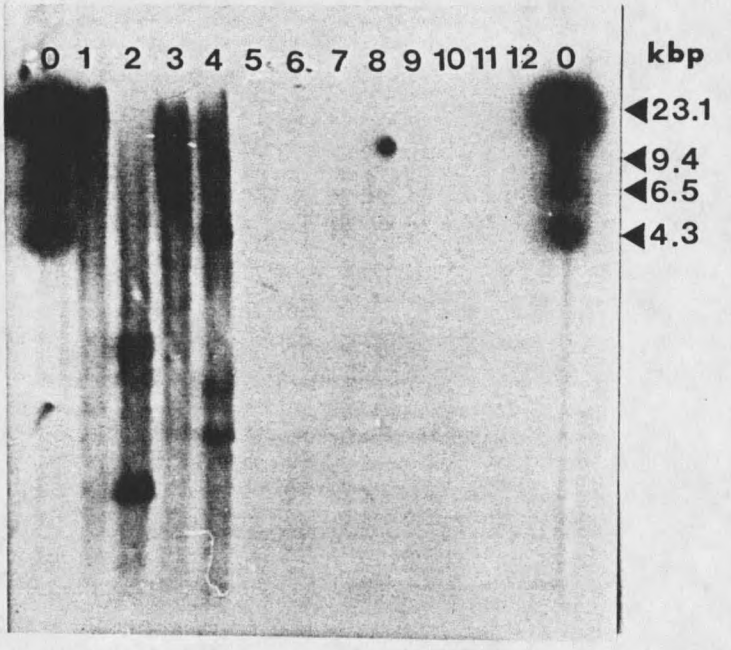

Figure 2. Barley specificity of repetitive elements. Filters contained barley (lanes 1-4), rye (lanes 5-8) and wheat (lanes 9-12) total genomic DNAs digested with BamHI (lanes 1, 5 and 9), HindIII (lanes 2, 6 and 10), DraI (lanes 3, 7 and 11) and EcoRV (lanes 4, 8 and 12) restriction endonucleases, and probed with element #25.

Physical Maps of Repetitive Elements

Physical restriction maps for the five repetitive clones are shown in Figure 3. Fragments smaller than 200 base pairs were not detected on the gels. An apparent lack of internal repetitive structure of restriction sites is observed in the maps of the clones except, perhaps, for clone #44 where HindIII sites seem to be associated with RsaI sites.

Sequence of Subfragments of Repetitive Elements

Sequenced fragments averaged 250 bp. A total of 2550 base pairs were sequenced, representing 13 % of the total sequence of the clones (Table 1). Locations of sequenced fragments are shown by double arrowed lines under the map of each clone (Fig. 3).

Computer analysis of the partial sequence data performed using Genepro (Riverside Sci. Enterprises 1988) did not reveal any simple repetition pattern within the restriction fragments studied. There were, however, several 6 to 10 base pair long direct repeats. Table 2 lists direct repeats at least six bp long where repeated sequences share more than 80 % homology. The two 6 bp long sequences reported here were perfect repeats; the other direct repeats were longer than 6 bp. Nineteen of the 44 short nucleotide sequences listed in Table 2 were found to be repeated in more than one clone.

Short direct repeats could have resulted from the

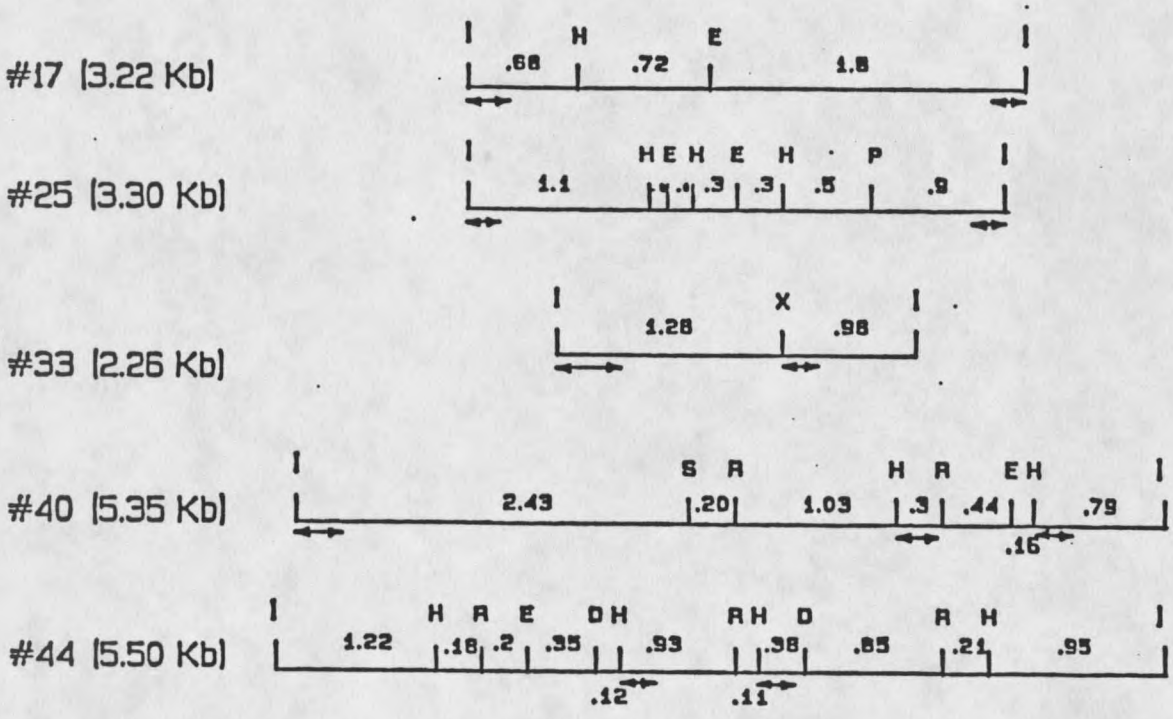

Figure 3. Physical maps of selected repetitive elements. Restriction sites shown are: EcoRI (I), EcoRV (E), HindIII (H), PstI (P), RsaI (R), DraI (D), SstI (S) and XbaI (X). Double arrowed lines represent length of sequence obtained for each fragment. Sizes of restriction fragments are in kilo base pairs.

Table 1. Partial sequencing data from repetitive elements. An example of short direct repeats is underlined for each fragment. Boldfaced sequences represent one of the restriction sites used for subcloning the fragments in a sequencing vector: GAATTC (EcoRI), TCTAGA (XbaI) and AAGCTT (HindIII). Positions of the sequenced subfragments in the restriction maps are indicated in base pairs.

#17 clone

GAATTCTTTT ACTTAGGCTT TAGCAAATCT TACTTGTGAT GTTATTTTCA CTTGTGGCTT 60
GATTTTAGTG CTTATGGTGT GTTTTGTGTT TGTATCGGTA GATCATCCGG AGTGCGAACG 120
TGTTACTTAG AAGCGCTCGA TCAAGACAAC CATCATCAAG GCAAGTCATT TGATCATGTT 180
ATTTCTATGT TTCGATGCAT GTAGTTCACT TCTTGCCAAT GCATGCTGCC TAGGTACTTG 240
AACTTAGTTA AGTGT 255

TTGTGGCACA TGATTGCGTC CGCAGATGGA GCGTGAACTT ATCACTGCTG GTTGTTCTGA 3091
AGCTGAGAAG GTGCGTTTTG CCGCACATCA ACTTGAAGGC CCTGTAGCTG CTTGGTGGGA 3151
GAACTACACC ACCACTTATC CCATTGCTGG AGTCACTTGG GAGGAATTC 3200

#25 Clone

GAATTCTTCC GATTCTCTTT TGACGATTCG AAAAGGGTTT GTCGAGGAGA CTTTGATCAA 60
CCTCATTGAC GGATTTCTTT CGATTGACCA TTTGAGATGG ATGAATCGAG GAGTCACAAA 120
CCTCTATTCC AAGCTTCACC TTCGTTGCTT AGAGAAAATG AATAAGGTTA GTTAGTTCCT 180
GTT 183

CCAAGTAAAT TGCATGTGCC ACTCTCTAAA CCTTCAAGAA ATAATCTGGT TATCCTCGAT 3192
ATGTGTTTAT GTGGTGACAG GGCTATCAGT ATCTTCCATG CTAGGCGTGT TATCCTCGAT 3252
ATGTGTTTAT TCACTATGAT TCACGAGAAA GGACTGGTAA TTGAATTG 3300

#33 Clone

GAATTCCCAG TTCCATCCTC AAATCAAAAA GATAATTGCA AACAAAACTC CCCCAGGATT 60
GATGTTGGTA TGGACGGCAC CCGAGTATTC GGCTAGTCAT GGAGTGTGAT GTAGCGTGGA 120
TGGGGGAGTC AAAAATTTAC TTTTCTNTTT GGGAACTGCC TATAGCATGT GTAGCGTGGA 180
AGATGATGAG AACTCTTAGT CATTGCATTG ACAATGAAAG CATGCCACCC AAAATTATTA 240
TCTCTGTTTT CAAAGCTCGA GCTCTGGCAC CTCAGCAAAT CAATGCTTCC CTCTGCGAAG 300
GGCCTGTCTA TTTATGTTCC TGTTAGTCAT CCTCTTCTTA TAAAGCACCA ATTAGAGAGC 360
ACTCTGTCAT TTTATGCTTC TTTAACTG 388

TCTAGAAAAA TTCTACGTAA ATTTTCATGT CATTCCGAGA ACTTTTATTT CTGCNCAAAA 1340
ACAACACCAT GGTAATTCTG CTGAAAACAG CGTTAGTCGA GTTAGTTCAT TCAAATCATG 1400
CAAATTAGAG TCCAAAAGAA GGGCAAAGGA GTTCGGAAAA GTAGATACGA CGGAGACGTA 1460
TCTACTCTCG CAGATT 1476

Table 1 (continued)

#40 Clone

```
GAATTCTAAA TAAGTATGCA AGCTCCATCG ATCCATTTAT GGACTGGTGC AAGCATCTGT    60
GAGTTGGAAC CAACGTTTTG ATGAGGTGAT CAAAGCGTTC GGGTCTATAC AAAATTTTGT   120
ATAAACTTGC ATTTACAAGA AAGTGAGTGG GAGCTCTATA AVATTTCTAA TATTATATGT   180
GGATGACATA TTGCTGATTG GAAACAT                                       207

AAGCTTTCAC CGACTTTATA TTCACACTTT CACTTTGTTA GTCGGTGTTC ACACTTTGCC  3720
ACTGTATCAT CATGCTACTT TATTTCTTTT TCTCGCTTTC TTCTCGTGTG TTTGTCTAGT  3780
TTGAGAAAAA ACCCAAAAGA TTCTTTCTTA TTTGCTTGTT GGGAGCTTTC CGTGTAAATA  3840
GTTTCTTTCT TTGGGTCAAG GAGAAGACCA TGGTTACAAT GTTAGTGGCT CTCGCATCTT  3900
AATTAGTTAC TGTCAAGAGC ATATTAC                                       3927

AAGCTTTGGG GGTGCGGTAA GCTGGTGCAG TTCCAAGCAA AGCGTTGTAG CTGATTCTAC  4620
ATGTGAAACA GAGTACATAG CTGCCTCAAA CGGCGGCTAA CGGAGGGTGT CTGGATGAAG  4680
AAGTTCATGA CAGATTGATA ACCCACAAGT ATAGGGGATC GAACAGTTTT CGAGGATAGA  4740
GTATTCAACC                                                          4750
```

#44 Clone

```
AAGCTTTTAT CGGTACAATT GCTGTAGGAG AGGAGATAGG AGTGTCTTGC GCTAGAGCTG  2130
CTAGCATTCA TTTTCCCGTG CTTCGGTACT TCTCACTATT TGTGGGAAAA TGTTTGATTG  2190
GTCGTGGGAA AGCTGGGTCA CTTAGTTACC CCAGATCTTG TGGTCTTGCG CGAACGCCTT  2250
TATAACGATA AAACTTATAG CTTGGGCGCT ATAGTAGCTC AACGTGACCT GACCTTCTAA  2310
GGGTGCTTTA GGA                                                     2323

AAGCTTGGAG GGAAATAACC AGCTCCGGGG TACTCTAGAG GGGACTGACC ATGCCACTGC  3170
TGTGTGACCT CCTTTGGCTC CCACTGATAA ACTGGCGTCT GGTACTCGAC TGGCGGCTCG  3230
GGCACGGGCA CCTGTGGTTG GGCCAAGCCC AATATGCGTA GATGTCGAGG GCATAATAGT  3290
GTACCTGCCT GCATGAAGAT TGAACAAAGA AGGAGCAGGC AAAGTAATAG TCTCACGAGT  3350
ACCCTGACTA ATACAGTTAT AATATCCTTC CACC                               3384
```

duplication events that took place in the course of the evolution of these repetitive elements. Once a sequence is duplicated it may diverge via point mutations. Therefore, it was of interest to search for imperfect direct repetitions of more than 6 bp where the repeated segments share at least 80 % homology. A high number of 7-10 bp long repetitions where one nucleotide might be different within the repeating unit was found. These repeats comprise 43 % of the total obtained sequence. The close location of some of the direct

Table 2. Short direct repeats and their copy number in each repetitive element. Asterisks represent the original clone where the sequence was found.

Oligonucleotide	Element #17	#25	#33	#40	#44
ATCAAGGCAG	*2	0	0	0	0
ATGTTATTT	*2	0	0	0	0
TTCACTTGT	*2	0	0	0	0
AGTCATTTGA	*1	0	3	0	0
CGTCCATC	*2	0	0	0	0
ACAACCATCA	*2	0	0	0	0
TGTAGTTCAC	*2	0	0	0	0
CAGCTTCAG	*2	0	0	0	0
CCAGCAGTG	*2	0	0	0	0
ATTCTTTCG	2	*2	2	1	0
TTGACCATT	0	*2	1	0	1
TCGAGGAGTC	0	*3	2	1	0
GTTAGTT	1	*2	1	2	1
TTATTTCT	1	*1	2	2	0
TTTACTT	2	*1	1	0	0
TTCTGCTGAA	0	0	*2	0	0
TAGTTCATT	0	0	*1	2	0
ATTCTACGT	0	0	*1	2	0
GAGAACTTTT	0	0	*2	0	0
CAAATCAA	0	0	*2	0	0
TTCACACTTT	1	0	0	*2	0
CACTTTG	0	0	0	*2	0
TTTCTTTTT	0	0	1	*2	0
CAAAAAGAT	0	0	2	*1	0
TAGCTG	0	0	0	*2	0
AAGCTGG	0	0	0	*2	1
AGCAAA	1	0	0	*2	0
ATGACAGATT	0	0	0	*2	0
AGTATTCAA	0	0	1	*2	0
TGGTGCAGT	2	0	0	*1	0
AAGTTCATG	2	0	1	*1	0
TGCAAGCATC	0	0	0	*2	0
GTGAGTGGGA	0	0	0	*2	0
TTGGAACCA	0	0	0	*2	0
GTGGGAAA	0	0	0	0	*2
GTCTTGCGC	0	0	0	0	*2
GCGCTATAG	0	0	0	0	*2
TCTCACTATT	0	0	0	0	*2
TGACCTTCT	0	0	0	0	*2
GTGCTTTAGG	2	0	0	0	*1
GGTACTCTA	0	0	0	0	*2
ACTGGCGGCT	0	0	0	0	*2
CGGGCACCTG	0	0	2	0	*2
TAATAGTCT	0	0	0	0	*2

repeats within a single sequence suggests that the duplication process occurred over a very short distance within these elements.

Similar distributions of direct repeats were found by Sakowicz et al. (1986) in repeated sequences of the Lupinus luteus genome. Also, Junghans and Metzlaff (1988) detected a high number of 4-8 bp long direct repeats not arranged in a repetitive fashion of a higher order when they cloned highly repetitive DNA from barley.

CHAPTER 3

EVOLUTIONARY STRUCTURE OF DISPERSED MIDDLE-REPETITIVE SEQUENCES

Introduction

A current basic problem in genetics is to explain how repetitive sequences have diverged during the course of evolution. Mutations such as single base changes, deletions or insertions are believed to be the major factors responsible for this process.

In the past few years, data on sequence organization in higher plant genomes have been published. Sequence organization patterns have been described but the significance of these patterns is still a mystery. The processes by which the size of the genome has grown during the evolution of cereals is of both intrinsic and practical interest.

The proposal that long blocks of tandemly repeated sequences could be periodically created and serve as precursor material for families of dispersed repeated sequences was first made by Britten and Kohne in 1968. Repeated sequences accumulate mutations and diverge from other members of the same family. DNA may move around to become inserted in new positions during chromosome evolution. DNA pieces spanning a junction between different sequences will be amplified and the newly amplified

repeating unit will be compound. Some of the diverged members of the family may acquire or lose sites for restriction enzymes.

In this chapter, the distribution of five Hordeum specific repetitive elements in the barley genome is examined and differences in repetitive element structure across species representing each of the four Hordeum sections evaluated.

Materials and Methods

Plant Material

The species utilized in this study included representatives for each of the four sections suggested by Bothmer and Jacobsen (1985) for the genus Hordeum: H. vulgare and H. bulbosum (section Hordeum), H. chilense (section Anisolepis), H. procerum (section Critesion), and H. depressum (section Stenostachys).

The Triticum aestivum c.v. Chinese Spring-Hordeum vulgare c.v. Betzes addition lines (Islam et al. 1981) were used to determine the chromosomal distribution of five repetitive elements in barley.

Plant DNA Extraction

Total plant DNA was extracted from 30 mg of lyophilized tissue of single pot-grown plants using modifications of the method of Murray and Thompson (1980) suggested by Saghai-Maroof et al. (1984).

Isolated DNA was quantified by fluorometry using the DNA specific fluorescent dye Hoechst 33258.

Restriction Endonuclease Digestion, Gel Electrophoresis

Ten ug aliquots of DNA from five Hordeum species were digested to completion with 100 units of HindIII and EcoRV, EcoRI and XbaI or HindIII and RsaI restriction endonucleases singly and in combination. Different pairs of enzymes were used to digest DNA to be probed with each of the five selected clones. Restriction enzymes with more than one site within the clone map were chosen when possible.

DNA prepared from each of the six available addition lines containing a full set of wheat chromosomes and barley chromosomes 1, 2, 3, 4, 6 or 7 respectively was digested with HindIII. Digestions were performed in the buffers recommended by the manufacturer (IBI or BRL).

Restriction fragments were size-fractionated by gel electrophoresis on a 11 x 9 cm 0.8% agarose gel in TBE (8.9 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA pH 8.0) at 2 V/cm overnight. Lanes of molecular size standards (bacteriophage Lambda digested with HindIII) were included in each gel.

Southern Transfers

Restricted DNAs were transferred to Zeta-Probe nylon membranes according to the methods of Southern (1975).

Probe Labeling

Plasmids carrying subcloned elements were isolated from E. coli hosts using the miniprep procedure of Birnboim and Doly (1979). Approximately 0.1 ug of miniprep DNA was labeled by primer extension (Feinberg and Vogelstein 1984) using deoxycytidine 5'[^{32}P]triphosphate and utilized without removing the unincorporated nucleotides. Prior to hybridization, the labeled probes were mixed with 0.2 ml of 0.2 N NaOH and denatured by heating to 100°C for 10 minutes. Salmon sperm DNA was added to the mix prior to heating.

Hybridization

Zeta-Probe membranes were prehybridized in 15-20 ml of 1.5 x SSPE, 0.1 % SDS and 0.5 % Blotto solution at 65°C in a water incubator for 4-24 hours (Reed and Mann, 1985). Denatured probes were added to the bagged filters and prehybridization mix and bags resealed and incubated at 68 °C overnight.

Washing and Autoradiography

Filters were washed for 15 minutes at room temperature successively in 300 ml of 2 x SSC/0.1% SDS, 0.5 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS solutions followed by three final washes of 0.1 x SSC/1.0% SDS at 65°C.

Washed filters were wrapped in plastic wrap and placed adjacent to a sheet of X-ray film in an exposure cassette with one intensifying screen.

Autoradiography was performed at -70°C for 12-24 hours.

Results and Discussion

Chromosomal Distribution of Repetitive Clones

The chromosomal distribution of repetitive sequences was estimated by evaluating the apparent number of barley chromosomes carrying barley-specific sequences hybridizing to each clone. This analysis showed the repetitive elements to be present in multiple copies on six of the seven chromosomes of the barley genome (Fig. 4). These blots also showed little crosshybridization to Chinese Spring DNA. Repetitive sequence DNA constitutes 70% or more of most grass genomes, and specific families of sequences show major changes in amount and structure among closely related species (Appels et al. 1987). In contrast to the long tandem arrays of DNA sequences which coincide with C-banded heterochromatin, dispersed repetitive sequences are more widely distributed among chromosomes (Appels et al. 1987). Early studies on wheat, rye, barley, and oats showed that moderately repeated sequences could be subdivided into two general classes (reviewed in Flavell 1982). One class was apparently genus-specific while the other crosshybridized with moderately repeated sequences from other genera.

Here, we deal with genus-specific middle repetitive DNA units that are dispersed in multiple copies on at least six

of the barley chromosomes. In the example shown in Figure 4, ^{32}P-labeled DNA probe #44 was hybridized to digests of DNA from the wheat-barley addition lines (Islam et al. 1981). The subsequent X-ray indicated the distribution of sequences across barley chromosomes 1, 2, 3, 4, 6 and 7. The arrays of repeats are dispersed among different regions of the karyotype, which indicates that arrays were duplicated or divided and translocated between chromosomes during evolution.

Variation in the Structure of Repetitive Elements

Southern blot hybridization analysis was performed to evaluate the variation in structure of sequences showing homology with the cloned sequences during evolution of the genus. Examples of hybridization patterns obtained from this series of experiments are shown in Figures 5, 6, 7, 8 and 9.

With probe #17 (Fig. 5) practically no hybridization was detected with species other than H. vulgare and H. bulbosum indicating lack of sequences with similarity to the probe or a high divergence of members of this repeat family in wild relatives of barley. However, a HindIII ladder was observed in H. bulbosum with bands increasing in size by intervals of 2000 bp.

Probe #25 (Fig. 6) gives complex hybridization patterns for H. vulgare and H. bulbosum and a common HindIII fragment of 2 kbp across all the species.

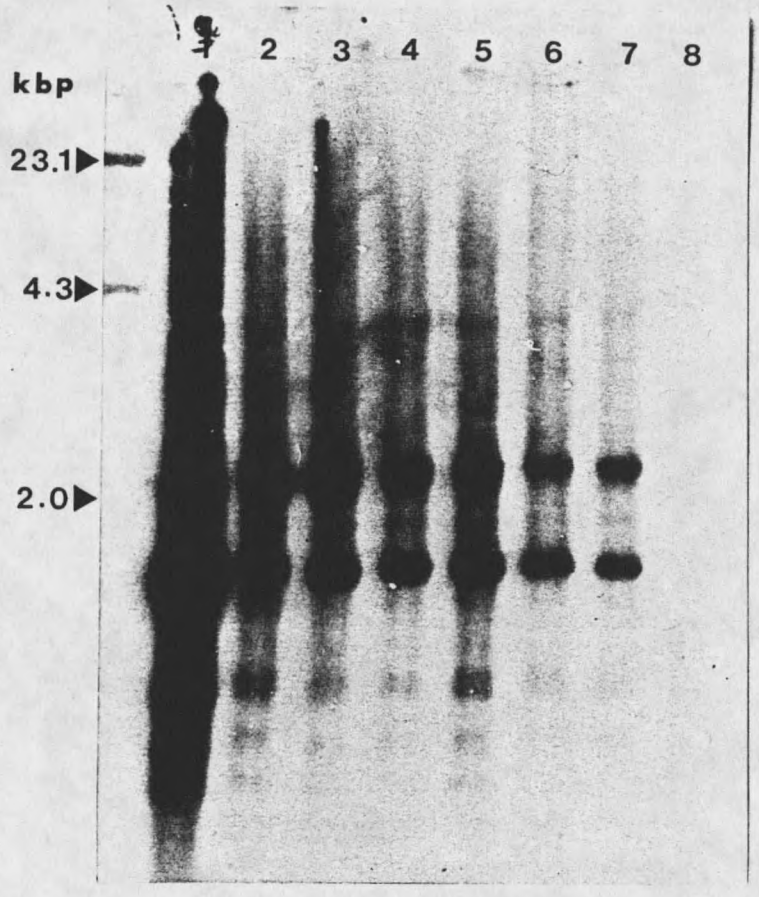

Figure 4. Southern blot of DNAs from wheat-barley addition lines containing a complete set of wheat chromosomes and barley chromosomes 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 6 (lane 6) and 7 (lane 7) respectively. DNA was digested by HindIII and probed with clone #44. Lanes 1 and 8 contain "Betzes" and "Chinese Spring" DNAs respectively. Lane 0 is the molecular size standard Lambda digested by HindIII.

Clone #33 (Fig. 7) showed poorly delimited zones of hybridization of large fragments for all the species, superimposed upon a few smaller bands for H. vulgare and H. bulbosum digests.

With probe #40 (Fig. 8) hybridization is considerably reduced for H. chilense, H. procerum and H. depressum, compared with the patterns of H. vulgare and H. bulbosum. Some prominent low molecular weight fragments are common to all the species, however.

No significant hybridization to probe #44 (Fig. 9) was observed for digests of H. chilense, H. procerum and H. depressum. An RsaI ladder was observed in H. bulbosum with a periodicity of 150 bp.

No unifying hypothesis has been sufficient to explain the variation observed in this experiment. Clones #44 and #17 appeared to be limited in distribution to H. vulgare and H. bulbosum. Ladders were observed for both clones in H. bulbosum genomic digests, suggesting tandem organization of sequences with occasional loss of restriction sites. The lack of hybridization to high copy number elements from the other Hordeum species suggests either very recent amplification in the lineage leading to H. vulgare and H. bulbosum, or extremely high mutagenesis and divergence of this sequence family in the more distantly related Hordeum members.

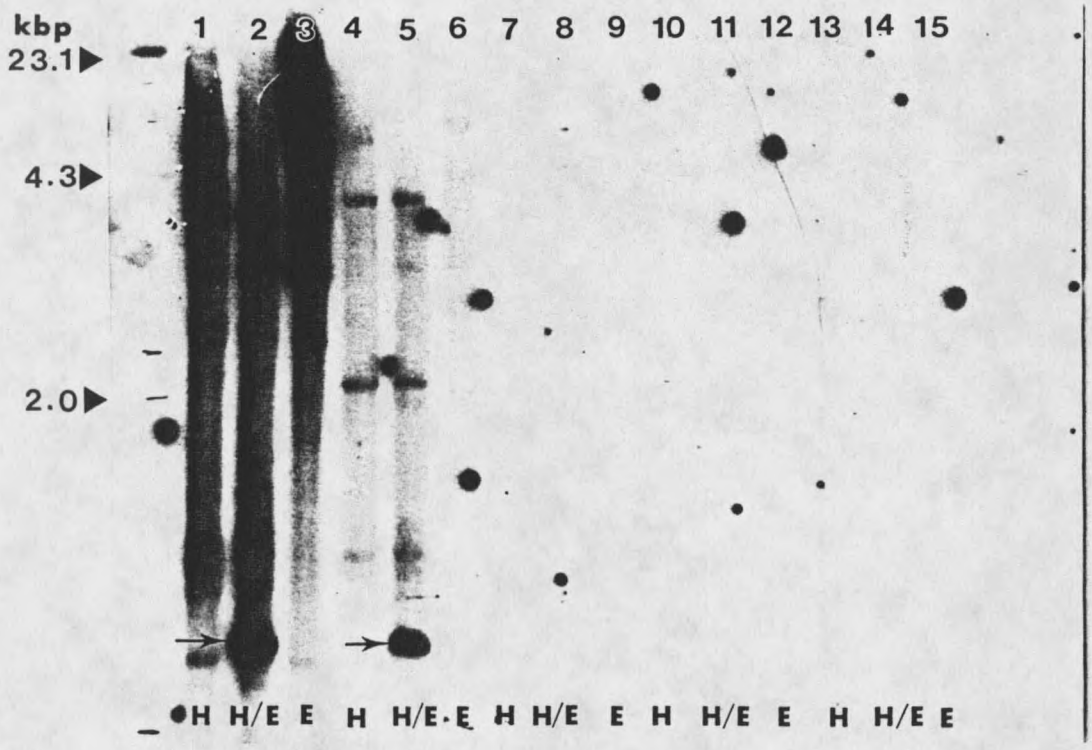

Figure 5. Autoradiograph of single and double digested genomic blots of five Hordeum species hybridized with probe #17: H. vulgare (lanes 1-3), H. bulbosum (lanes 4-6), H. chilense (lanes 7-9), H. procerum (lanes 10-12) and H. depressum (lanes 13-15). DNA was digested with HindIII (H) and/or EcoRV (E). An apparent ladder pattern with fragments of 2, 4, and 6 kbp, respectively, is observed in lanes 4 and 5. Some of the reamplified variants in high copy number are indicated by arrows.

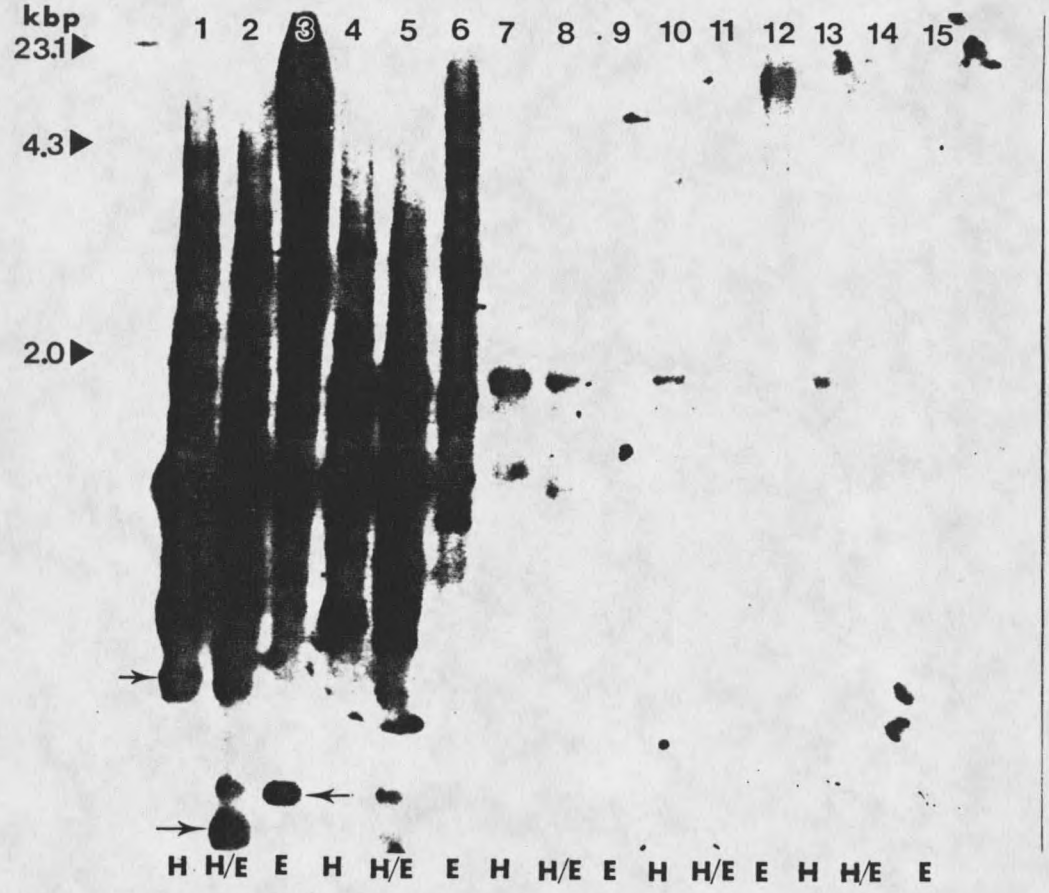

Figure 6. Autoradiograph of single and double digested genomic blots of five Hordeum species hybridized with probe #25: H. vulgare (lanes 1-3), H. bulbosum (lanes 4-6), H. chilense (lanes 7-9), H. procerum (lanes 10-12) and H. depressum (lanes 13-15). DNA was digested with HindIII (H) and/or EcoRV (E). Some of the reamplified variants in high copy number are indicated by arrows.

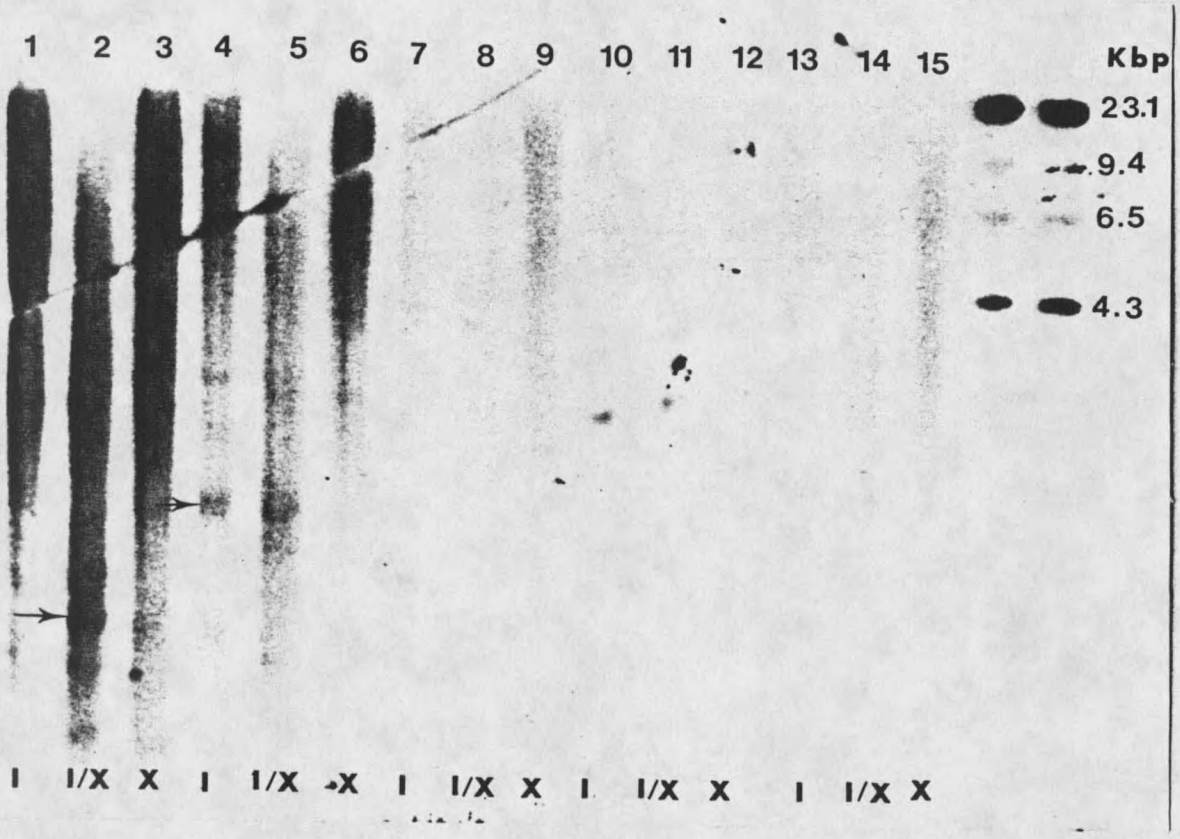

Figure 7. Autoradiograph of H. vulgare (lanes 1-3), H. bulbosum (lanes 4-6), H. chilense (lanes 7-9), H. procerum (lanes 10-12), and H. depressum (lanes 13-15) DNAs digested with EcoRI (I) and/or XbaI (X) and probed with clone #33. Some of the reamplified variants in high copy number are indicated by arrows.

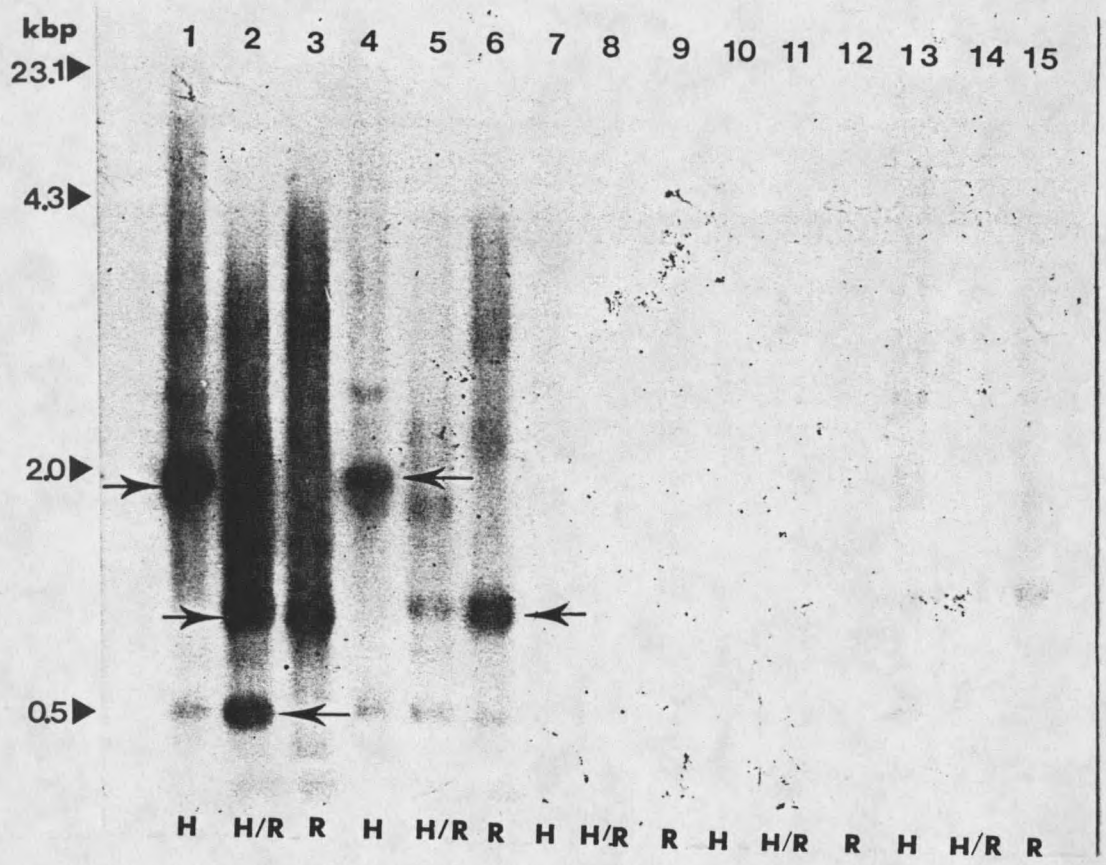

Figure 8. Autoradiograph of H. vulgare (lanes 1-3), H. bulbosum (lanes 4-6), H. chilense (lanes 7-9), H. procerum (lanes 10-12), and H. depressum (lanes 13-15) DNAs digested with HindIII (H) and/or RsaI (R) and probed with clone #40. Some of the reamplified variants in high copy number are indicated by arrows.

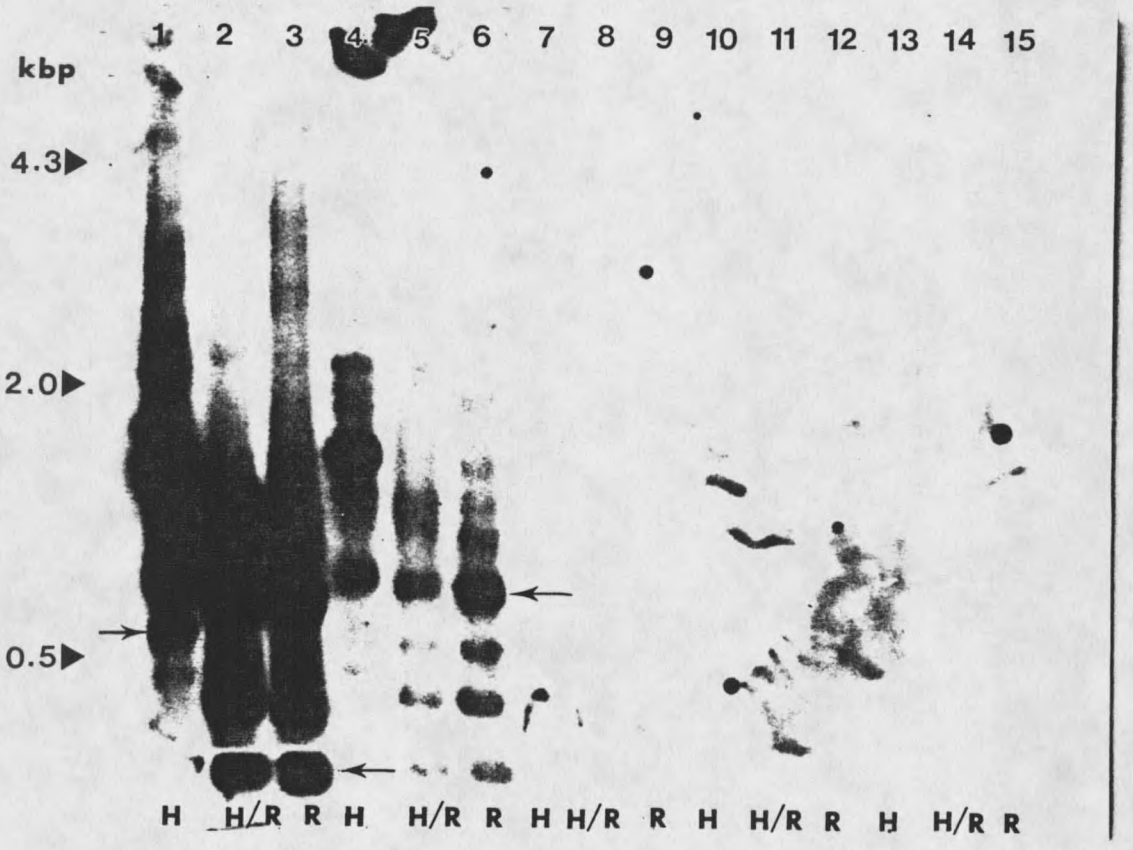

Figure 9. Autoradiograph of H. vulgare (lanes 1-3), H. bulbosum (lanes 4-6), H. chilense (lanes 7-9), H. procerum (lanes 10-12), and H. depressum (lanes 13-15) DNAs digested with HindIII (H) and/or RsaI (R) and probed with clone #44. A RsaI ladder is observed in lane 6 with fragments at intervals of 150 bp. Some of the reamplified variants in high copy number are indicated by arrows.

Clones #25 and #40 were found in high apparent copy numbers in H. vulgare and H. bulbosum, and produced much lower signals when used against genomic digests from the other Hordeum species. Common 2 kbp HindIII fragments were observed when probe #25 was hybridized to these five Hordeum species, and 800 and 500 bp RsaI common fragments were observed when probe #40 was utilized. This observation may suggest that a common 2 Kbp Hind III fragment with homology to probe #25 comprised a portion of the repetitive element family from which this clone was selected. Similarly, the 500 and 800 bp RsaI fragments which are common across all Hordeum species suggest the existence of a common primitive ancestral sequence.

No restriction endonuclease resolved the patterns from clone #33 into simple bands. The simplest explanation for this observation is that we were unable to find restriction sites which lay within the primary amplified unit. This may provide an example of a small repetitive element which has been dispersed to many locations throughout the genome without tandem amplification.

These results suggests three routes by which amplification of dispersed elements may have occurred. First, some sequences, as those with similarity to clones #17 and #44, recently diverged from a common ancestor by both tandem amplification and translocation to new positions in the genome. Tandem amplification followed by occasional

loss of single cleavage sites by mutation may have resulted in the ladder patterns similar to those found in H. bulbosum. The absence of ladders in the hybridization patterns of digests of the other genomes demonstrates either that those repeat units in other species did not possess cleavage sites for the enzymes used or that sequences in the same family were not found in tandem arrays.

Second, ancestral common fragments with homology to probes #25 and #40 may have undergone several rounds of amplification and rearrangement in the H. vulgare and H. bulbosum genomes while remaining "sessile" in the lineage leading to H. chilense, H. procerum and H. depressum genomes. The presence of relatively low copy number bands of discrete mobility suggests a common ancestral sequence of at least 2 kbp in the case of the family related to clone #25 and of 500 bp in the case of clone #40. In this process of divergence of Hordeum species, some different sets of sequences may be amplified in different sections of the Hordeum genus. As divergence time increases, interspecies sequence similarities decrease for repeated and non-repeated DNAs.

Finally, broad smears in the hybridization pattern of species probed with clone #33 may suggest the absence of relevant cleavage sites within the basic amplified unit which shows similarity to the probe. The simplest explanation for this observation may be that this clone

shares homology with a small dispersed element which has been scattered as a repeated element throughout the H. vulgare genome. Some multimers are present in high frequency (arrows in Figs. 5, 6, 7, 8, and 9) which is strong evidence for reamplification of members of this particular family of repeats. Prominent bands can then be explained by the presence of multiple copies of such complex units in the genome.

The variation observed in the arrangements of these sequences demonstrates great diversity within the genus Hordeum. Events such as convergence of repeated sequences from related families or loss of these sequences might also take place in the process of divergence of repetitive DNA, as well. Evidence supporting these mechanisms is not provided from the type of analysis described in this chapter.

If closely related sequences are amplified in different combinations then repeating units should have sub-repeats in common. If a diverged repeated family is present in two closely related species and different members of the family become reamplified in the two species, common repeated sequences should be relatively short and interspersed with non-repeated or other repeated unrelated sequences. The average repeating unit length in compound repetitive sequences appears to be in the region of a few hundred bp (Rimpau et al. 1978; Flavell and Smith 1976). Except for

probe #40 (Fig. 8), only H. vulgare and H. bulbosum shared bands of low molecular weight. For the rest of the species there was an apparent lack of common low molecular weight units. If the common repeats existed in ancestral genomes interspersed with non-repeated DNA, amplification of compound sequences occurred in H. vulgare and H. bulbosum genomes after the divergence between these two species and the rest of the genus.

CHAPTER 4

RELATIONSHIPS AMONG 20 *HORDEUM* SPECIES BASED ON SOUTHERN BLOT GENOTYPES

Introduction

During the last decade there has been an increased interest in utilizing the genetic resources available in wild relatives of cultivated plants. For optimal utilization of alien genetic material in plant breeding, it is important to have a knowledge of variation patterns and inter-relationships within plant groups that include cultivated species (Feldman and Sears 1981).

Although most authorities have agreed upon the delimitation of the genus *Hordeum*, there have been various proposals which included other genera in *Hordeum* (Hackel 1887), as well as those which transferred species of *Hordeum* to other genera (e.g., *Critesion*) (Löve 1980, 1982, 1984; Dewey 1984). However, these proposals are not generally accepted at present (Bothmer and Jacobsen 1985).

Species relationships in the genus *Hordeum* remain a subject of disagreement, (reviewed by Bothmer and Jacobsen 1985). The division of *Hordeum* into sections has not yet been as generally agreed upon. Various criteria have been used by different workers for delimiting sections within the genus, but no one model of classification is generally accepted. Åberg (1940) and Bell (1965) recognized four

sections, whereas Nevski (1941) and Tzevelev (1976) recognized five. Bothmer and Jacobsen (1985) proposed 4 sections: section Hordeum consisting of H. vulgare, H. bulbosum and H. murinum; section Anisolepis consisting of exclusively diploid species native to South and North America; section Critesion (Rafinesque) Nevski comprising six species, two diploids, one tetraploid and three hexaploids occurring in southern South America, North America and easter Siberia; and section Stenostachys Nevski, the largest group in the genus and also the one with the largest area of distribution.

Initial studies were based on morphology, life forms, reproductive systems, dispersal mechanisms, geographical distribution and ecology (Nevski 1941; Lundqvist 1962; Bothmer and Jacobsen 1979; Stebbins 1975). Cytological approaches and compatibility in crosses (Linde-Laursen et al. 1980; Bothmer et al. 1980; Wang and Hsiao 1986) added extra taxonomic parameters. Molecular analysis of DNA sequences has been used to infer phylogenetic relationships in other grass genera (Appels and Honneycut 1986).

DNA analysis has proven useful for inferring relationships in Hordeum. Chloroplast DNA variation has been used to study the origin of Hordeum (Poulsen 1983). Gupta et al. (1989) and Molnar et al. (1989) found highly-reiterated DNA to be useful in assaying relationships among different Hordeum species.

In this chapter five repetitive elements from Hordeum were use to infer relationships among 20 Hordeum species based on Southern blot hybridization patterns. Relative abundances of these elements were estimated. Cladistic analysis utilizing hybridization genotypes were carried out to reveal relationships among 20 Hordeum species.

Materials and Methods

Plant Material

The 20 Hordeum species studied, their ploidy level, life form and origin (according to Bothmer and Jacobsen 1985) are listed in Table 3.

H. brachyantherum and H. parodii 6x were included along with species listed in Table 3 for dot blot experiments.

Genomic Blot Preparation

Ten ug aliquots of DNA from 20 species were digested to completion with HindIII restriction endonuclease. Restriction fragments were size-fractionated by gel electrophoresis (Fig. 10). Southern transfer and hybridization procedures were identical to those detailed in chapter 3.

Estimation of relative abundances

Dot blot experiments were prepared with serial dilutions of 10 ug to 5 ng of total cellular DNA from twenty-two Hordeum species according to methods described by

Blake (1987). Dot blots were probed with repetitive clones and relative abundances estimated by determination of the endpoint at which a signal could be detected (Blake 1987).

Table 3. List of species used in this study along with their ploidy level, life form and origin, according to Bothmer and Jacobsen (1985).

Section/species	Ploidy level	Life form	Origin E/A[a]
Hordeum			
vulgare	2X	annual	E
bulbosum	2X	perennial	E
leporinum	2X	annual	E
leporinum	4X	annual	E
murinum	2X	annual	E
glaucum	2X	annual	E
Anisolepis			
muticum	2X	perennial	A
stenostachys	2X	perennial	A
chilense	2X	perennial	A
flexuosum	2X	perennial	A
Critesion			
lechleri	6X	perennial	A
procerum	6X	perennial	A
arizonicum	6X	perennial	A
Stenostachys			
marinum	2X	annual	E
geniculatum	4X	annual	E
bogdani	2X	perennial	E
roshevitzii	2X	perennial	E
depressum	2X	annual	A
parodii	2X	perennial	A
magellanicum	2X	perennial	A

(a) Origin of species in Eurasia (E) or America (A) as reported by von Bothmer and Jacobsen (1985).

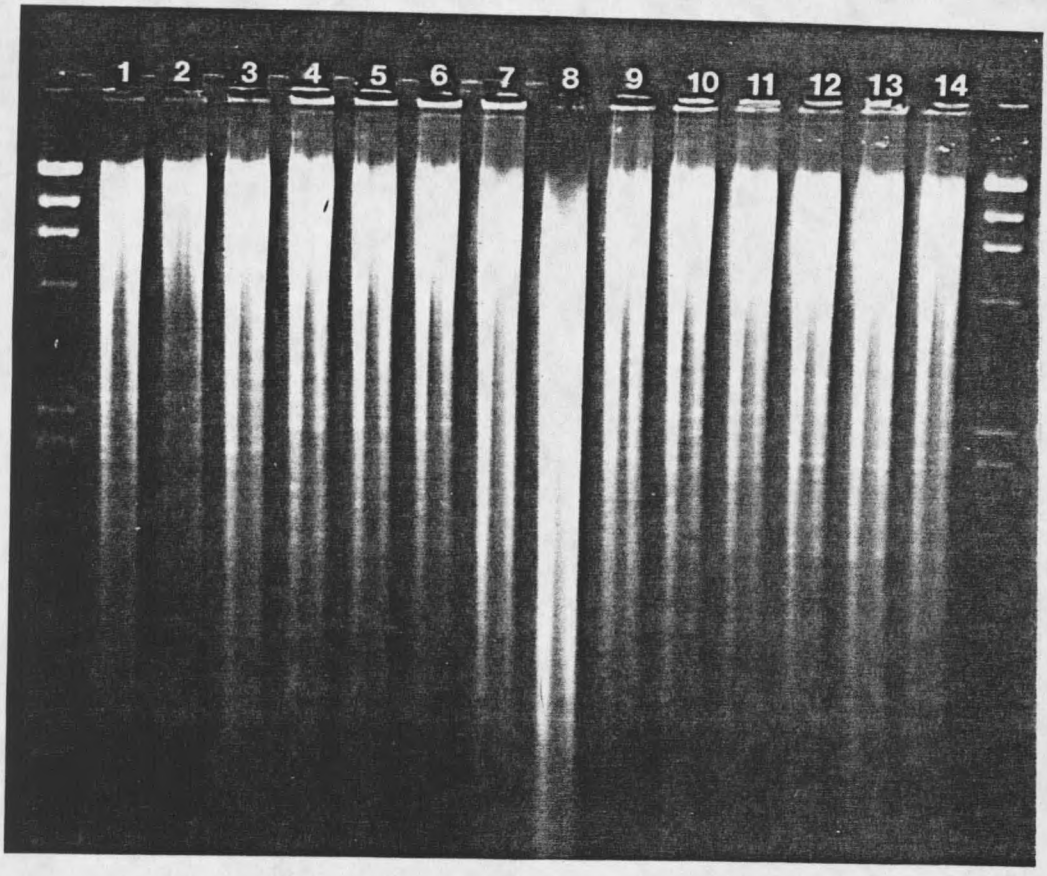

Figure 10. Digestion of genomic DNA with HindIII restriction endonuclease. Ten ug of total genomic DNA from the following species was loaded in each lane: H. vulgare (lanes 1 and 3), H. bulbosum (lane 2), H. murinum (lane 4), H. leporinum (lane 5), H. leporinum 4x (lane 6) H. glaucum (lane 7), H. muticum (lane 8), H. stenostachys (lane 9), H. chilense (lane 10), H. flexuosum (lane 11), H. lecheri (lane 12), H. procerum (lane 13), and H. arizonicum (lane 14).

Cladistic Analysis of Hybridization Genotypes

A data matrix was assembled including all bands observed with each probe in Southern blot hybridizations to DNA from 20 Hordeum species. Each hybridization band was present in more than one taxon and hence all were informative for the construction of the cladogram. The data matrix comprised 20 taxa and 61 characters and a binary code was used to assign the absence (0) or presence (1) state of a particular band to each taxon.

The data matrix was analyzed using Swofford's (1985) computer package PAUP (phylogenetic analysis using parsimony, version 2.4). Options employed included global branch swapping, which permits construction of all possible subtrees until a minimum length is achieved. All characters used in this analysis have two states. No character state polarity was considered for any of the characters and all were therefore unordered in all analyses. Cladograms were rooted in their middle points because of the lack of data from an outgroup due to the genus specificity of the probes. The cladograms were combined into strict consensus trees (Adams 1972) that contain only those monophyletic groups common to all cladograms.

Forward changes, reversals (losses of particular restriction fragments) and parallelisms (independent gains or losses of particular fragments) are shown in the cladogram. Although initially a 1 in the data matrix

indicated the presence of a restriction fragment, the derived condition is the loss of a fragment for characters 4, 5, 7, 16, 18, 20, 21, 37, and 55.

Results

Relative Copy Number Estimation

From the dot blot analyses it was possible to infer that sequences similar to the cloned elements are conserved across species although the amount of crosshybridization differs dramatically among these 22 species (Fig. 11). Apparent relative copy numbers for each of the clones across 22 Hordeum species are summarized in Table 4. A value 1x was given to the species with the lowest hybridization signal. Values proportional to that were assigned to the rest of the species according to the smallest amount of DNA that showed hybridization under our conditions.

Southern Analysis

Considerable variation in the hybridization patterns of HindIII-digested DNA was detected among species with each probe (Figs. 12, 13, 14, 15, and 16). The similarity of hybridization patterns and signal intensity of H. vulgare and H. bulbosum was noticeable as was the similarity of H. marinum and H. geniculatum. Each of these species showed intense, complex hybridization patterns with each clone.

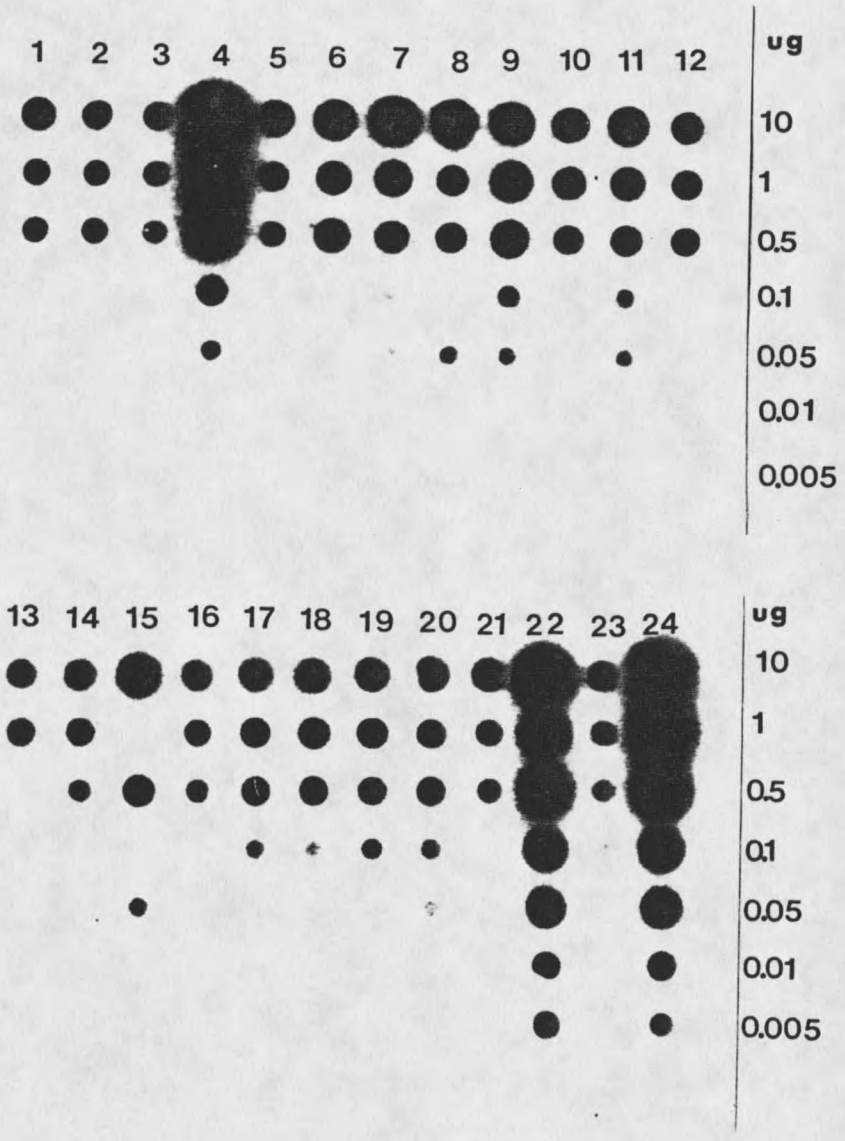

Figure 11. Dot blots of 22 Hordeum species probed with clone #33. H. arizonicum (lanes 1 and 5), H. bogdani (lane 2), H. brachyantherum (lane 3), H. bulbosum (lanes 4 and 22), H. chilense (lane 6), H. depressum (lane 7), H. flexuosum (lane 8), H. geniculatum (lane 9), H. glaucum (lane 10), H. lechleri (lane 11), H. leporinum (lane 12), H. leporinum 4x (lane 13), H. magellanicum (lane 14), H. marinum (lane 15), H. murinum (lane 16), H. muticum (lane 17), H. parodii (lane 18), H. parodii 6x (lane 19), H. procerum (lane 20), H. roshevitzii (lane 21), H. stenostachys (lane 23), and H. vulgare (lane 24). The amount of DNA used for every species in each line of dots is indicated in micrograms.

Variation in banding patterns was more dramatic among species of different taxonomic sections than among species within the same section. This feature permitted the differentiation of Hordeum sections based on Southern analyses.

Table 4. Relative abundances of middle-repetitive elements across Hordeum species. A value 1x was given to the species with the lowest hybridization signal. Values proportional to that were assigned to the other species according to the smallest amount of DNA in the dot that showed hybridization under our conditions.

Section/species	Clone #17	#25	#33	#40	#44
Hordeum					
vulgare	100x	2000x	500x	2000x	2000x
bulbosum	100x	2000x	500x	2000x	2000x
leporinum	10x	100x	5x	20x	1x
leporinum 4x	1x	10x	5x	10x	1x
murinum	5x	200x	10x	200x	200x
glaucum	10x	20x	5x	20x	20x
Anisolepis					
muticum	10x	200x	10x	200x	200x
stenostachys	1x	1x	5x	20x	100x
chilense	10x	100x	10x	200x	20x
flexuosum	50x	200x	10x	200x	20x
Critesion					
lechleri	10x	20x	10x	20x	1x
procerum	10x	200x	10x	200x	1x
arizonicum	5x	1x	1x	10x	1x
Stenostachys					
marinum	10x	2000x	10x	200x	1x
geniculatum	50x	200x	10x	200x	20x
bogdani	5x	1x	1x	1x	1x
roshevitzii	1x	200x	5x	10x	1x
brachyantherum	10x	1x	1x	20x	1x
depressum	10x	100x	10x	200x	20x
parodii	10x	200x	10x	200x	200x
parodii 6x	10x	200x	10x	200x	200x
magellanicum	5x	1x	5x	20x	1x

Probe #17 (Fig. 12) appeared to separate the four proposed sections well. However, H. glaucum showed a much weaker signal from this probe than the rest of the species in the section Hordeum. Within section Stenostachys, H. marinum and H. geniculatum showed a higher homology to the probe, as pointed out before, while H. bogdani exhibited different size bands than the rest of the species of its group.

With probe #25 (Fig. 13) section Anisolepis and section Critesion were distiguishable from each other by a single hybridization fragment and distinguishable from sections Hordeum and Stenostachys. H. roshevitzii and H. depressum showed a smaller 2.5 kbp band than the rest of the Stenostachys species.

Probe #33 (Fig. 14) produced a very distinctive pattern for the species of Stenostachys section which was close but not identical to that found for Anisolepis and Critesion sections. The banding patterns for H. vulgare and H. bulbosum were distinct relative to other members of the section Hordeum.

Probe #40 (Fig. 15) distinguished among sections Hordeum and Stenostachys, and separated these from the unresolved Anisolepis and Critesion sections.

Probe #44 (Fig. 16) did not provide much information in grouping species into sections. However, within section Stenostachys, H. marinum and H. geniculatum exhibited

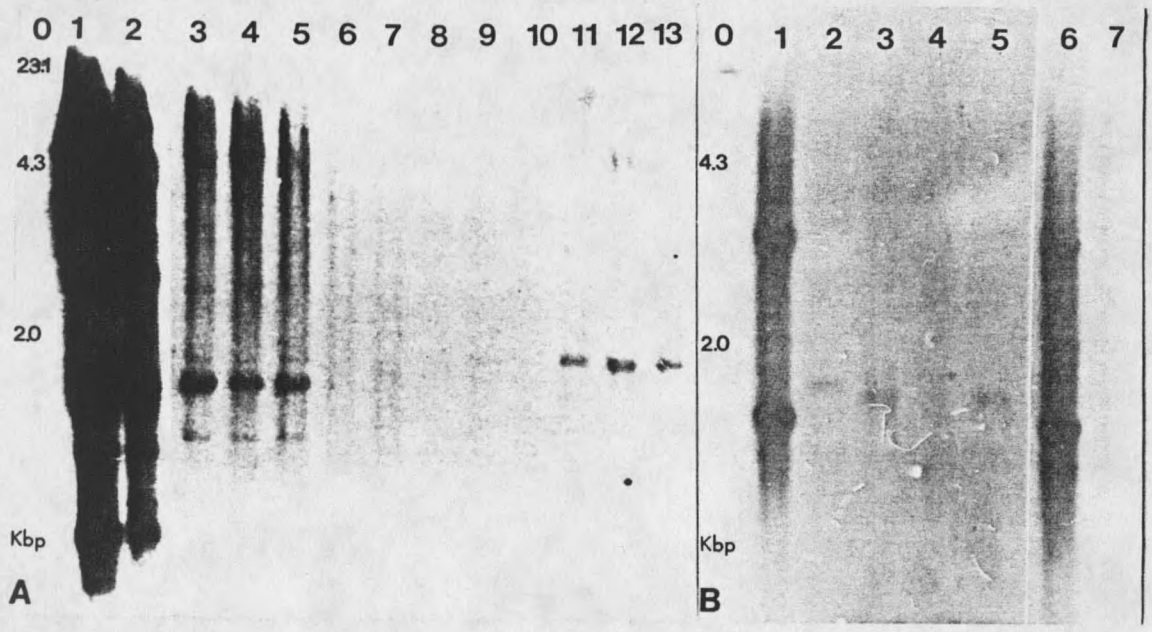

Figure 12. Southern blots of Hordeum species for probe #17. (A) Lanes 1-6, Hordeum section: H. vulgare, H. bulbosum, H. murinum, H. leporinum, H. leporinum 4x, and H. glaucum; lanes 7-10, Anisolepis section: H. muticum, H. stenostachys, H. chilense, and H. flexuosum; lanes 11-13, Critesion section: H. lechleri, H. procerum, and H. arizonicum. (B) Section Stenostachys, lanes 1-7: H. marinum, H. bogdani, H. roshevitzii, H. depressum, H. parodii, H. geniculatum and H. magellanicum. Lanes 0 contain the molecular size standard Lambda digested with HindIII.

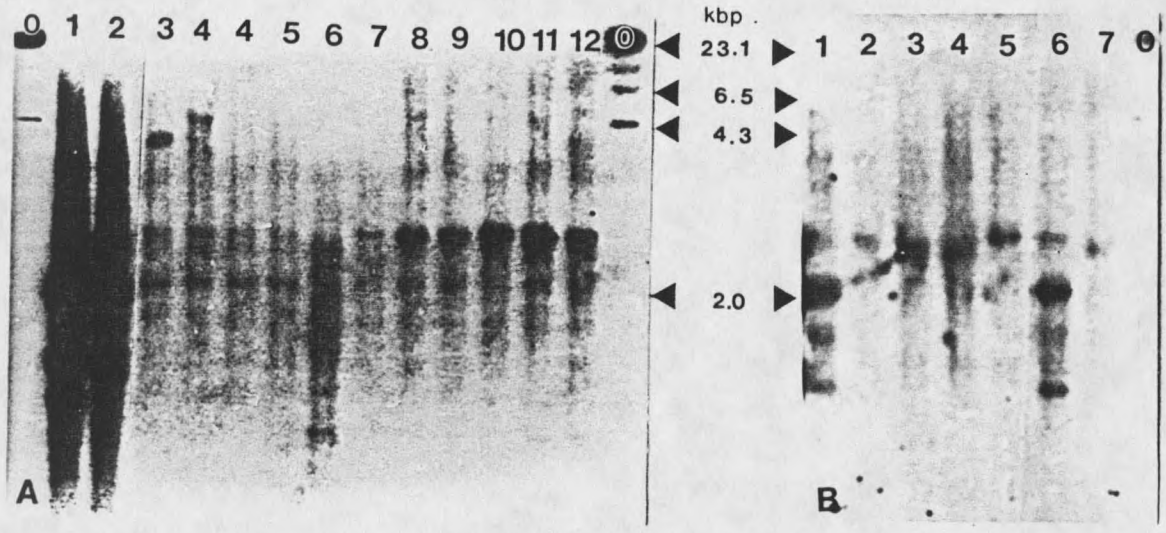

Figure 13. Southern blots of Hordeum species for probe #25. (A) Lanes 1-6, Hordeum section: H. vulgare, H. bulbosum, H. murinum, H. leporinum, H. leporinum 4x, and H. glaucum; lanes 7-10, Anisolepis section: H. muticum, H. stenostachys, H. chilense, and H. flexuosum; lanes 11-13, Critesion section: H. lechleri, H. procerum, and H. arizonicum. (B) Section Stenostachys, lanes 1-7: H. marinum, H. bogdani, H. roshevitzii, H. depressum, H. parodii, H. geniculatum and H. magellanicum. The molecular size standard Lambda digested with HindIII was included in lanes 0.

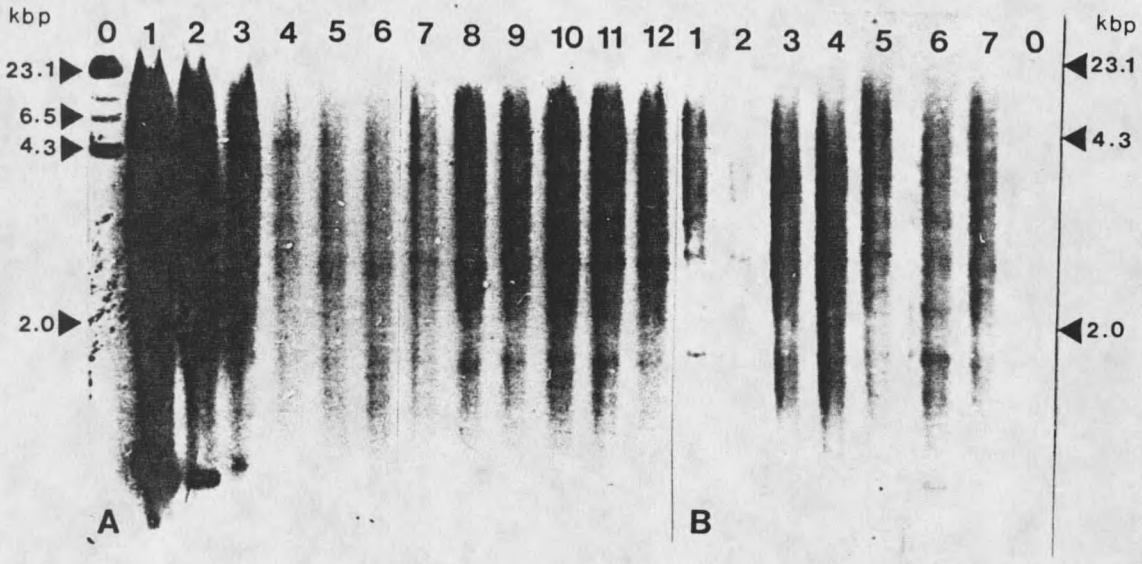

Figure 14. Southern blots of Hordeum species for probe #33. (A) Lanes 1-6, Hordeum section: H. vulgare, H. bulbosum, H. murinum, H. leporinum, H. leporinum 4x, and H. glaucum; lanes 7-9, Anisolepis section: H. stenostachys, H. chilense, and H. flexuosum; lanes 10-12, Critesion section: H. lechleri, H. procerum, and H. arizonicum. (B) Section Stenostachys, lanes 1-7: H. marinum, H. bogdani, H. roshevitzii, H. depressum, H. parodii, H. geniculatum and H. magellanicum. The molecular size standard Lambda digested with HindIII was included in lanes 0.

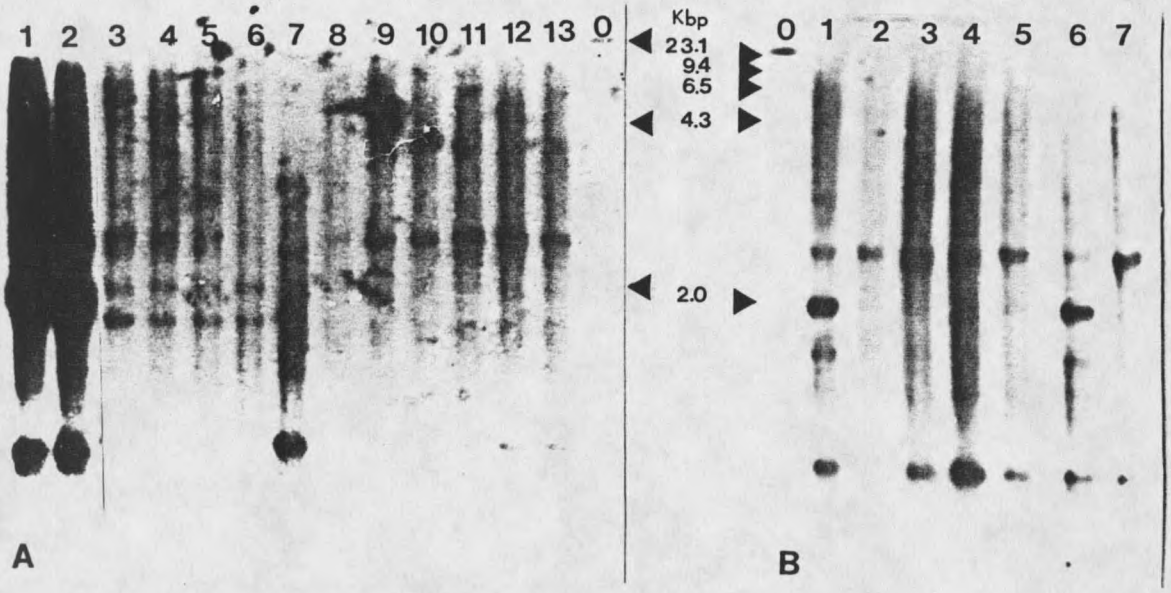

Figure 15. Southern blots of Hordeum species for probe #40. (A) Lanes 1-6, Hordeum section: H. vulgare, H. bulbosum, H. murinum, H. leporinum, H. leporinum 4x, and H. glaucum; lanes 7-10, Anisolepis section: H. muticum, H. stenostachys, H. chilense, and H. flexuosum; lanes 11-13, Critesion section: H. lechleri, H. procerum, and H. arizonicum. (B) Section Stenostachys, lanes 1-7: H. marinum, H. bogdani, H. roshevitzii, H. depressum, H. parodii, H. geniculatum and H. magellanicum. The molecular size standard Lambda digested with HindIII digested was included in lanes 0.

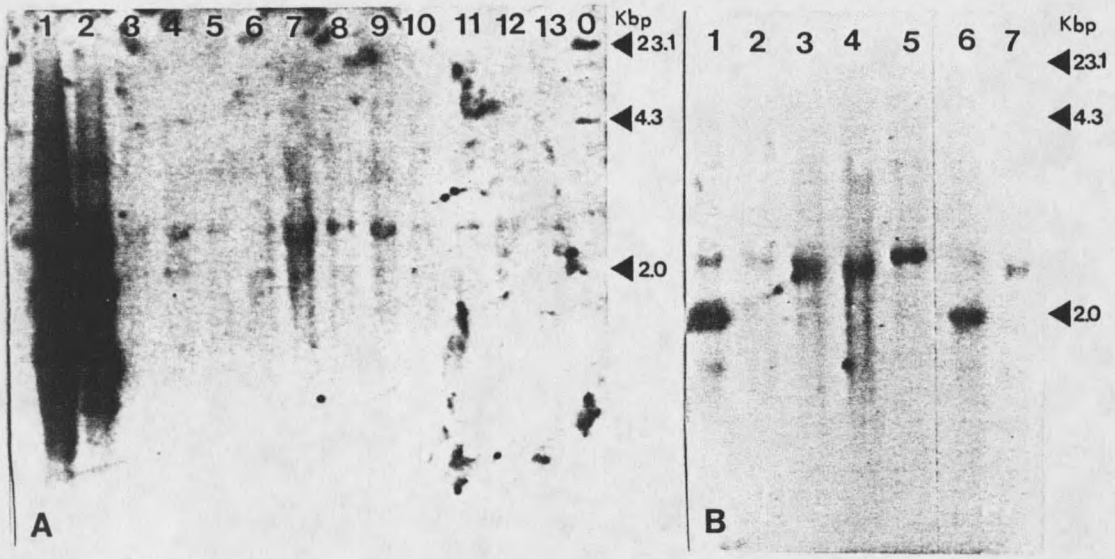

Figure 16. Southern blots of Hordeum species for probe #44. (A) Lanes 1-6, Hordeum section: H. vulgare, H. bulbosum, H. murinum, H. leporinum, H. leporinum 4x, and H. glaucum; lanes 7-10, Anisolepis section: H. muticum, H. stenostachys, H. chilense, and H. flexuosum; lanes 11-13, Critesion section: H. lechleri, H. procerum, and H. arizonicum. (B) Section Stenostachys, lanes 1-7: H. marinum, H. bogdani, H. roshevitzii, H. depressum, H. parodii, H. geniculatum and H. magellanicum. The molecular size standard Lambda digested with HindIII was included in lanes 0.

greater similarity to each other than to other members of the group. H. vulgare and H. bulbosum were also clustered relative to other members of their section.

Cladistic Analysis

Information from hybridization of five mid-repetitive probes to 20 Hordeum taxa was expressed in the form of a data matrix for absence/presence of hybridization bands and analyzed using PAUP program (Swofford 1985).

One of the 20 equally parsimonious cladograms produced by PAUP from the data is shown in Fig. 17. H. vulgare and H. bulbosum related to each other closer than to any of the other section Hordeum species. H. glaucum was placed one branch apart from the other species in its group. Sections Anisolepis and Critesion clustered together apart from section Stenostachys, but were distinguished from each other. Species of section Stenostachys were classified together in a group where the close relation between H. marinum and H. geniculatum is evident. The corresponding consensus tree is shown in Fig. 18.

Discussion

Little information was gained from copy number analysis regarding the grouping of species into sections. However, strong hybridization to all the probes was detected in the dot blots of DNA from H. vulgare and H. bulbosum, indicating

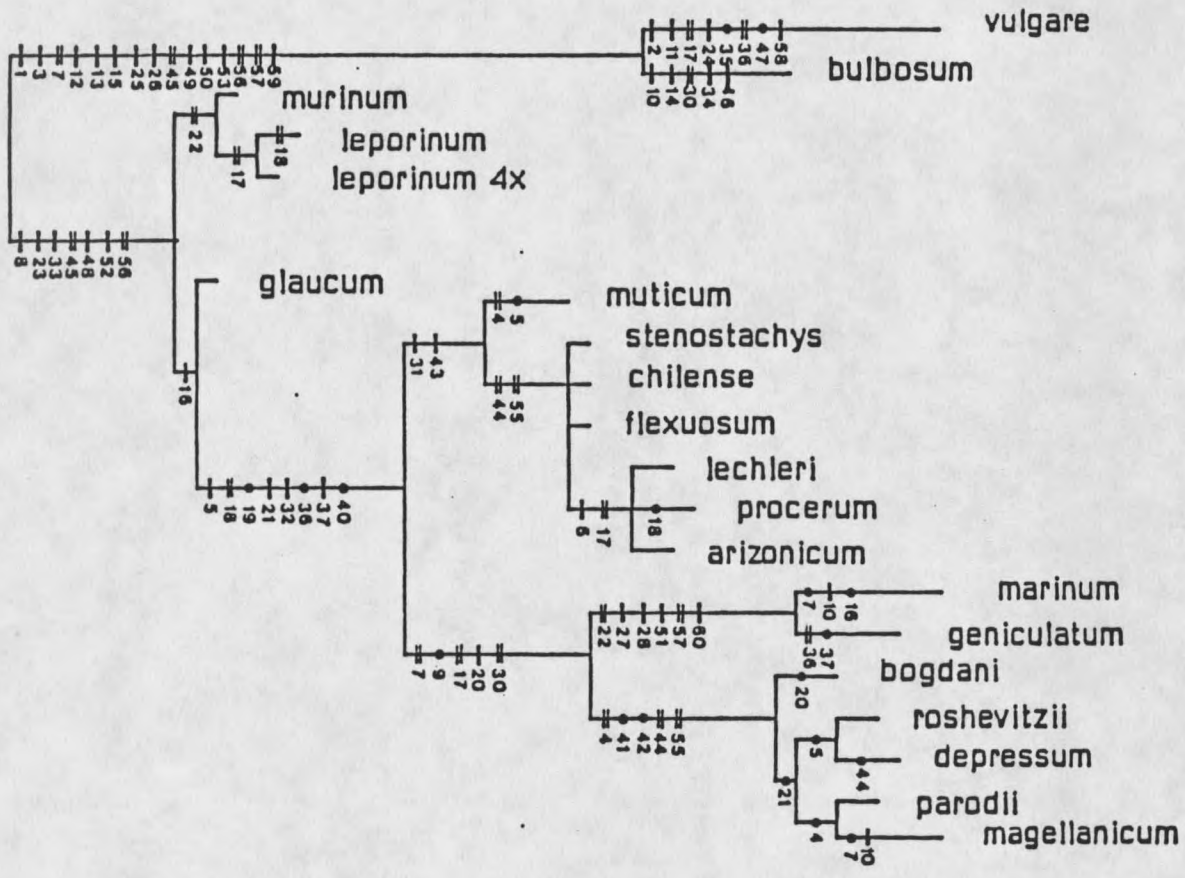

Figure 17. One of the 20 most parsimonious trees generated by PAUP for Southern data of 20 Hordeum species. CI=0.691. The numbers refer to the restriction fragments included in the data matrix. Synapomorphies, reversals and parallelisms are shown by bars, dots and parallel lines, respectively.

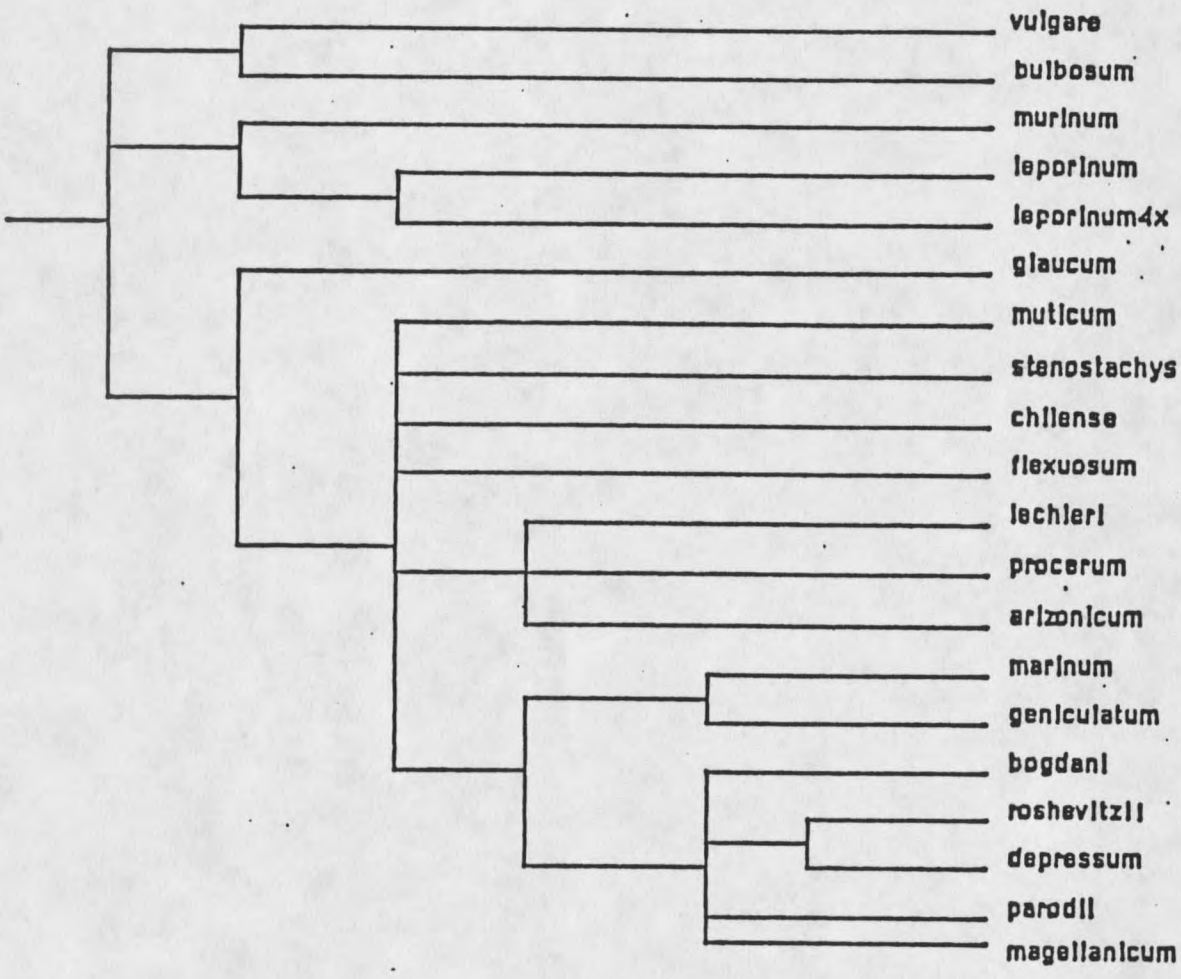

Figure 18. Strict consensus tree for Southern data of 20 Hordeum species.

an abundance of sequences with similarity to the probes. These two species seem to have similar numbers of each cloned sequence, and considerably more of these sequences than the rest of the species in the genus.

Hybridization was detectable but weaker in H. leporinum (2x and 4x), H. glaucum, H. lechleri, H. arizonicum, H. bogdani, H. brachyantherum, and H. magellanicum, indicating either reduced abundance of similar sequences or reduced sequence homology. Although DNA sequences related to the mid-repetitive probes are present in all the species investigated, variation in their copy number or percentage of homology (not distinguished by this analysis) among groups was not obvious. The five clones evaluated appear to be homologous to elements which have increased in copy number relatively recently in the lineage resulting in cultivated barley.

Although only one accession for each species was used in this study, we examined six cultivars of Hordeum vulgare with six restriction endonucleases to estimate intra-specific variation. Two of the cultivars showed an extra band when digested with BamHI and HindIII and probed with two of the clones while the rest were uniform. Thus, intraspecific variation appeared to be slight. Figure 19 shows an example for three cultivars and three restriction endonucleases.

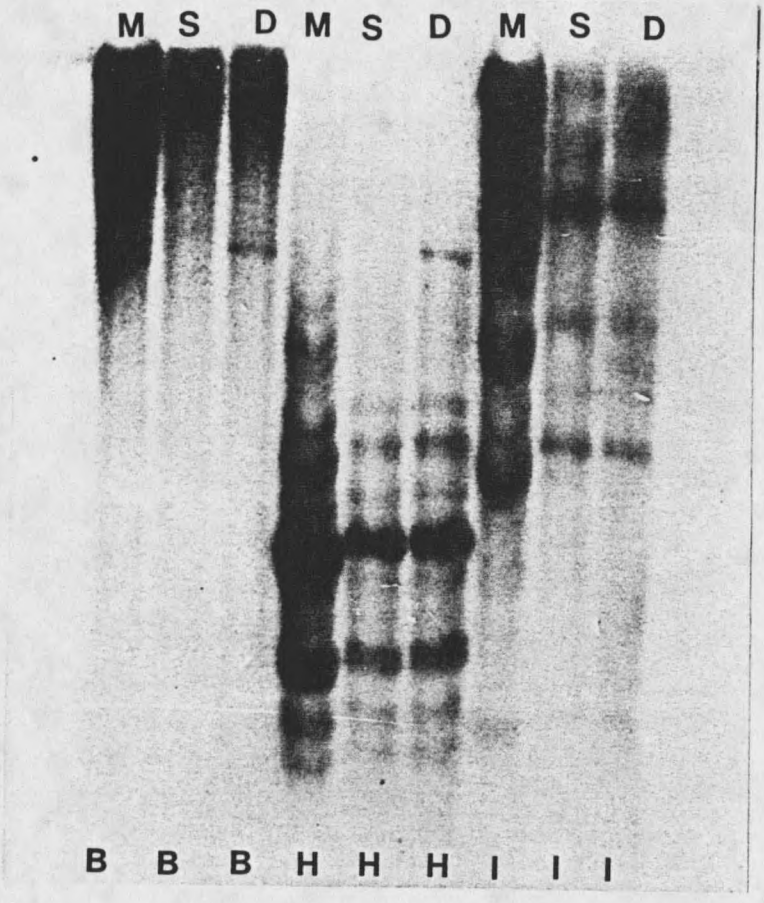

Figure 19. Southern blot of 3 barley cultivars probed with clone #44. Letters indicate the following DNA samples and restriction endonucleases: Morex (M), Steptoe (S) and Dicktoo (D); and BamHI (B), HindIII (H) and EcoRI (I).

Substantial variation was detected at the interspecific level. Five distinct sets of hybridization patterns appeared to be conserved in the section Hordeum, with H. vulgare and H. bulbosum giving unique and strong variant patterns (Figs. 12, 13, 14, 15, and 16).

Identical patterns were found for the species of section Stenostachys with probes #33 (Fig. 14 B) and #40 (Fig. 15 B). Two different typical patterns for sections Anisolepis and Critesion were shown with probe #17 and #25 (Figs. 12 A and 13 A). Furthermore, in H. marinum and H. geniculatum, regarded to be conspecific by Bothmer and Jacobsen (1985), prominent extra bands corresponding to fragments of 3.0 kbp, 2.4 kbp and 1.0 kbp (probe #17), 1.8 kbp and 1.3 kbp (probe #25), 2.0 kbp (probe #33), 2.0 kbp (probe #40) and 2.0 kbp, 1.5 kbp and 1.0 kbp (probe #44) appeared to be superimposed upon the patterns for the species of section Stenostachys. These bands have been gained in H. marinum and H. geniculatum recently by amplification of the original sequence, after the divergence of the section Stenostachys. Alternatively these sequences may have been lost due to deletion in the parent of the remaining members of this section.

Computerized analysis of this information resulted in production of a dendogram of the taxa generally compatible with previous knowledge regarding genus Hordeum (Fig. 17). H. glaucum is placed apart from the rest of the species of

section Hordeum; however, this "species" has been treated as conspecific to H. murinum and H. leporinum by Bothmer and Jacobsen (1985).

A consistency index (CI) (Kluge and Farris 1969) was computed as an estimate of homoplasy in the data. For a data matrix of this type, with binary coding, the consistency index (CI) is equivalent to the number of characters divided by the number of steps in the cladogram and is a rough measure of parallelism or convergence. Ideally, with no homoplasy, the consistency index is 1. For the cladogram in Fig. 17, the consistency index is 0.691, a value indicating 31% homoplasy. Our data matrix comprises 20 taxa and 61 characters. Thirty-nine characters (about 60% of the total) had CI's of 1.0, eleven had CI's of 0.5, four of 0.33, two of 0.25 and five were constant characters.

Although the consensus tree is not to be taken as a phylogenetic tree, affinities found in the cladogram may reflect true phylogenetic relationships (Bonen and Doolittle 1976). If the consensus tree (Fig. 18) is accepted as a cautious hypothesis of relationships, some conclusions may be drawn. First, H. vulgare and H. bulbosum are more closely related to each other than either is to the other taxa. Gupta et al. (1989) using a highly repeated ribosomal probe from Secale cereale found that H. vulgare differed markedly from the other species of the section Hordeum, and that H. bulbosum showed a greater similarity to the probe

than the other species of its section. Results presented here further demonstrate the close relationship between H. vulgare and H. bulbosum, considered previously closely related from cytogenetic studies (Kasha and Sadasivaiah 1971). Our data do not support the distinction of H. vulgare as the sole species in a separate genus (Hordeum) as was suggested by Löve (1984) and Dewey (1984). By our criteria, cultivated barley appears quite closely related to H. bulbosum. The Critesion (Löve 1984) and Hordeum genera could also be reunited on the base of their unique spikelet morphology (Kellogg 1989). It is clear however that H. vulgare and H. bulbosum are more closely related to each other than to the other species of the section Hordeum (H. murinum, H. leporinum, and H. glaucum) and therefore their classification in a section distinct from the other species of the section Hordeum may be justified.

Second, and in agreement with Bothmer and Jacobsen (1985), the two H. leporinum species (2x and 4x) appear as sister taxa, and clustered closely with H. murinum. The same is observed for H. marinum and H. geniculatum, treated as conspecific as well by the same authors.

Third, sections Anisolepis, Critesion and Stenostachys appear more closely clustered to each other in the consensus cladogram than to section Hordeum. Sequence divergence is known to have accompanied amplification events during the evolution of cereals (Flavell 1985). The sequences utilized

in this study were selected on the basis of being highly reiterated in cultivated barley and of low copy number in wheat and rye. Sequences selected in this manner are likely to be more diverged in cultivated barley than in related species. This would result in the identification of elements which are less derived in the wild relatives, and thus a closer clustering of the wild relatives than may be actually warranted. Although the four species representing the section Anisolepis are not resolved with respect to each other, the section is very-well delimited from Critesion and Stenostachys sections.

The evolutionary structure of the genus will be correctly resolved not only by morphological cladistic or genome analysis, but also by DNA similarities. These experiments demonstrated that DNA hybridization data of repetitive sequences provides valuable information in formulating phylogenetic hypotheses.

Restriction profiles of total DNA from these 20 species provided measures of genetic similarities which were useful in delineating sections in the genus Hordeum.

CHAPTER 5

CLADISTIC ANALYSIS OF SEQUENCE AMPLIFICATION PATTERNS FOR 23 *HORDEUM* SPECIES

Introduction

There is general agreement among systematists that classifications should provide an estimate of phylogenetic history (Stevens 1987). The phylogeny of *Hordeum* species has been estimated by chromosome pairing studies of hybrids at different ploidy levels (Löve 1984; Dewey 1984), karyotype analysis using chromosome measurements, chromosome banding patterns (Linde-Laursen *et al.* 1980), and *in situ* hybridization with specific probes (Rayburn and Gill 1987).

Appels *et al.* (1987) and West *et al.* (1988) reviewed evidence bearing on the evolution of nuclear genes in the genera *Triticum*, *Hordeum*, and *Secale*. Restriction enzyme analysis of the chloroplast DNA (Poulsen 1983) and rDNA polymorphisms (Molnar *et al.* 1989; Gupta *et al.* 1989) have also contributed. Recently, homologous DNA sequences from a wide array of animals were analyzed to estimate phylogenetic relationships by polymerase chain reaction sequence amplification and direct sequencing (Meyer *et al.* 1988).

The PCR technique was conceived and developed at Cetus Corporation by Mullis and Faloona (1987). It is an *in vitro* method for the primer-directed enzymatic amplification of specific DNA sequences. The specificity of PCR amplification

is based on two oligonucleotide primers which flank the DNA segment to be amplified and anneal to opposite strands.

In this chapter oligonucleotide primers were selected from dispersed middle repetitive element sequences, and used to amplify elements from 23 Hordeum species using the polymerase chain reaction (PCR) approach.

Materials and Methods

Plant Material

The 23 Hordeum species analyzed included those listed in Tables 3 and 5. Except for five accessions of Hordeum chilense (gift of Dr. A. Martin) and eight of H. vulgare, all species were represented in this analysis by one accession. The use of more accessions for the rest of species was limited to availability from the USDA world collection. Total plant DNA was extracted as previously described (chapter 3).

Table 5. Additional species included in the PCR experiments. Ploidy level, life form and origin as in Table 3.

Section/species	Ploidy level	Life form	Origin E/A
Stenostachys			
californicum	2X	perennial	A
brachyantherum	4X	perennial	A
parodii	6X	perennial	A

Selection of Primers

To design primers, we initially studied partial sequences obtained for each of the 5 nuclear barley specific repetitive clones selected in chapter 2. Twelve to twenty base pair sequences were selected from each clone which flanked 100 to 200 base pair sequences that shared short repeats among clones. Primers were also selected for relative GC richness and a lack of internal and interprimer homology.

Amplification of Repetitive Sequences

Polymerase chain reaction sequence amplification was performed as described by Erlich et al. (1986) using approximately 100 ng of genomic DNA and 20 ng of each primer. The cycling protocol was of 1 minute at 94 ^{o}C for denaturation of template DNA, 2 minutes at 37 ^{o}C for annealing of the primers to the DNA template and 4 minutes at 72 ^{o}C for extension of the primers. This cycle was repeated thirty times.

DNA Electrophoresis

Amplified DNAs were separated by electrophoresis in 1.4% agarose gels at 100V for two hours. Gels were stained with ethidium bromide and photographed under UV light.

Cladistic Analysis

Data was analyzed entirely, and in subsets, as follows:
a. all the PCR fragments present in all the taxa,
b. all the PCR fragments present in all the taxa that were also monomorphic within the 5 H. chilense accessions and the 8 H. vulgare cultivars, but polymorphic among species,
c. by removing known allopolyploid taxa.

The presence (1) or absence (0) of each PCR fragment was scored for every accession and a data matrix constructed. These characters (64) of PCR fragments were unordered during analysis. Data was analyzed with the computer program PAUP (Swofford 1985) using the options of global branch swapping on multiple parsimonious trees. Midpoint rooting was used because the difficulty in determining or using an outgroup. The mid-repetitive elements cloned in chapter 2 were specific to Hordeum species (little or no hybridization was detected with species of Triticum or Secale) and identified interspecific variation in both copy number and structure. For this reason no outgrouping from related genera could be used.

Forward changes, reversals (losses of particular amplification fragments) and parallelisms (independent gains or losses of particular fragments) are shown in the cladogram. Although initially a 1 in the data matrix indicated the presence of a fragment, the derived condition is the loss of a fragment for characters 6, 18, 46, 49.

Results

Amplification Patterns

Several different amplification protocols were assayed to identify one which would provide more consistent discrimination among species, but no improvement was attained. As a consequence the reported data were obtained using the standard reaction protocol previously described.

To check whether or not the fragments resulting from the amplification experiments corresponded to sequences belonging to the original repetitive sequences, transfers of PCR gels and hybridization with original clones were performed as described in chapter 3. Major PCR bands resulted in strong hybridization bands, while some minor bands seemed to result from amplification of sequences less related to the original repetitive element (Fig. 20).

Different amplification patterns were observed for different species/primer combinations. Substantial interspecific variation was observed when each of the primer sets was tested across species (Fig. 21).

Hordeum section was distinctly delimited by the PCR analysis (clearly visible for the primers set in Fig. 21 where arrow shows a section specific PCR fragment). Poorer resolution was observed for the other sections in the genus. Analysis of barley cultivars and H. chilense accessions revealed many variable bands within species (Fig. 22).

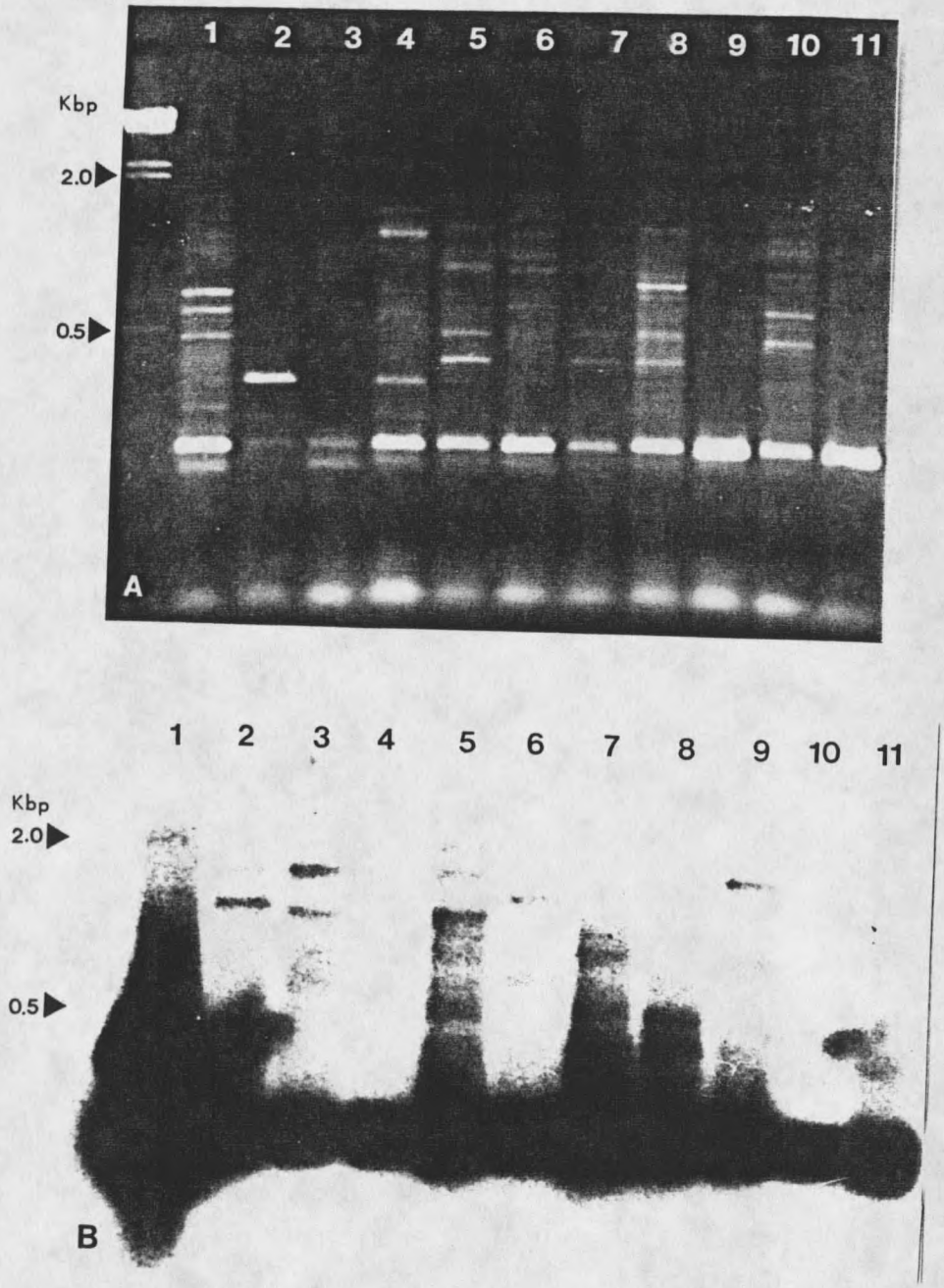

Figure 20. Identification of amplification fragments with similarity to the cloned repetitive elements. A: Gel of 11 Hordeum species amplification patterns when primers chosen from #33 element sequence were used for the PCR experiment. B: Autoradiograph of blot of A probed with labeled clone #33. Lanes 1-11: H. magellanicum, H. marinum, H. murinum, H. muticum, H. parodii, H. parodii 6x, H. procerum, H. roshevitzii, H. bulbosum, H. stenostachys and H. vulgare.

Cladistic Analysis of Total PCR Data

For a data matrix including all the PCR data for the 23 taxa, PAUP gave 25 most equally parsimonious trees at 123 steps which were all similar to the one shown in Fig. 23. In this analysis the 6 species belonging to the section Hordeum were clustered together. H. geniculatum, often recognized as a subspecies of H. marinum was consistently grouped with this species. The Anisolepis species H. chilense and H. flexuosum were associated together but did not show more affinity to the other two Anisolepis species, H. stenostachys and H. muticum, than to the Stenostachys section species. The same was observed for the Critesion section species H. arizonicum, H. procerum and H. lechleri which were interspersed among Stenostachys section species.

A strict consensus tree (Adams 1972) was computed for this data set in order to identify the clades which were consistently resolved. For total PCR data-total taxa, the consensus tree is shown in Fig. 24. The lack of resolution for H. brachyantherum, H. californicum, H. lechleri, H. muticum and H. procerum is evident.

Cladistic Analysis of Amplification Fragments Monomorphic Within Species

Homoplasy may occur throughout the tree if the state of presence of a character (PCR fragment) is assigned to a species when a particular size band is observed in the pictures of the gels. Although two species may share

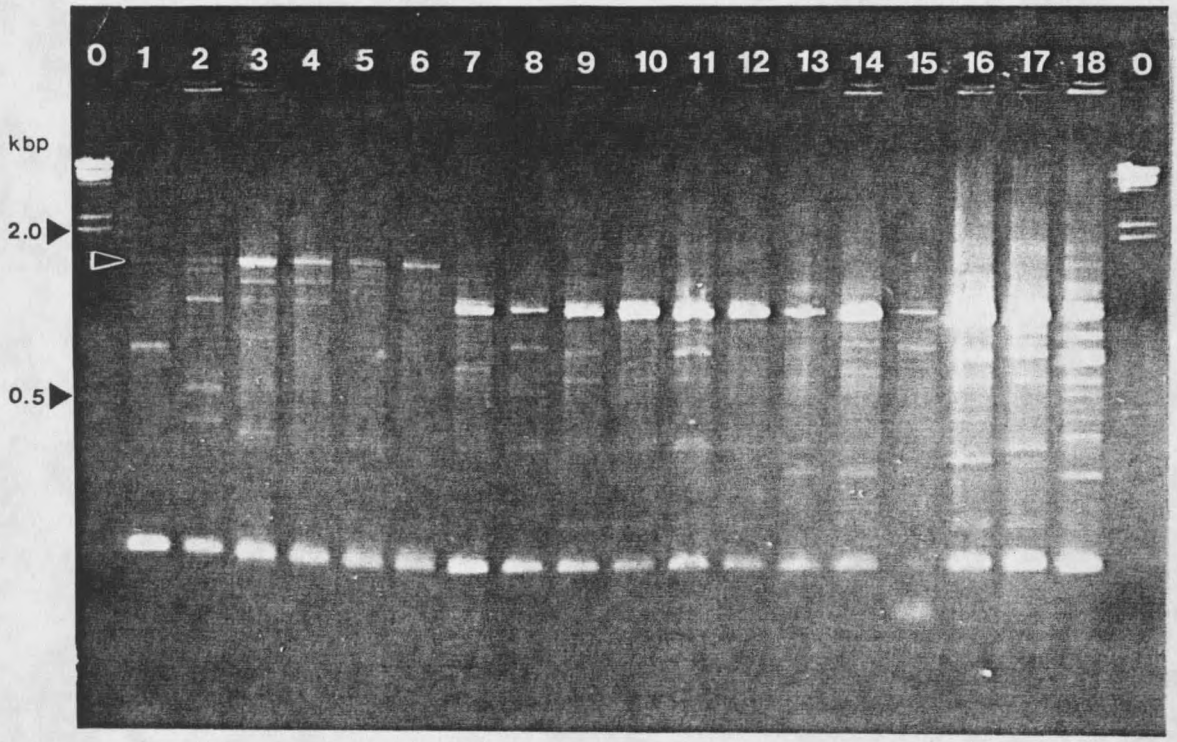

Figure 21. Interspecific variation of PCR patterns for one of the five primers sets and 18 Hordeum species. Lanes 1 to 6, Hordeum section: H. vulgare, H. bulbosum, H. murinum, H. leporinum, H. leporinum 4x and H. glaucum; lanes 7 to 9, Anisolepis section: H. stenostachys, H. chilense and H. flexuosum; lanes 10 to 12, Critesion section: H. lechleri, H. procerum, and H. arizonicum and lanes 13 to 18, Stenostachys section: H. marinum, H. geniculatum, H. bogdani, H. depressum, H. parodii and H. magellanicum. Molecular size standards (lambda phage, digested by HindIII) are included in lanes 0. Arrow indicates section Hordeum specific amplification band.

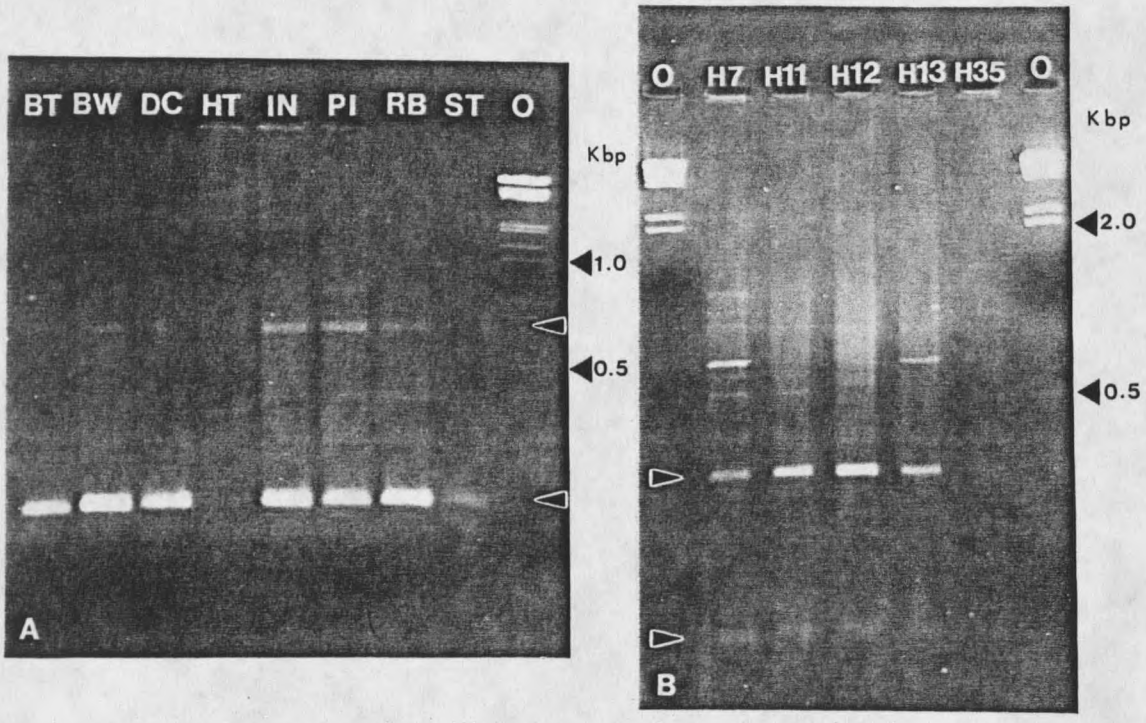

Figure 22. Intraspecific variation of PCR patterns for the same set of primers in Fig. 1. (A) Barley cultivars: Betzes (BT), Bowman (BW), Dicktoo (DC), Hector (HT), Ingrid (IN), Piroline (PI), Robust (RB), Steptoe (ST). (B) H chilense accessions: H7, H11, H12, H13, and H35. Lanes 0 represent molecular size standard (Lambda HindIII digested). Arrows show PCR bands monomorphic within species.

fragments of the same electrophoretic mobility, they need not be homologous. The somewhat subjective interpretation of amplification patterns has certain limitations, as do most other biosystematic criteria. Analysis of barley cultivars and H. chilense accessions revealed many variable bands within species (Fig. 22). The PCR data from 5 H. chilense accessions and 8 H. vulgare cultivars were utilized to create a new matrix of data in which only PCR fragments monomorphic within a species were analyzed. The number of characters became considerably reduced from 64 to 19. Since data from accessions other than H. vulgare and H. chilense were unavailable, some potentially informative amplification fragments were excluded from this second analysis. However this approach illustrates the effect that this method of analysis produces in the grouping of species.

The number of characters included in a data matrix affects the amount of homoplasy detected by cladistic analyses. If the number of characters is large, the effect of homoplasy will decrease and real relationships among taxa will be better detected. If the characters are too few, several equally or almost equally parsimonious trees will result. For the monomorphic PCR fragments study the number of characters was low and hence 50 parsimonious cladograms were produced in 36 steps. The corresponding consensus tree (Fig. 25) illustrates the clades consistently resolved in these cladograms. Since the accessions utilized for this

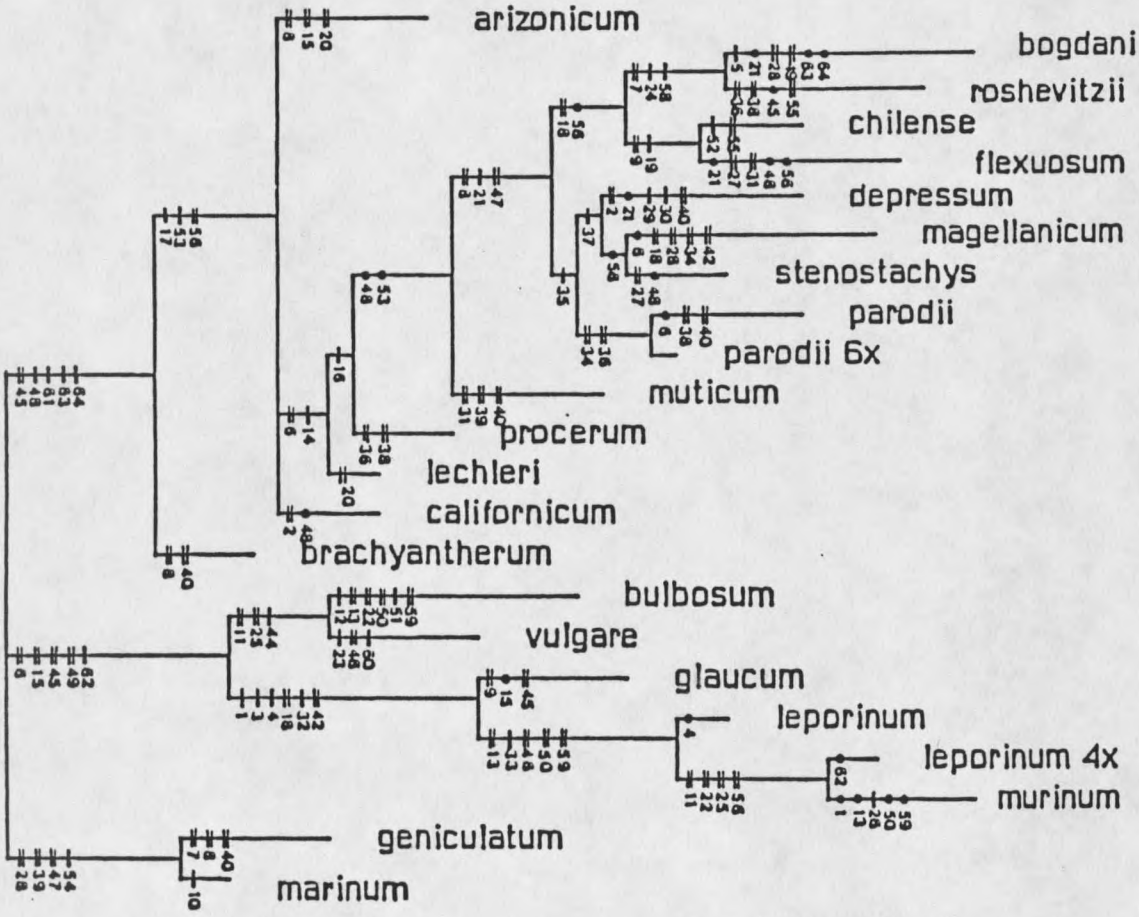

Figure 23. One of the most parsimonious trees for 23 Hordeum species when all PCR fragments were included in the data matrix. CI=0.504. The numbers refer to the amplification fragments included in the data matrix. Synapomorphies, reversals and parallelisms are shown by bars, dots and parallel lines, respectively.

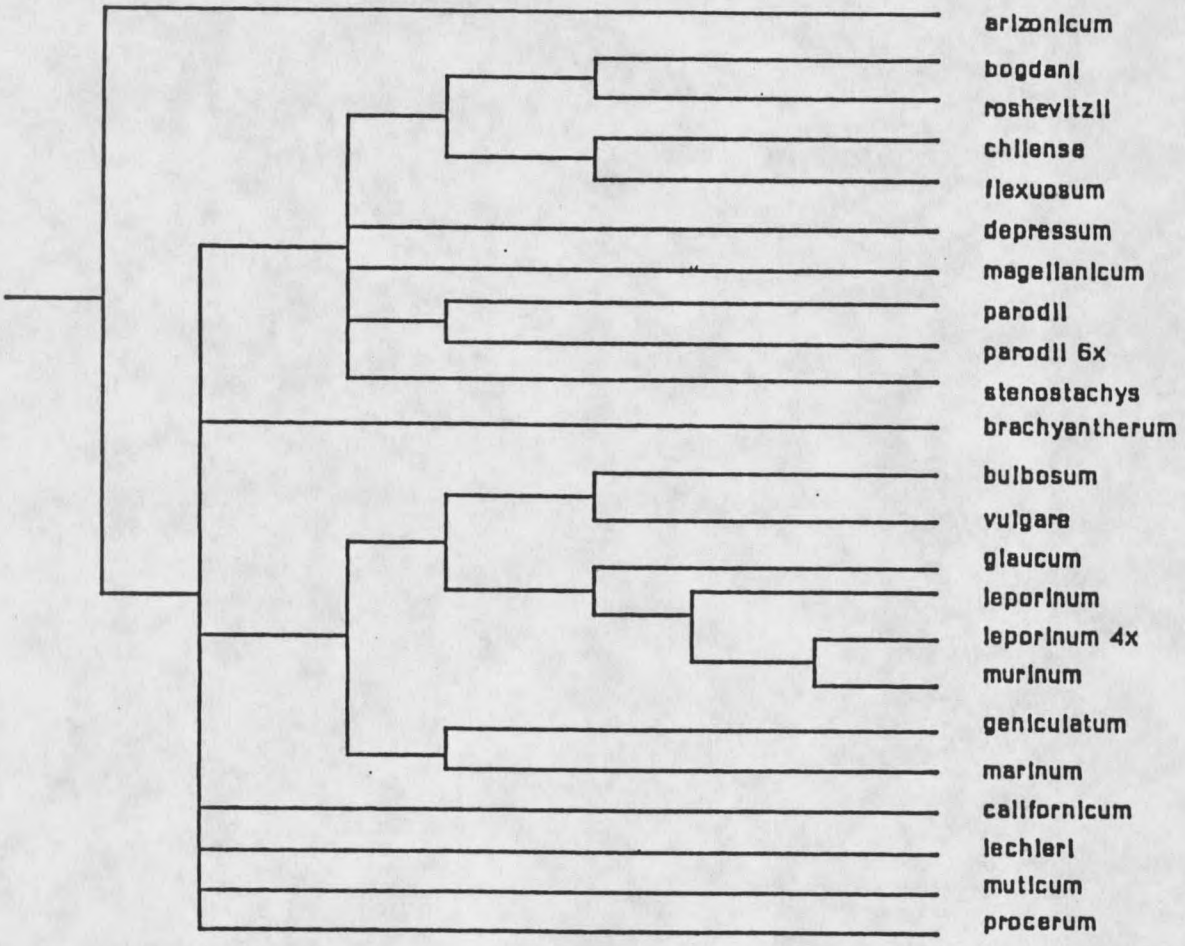

Figure 24. Strict consensus tree for complete PCR data from 23 Hordeum species.

attempt were from Hordeum and Anisolepis sections (according to Bothmer and Jacobsen 1985), only Hordeum and Anisolepis sections were resolved. Intraspecific monomorphic fragments that show interspecific polymorphism are useful markers for inferring relationships in these sections.

Cladistic Analysis of PCR Data from Only Monophyletic Taxa

The most fundamental assumption of cladistics is that evolution proceeds only by splitting of lineages (Farris 1983). Application of cladistic algorithms to the genus Hordeum would be valid only if the genus had evolved solely in this way. In reality, evolution of the genus has been affected by at least one source of reticulation: allopolyploidy (Grant 1971).

H. lechleri, H. arizonicum and H. procerum, the three representatives of the section Critesion used here, are of alloploid origin. The derived polyploids bridge gaps of varying width between their parents, producing the impression that the parents are more closely related than they really are. At least one of the supposed ancestors of H. procerum, H. stenostachys, is present in our study. The ancestors of H. lechleri are unknown. Because the heterogenomic taxa include characteristics of two or more lines, they introduce a high level of homoplasy into the matrix. Studies on groups that include known hybrids have shown that the presence of the hybrids results in reduced