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Ribosomal DNA repeat unit polymorphism in 25 *Hordeum* species *

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Summary. Tandemly repeated DNA sequences containing structural genes encoding ribosomal RNA (rDNA) were investigated in 25 species of *Hordeum* using the wheat rDNA probe pTA71. The rDNA repeat unit lengths were shown to vary between 8.5 and 10.7 kb. The number of length classes (1–3) per accession generally corresponded to the number of nucleolar organizing regions (NORs). Intraspecific variation was found in *H. parodii, H. spontaneum* and *H. leporinum,* but not in *H. bulbosum.* Restriction analysis showed that the positions of EcoRI, SacI and certain BamHI cleavage sites in the rRNA structural genes were highly conserved, and that repeat unit length variation was generally attributable to the intergenic spacer region. Five rDNA BamHI restriction site maps corresponded to the following groups of species: Map A – *H. murinum, H. glaucum, H. leporinum, H. bulbosum, H. marinum, H. geniculatum;* Map B – *H. leporinum;* Map C – *H. vulgare, H. spontaneum, H. agriocrithon;* Map D – *H. chilense, H. bogdanii;* and Map E – remaining 14 *Hordeum* species. The repeat unit of *H. bulbosum* differed from all other species by the presence of a HindIII site. The closer relationship of *H. bulbosum* to *H. leporinum, H. murinum* and *H. glaucum* than to *H. vulgare* was indicated by their BamHI restriction maps.

Key words: *Hordeum* – Barley – Ribosomal DNA – Polymorphism – Restriction analysis

Introduction

The classification of species in the genus *Hordeum* is still a matter of debate (reviewed by Bothmer and Jacobsen 1985). Since DNA homology is a measure of relatedness between species, direct study of DNA should resolve contentious issues. Much of the genome of eukaryotes consists of highly repeated DNA sequences (for review, see Long and Dawid 1980). Therefore, specific probes for repetitive DNA are of considerable interest in phylogenetic studies (Flavell 1982). For example, the tandem arrays of ribosomal DNA (rDNA) repeat units, located in the nucleolar organizer regions (NORs) of chromosomes, combine highly conserved gene regions, encoding ribosomal RNA with more variable intergenic spacer regions (IGS) (for reviews, see Appels and Honeycutt 1986; Rogers and Bendich 1987).

Polymorphism has been detected in the length of the rDNA repeat units of barley (*H. vulgare*) (Gerlach and Bedbrook 1979) and of its wild ancestor, *H. spontaneum* (Saghai-Maroof et al. 1984; Breiman et al. 1987), using the wheat rDNA probe pTA71. In the present study, using this probe, the extent of rDNA repeat unit polymorphism has been examined in 25 species of *Hordeum.* Intraspecific variation was investigated using the inbreeding species *H. leporinum, H. parodii* and *H. spontaneum,* and the obligate outbreeder *H. bulbosum.*

Materials and methods

Plant material

The species of *Hordeum* investigated are listed in Table 1. They include 61 accessions representing 25 *Hordeum* species, including two accessions of barley cv 'Betzes' (2x and 4x). Seed was obtained from Plant Gene Resources of Canada, Agriculture Canada, Ottawa.

DNA extraction and Southern blots

Total cellular DNA was isolated from leaves (2.5 g fresh wt) of individually pot-grown plants. The tissue was homogenized in 30 m*M* TRIS-HCl (pH 8.0), 30 m*M* EDTA, 6% SDS and ex-

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tracted at room temperature with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The aqueous phase was further extracted with chloroform/isoamyl alcohol (24:1, v/v). Sodium acetate (pH 5.5) was added to the aqueous supernatant to give a 0.3 *M* solution, and the DNA was precipitated with 2 vol of ethanol at −80 °C. After centrifugation, the pellet was suspended in 2 m*M* TRIS-HCl (pH7.7), 5 m*M* NaCl, 0.1 m*M* EDTA and further purified by CsCl/ethidium bromide density gradient centrifugation (Maniatis et al. 1982). Final DNA yield was estimated spectrophotometrically. Routinely 1–2 µg DNA was digested for 1 h at 37 °C, using 10 units of restriction endonuclease in buffer recommended by the supplier (Boehringer Mannheim, Canada). Each DNA sample was digested singly with BamHI, EcoRI, HindIII and SacI. Restriction fragments were separated by electrophoresis in 0.8% agarose gels in 89 m*M* TRIS, 2.5 m*M* EDTA, 89 m*M* boric acid. After denaturation in 0.5 *N* NaOH, 1.5 *M* NaCl for 30 min, and neutralization in 1.5 *M* NaCl, 1 *M* TRIS-HCl (pH 8.0), the DNA was transferred by blotting (Southern 1975) to Biodyne membranes (Pall) and dried at 80 °C for 2 h under vacuum.

DNA probing

The probe, pTA71, consisted of an 8.95-kb EcoR1 fragment of wheat DNA originally isolated in pACYC184, cited as pAC184 (Gerlach and Bedbrook 1979), since subcloned in pUC19 (as supplied by M. O'Dell, Institute of Plant Science Research, Cambridge). The cloned insert is a full-length nuclear rDNA repeat unit, and includes structural genes for 26S, 18S and 5.8S rRNA, as well as spacers. The probe was labelled by nick translation (Rigby et al. 1977) using deoxycytidine 5′-(^{32}P) triphosphate (3,000 Ci mmol^{-1}) from Dupont Canada. DNase I and DNA polymerase I were supplied by Boehringer Mannheim, Canada. Hybridization conditions were as described by Botchan et al. (1976), except that 50% formamide was included in the hybridization mixture and the temperature used was 42 °C. The final wash was for 90 min at 42 °C in 0.1 × SSC buffer (Maniatis et al. 1982). Autoradiography was carried out with Kodak XAR-2 film.

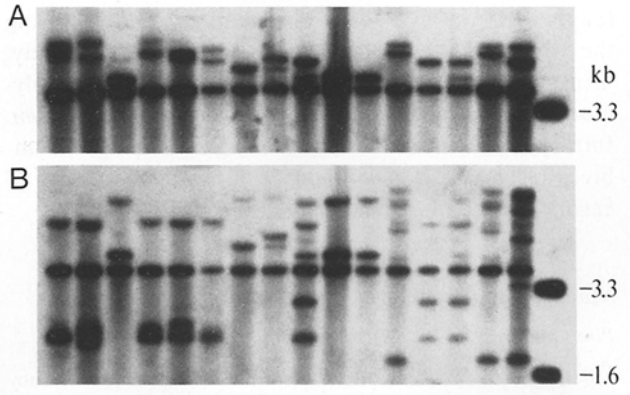

Fig. 1 A and B. Variation in **A** SacI and **B** BamHI restriction fragments which hybridize to pTA71 among (*lane 1*, left) *H. procerum*, *2* – *H. pubiflorum*, *3* – *H. glaucum*, *4* – *H. arizonicum*, *5* – *H. brachyantherum*, *6* – *H. parodii* CHC 1332, *7* – *H. marinum*, *8* – *H. geniculatum*, *9* – *H. leporinum* CHC 2325, *10* – *H. leporinum* CHC 2233, *11* – *H. murinum*, *12* – *H. agriocrithon*, *13* – *H. leporinum* CHC 2249, *14* – *H. leporinum* CHC 2369, *15* – *H. spontaneum* CHC 0526, *16* – *H. vulgare* cv 'Betzes' (diploid), and *17* – pBR322 size markers

Cytological techniques

Somatic chromosomes were studied using root tip mitoses. Root tips from young seedlings were excised and treated with ice-cold water for 24 h, before being fixed in 3:1, ethanol:acetic acid fixative for at least 24 h. The tips were squashed in acetocarmine and metaphase plates were examined for satellited chromosomes.

Results

Variation in rDNA repeat unit length

The hybridization patterns in Southern blots of EcoRI and SacI digests indicated 17 repeat unit length variants, in 29 combinations or phenotypes (Table 1). Each band in an EcoRI pattern indicated a length variant, while SacI patterns showed that each variant consisted of a common 3.9 kb component and a segment of variable size (Fig. 1). The 14 accessions of *H. bulbosum* were identical and possessed repeat units of 8.5 kb only (Fig. 2). The 16 accessions of *H. spontaneum* tested always contained two repeat units, one of 10.2 kb, while the second repeat unit was either 9.0, 9.3, 9.6 or 9.7 kb (Figs. 1 and 2). Only two of the six accessions of *H. leporinum* were identical in their combination of repeat unit lengths and each of the three accessions of *H. parodii* was unique (Table 1).

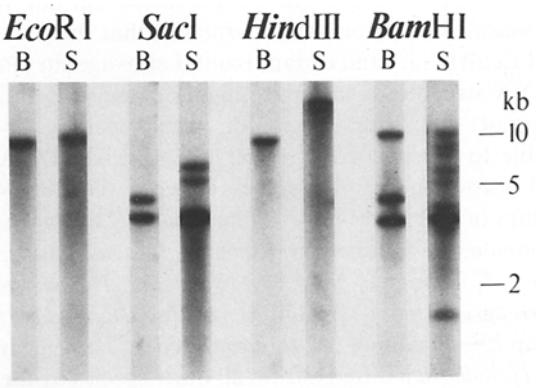

Fig. 2. Representative autoradiograms of *H. bulbosum* (B) and *H. spontaneum* (S) DNA digested with EcoRI, SacI, HindIII and BamHI and probed with pTA71. *H. bulbosum* produced a single band, with either EcoRI or HindIII representing its entire rDNA repeat unit length. Bands corresponding to fragments of 4.6 kb and 3.9 kb in SacI digests total to the repeat unit length. Bands corresponding to fragments of 8.5 kb and of 4.6 kb and 3.9 kb indicate full repeats and fractionated repeats following BamHI digestion. *H. spontaneum* DNA was not digested by HindIII but gave a double band corresponding to fragments of 9.3 kb and 10.2 kb following EcoRI digestion. Each repeat unit length contributes a common 3.9-kb fragment and either a 5.4- or a 6.3-kb fragment after SacI digestion. Both repeat unit lengths contribute 1.8-kb and 3.9-kb BamHI fragments. The 3.6- and 4.5-kb fragments represent the balance of the 9.3- and 10.2-kb repeat units respectively. Additional bands corresponding to fragments of 5.5, 6.4, 7.7, 8.4 and 10.1 kb indicate lack of cleavage in some repeats

Table 1. Ribosomal DNA repeat unit length variants in *Hordeum* species

Section	Species	Ploidy	No. of accessions	rDNA repeat unit length (kb) and (BamHI map)	Repeat length phenotype	No. of satellited chromosomes[a]	CHC accession no.
Hordeum	*H. murinum*	2x	1	8.5(A)	1	1	1384
	H. bulbosum	4x	14	8.5(A)[b]	1	1 (×2)[f]	note[d]
	H. glaucum	2x	1	8.5(A) 9.3(A)	2	2	–
	H. leporinum	2x	2	8.5(A)	1	1	2233, 2473
		4x	1	8.9(A) 9.4(A)	3	2	0869
		4x	1	8.5(A) 9.0(B)	4	2	2325
		4x	1	8.5(A) 9.1(B)	5	2	2369
		4x	1	9.1(B)	6	2	2249
	H. vulgare	2x	1	9.1(C) 10.2(C)	7	2	–
		4x	1	9.1(C) 10.2(C)	7	2 (×2)[f]	–
	H. spontaneum	2x	2	9.0(C) 10.2(C)	8	2	0543, 2111
		2x	2	9.3(C) 10.2(C)	9	2	0577, 0946
		2x	8	9.6(C) 10.2(C)	10	2	note[e]
		2x	4	9.7(C) 10.2(C)	11	2	1127, 2103, 2109, 8070
	H. agriocrithon	2x	1	9.6(C) 10.2(C)	10	2	2731
Anisolepis	*H. stenostachys*	2x	1	9.8(E)	12	2	1295
	H. muticum	2x	1	10.3(E)	13	1	1754
	H. chilense	2x	1	8.7(D) 9.6(DorE) 10.3(X)[c]	14	2	1669
	H. flexuosum	2x	1	9.8(E) 10.1(E)	15	1	1353
Critesion	*H. pubiflorum*	2x	1	9.6(E) 10.7(E)	16	1	1547
	H. lechleri	6x	1	9.4(E) 10.3(X)	17	4	1722
	H. procerum	6x	1	9.6(E) 10.2(X) 10.7(X)	18	3	1136
	H. arizonicum	6x	1	9.4(E) 10.7(E)	19	2	–
Stenostachys	*H. marinum*	2x	1	8.8(A)	20	1	8521
	H. geniculatum	4x	1	8.8(A) 9.2(A)	21	2	–
	H. bogdanii	2x	1	9.0(D) 9.8(D)	22	1	1117
	H. roshevitzii	2x	1	9.8(E)	13	1	–
	H. californicum	2x	1	10.2 (E)	23	1	1855
	H. brachyantherum	4x	1	9.3(E) 9.6(E)	24	2	1846
	H. depressum	2x	1	9.0(E)	25	1	1912
	H. mustersii	2x	1	9.5(E)	26	1	1358
	H. parodii	2x	1	9.1(E) 10.2(E)	7	1	1332
		6x	1	9.3(E) 10.1(E)	27	4	1575
		6x	1	9.2(E) 9.5(E) 10.2(X)	28	4	1632
	H. magellanicum	2x	1	9.6(E)	29	1	1463
Total	25		61				

[a] Number of satellited chromosomes per haploid genome
[b] *H. bulbosum* also has a HindIII site not found in other accessions surveyed
[c] BamHI phenotype X indicates that the corresponding repeat unit length rDNA produced insufficient observable BamHI fragments for the map to be determined
[d] *H, bulbosum* Canadian-Scandinavian *Hordeum* Collection (CHC) accession nos. 0008, 0049, 0438, 0447, 0454, 0458, 0480, 0809, 0837, 0864, 0890, 0903, 0986, 1089
[e] *H. spontaneum* CHC accession nos. 0397, 0473, 0485, 0526, 0618, 0627, 0851, 1135
[f] Autotetraploid *H. bulbosum* and *H. vulgare* have duplicates of each unique satellited chromosome

No clear bands of hybridization were detected following HindIII digestion of the DNA of any species, other than *H. bulbosum*. *H. bulbosum* gave a banding pattern identical to its EcoRI pattern, indicating that each repeat unit contains one HindIII site (Fig. 2).

Variation in BamHI restriction sites

Complex hybridization patterns were obtained with genomic DNA digested with BamHI (Figs. 1 and 2). Five BamHI restriction site maps (A–E) were obtained, each of which included a 3.9 kb fragment from the structural gene region (Fig. 3). Bands were also observed which corresponded to the sizes of adjacent BamHI fragments combined, consistent with sites being either absent or not cleaved. BamHI restriction maps could be used to group different *Hordeum* species (Table 1, Fig. 4).

Number of satellited chromosomes

Chromosome preparations were made to relate the rDNA repeat unit length variants with number of NORs.

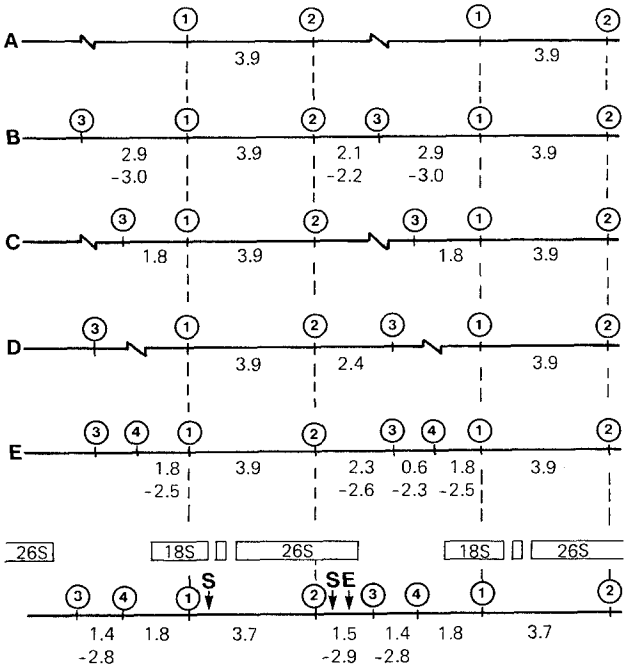

Fig. 3. BamHI restriction maps A, B, C, D and E as determined for certain *Hordeum* species compared to that published by Appels et al. (1980) for the 9.5- and 10-kb repeats of *H. vulgare* cv 'Clipper'. BamHI sites are indicated 1–4. The interrupted line indicates the fragment which varied in length according to the length of each specific repeat unit length variant. There is heterogeneity in *H. vulgare* with respect to the location of the third site in the map of Appels et al. (1980) and in the third and fourth sites of map E. The fragments flanking the 3.9-kb fragment were positioned 3′ or 5′ of the 3.9-kb fragment by assuming that the 18S BamHI site has little methylation as in *H. vulgare* (Gerlach and Bedbrook 1979), and the 26S BamHI site is highly methylated as in *H. vulgare* (Gerlach and Bedbrook 1979) and other species (Jorgensen et al. 1987), and as in *H. spontaneum* (Breiman et al. 1987), gives rise to the dominant combination fragment resulting from lack of cleavage at that site. The order of the 0.6–2.3 and the 1.8–2.5 kb fragments in map E has not been resolved. Map E predicts a fragment of 0.6–2.3 kb which was never observed, possibly because of poor homology with the wheat probe in this most rapidly diverging region of the repeat unit (Appels and Dvorak 1982). Positions of the EcoRI site (E) (Appels et al. 1980) and SacI/SstI sites (S) (Saghai-Maroof et al. 1984; Jorgensen et al. 1987) are indicated

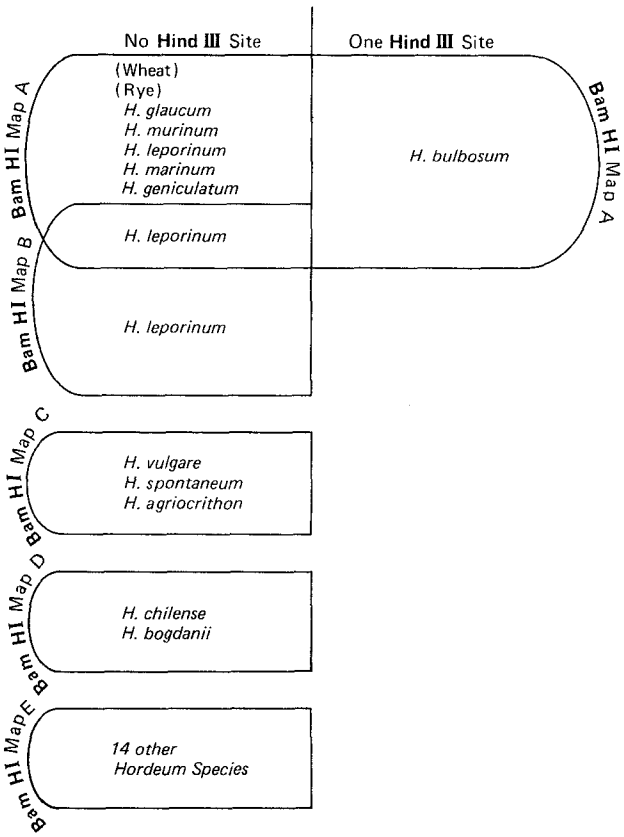

Fig. 4. Venn diagram showing the distribution of six rDNA BamHI plus HindIII restriction maps among *Hordeum* species

The number of rDNA repeat length variants and the number of satellited chromosomes corresponded in 52 of 61 accessions.

Discussion

Variation in rDNA repeat units among single accessions of 21 species of *Hordeum* have been studied. Multiple accessions have been examined in an additional four species to determine intraspecific variation.

Two types of polymorphism were observed. The first was in rDNA repeat unit length. In 11 species, only one repeat unit length was detected; together these represented nine length classes. Combinations of repeat unit length classes occurred in 15 species, in 12 of which the combination was unique. Variation was also determined at the intraspecific level. In *H. spontaneum,* two repeat unit length classes were detected in each accession, one of which was invariant. The 10.2-kb repeat unit, which was invariant in our accessions, was not always found in the accessions tested by Saghai-Maroof et al. (1984). Thus, there is great diversity in the genus *Hordeum* in repeat unit lengths.

The strong correlation between the number of repeat unit length classes detected and the number of pairs of satellited chromosomes, or NORs, suggests that in most cases the different classes are maintained at different NORs and have evolved independently, as has been postulated for other species (Appels and Honeycut 1986).

We have demonstrated that the structural gene region contained in a 3.9-kb SacI or a 3.9-kb BamHI segment of the rDNA repeat unit in barley (Fig. 3) is highly conserved in *Hordeum* species, length variation being attributed to the remainder of the repeat unit, which includes the IGS region. Variable numbers of sub-repeat units within the IGS region account for length variation in many genera (for a review, see Rogers and Bendich 1987).

However, there may also be length variation outside the sub-repeat region.

The second type of polymorphism that we observed in rDNA repeat units was in the number and relative position of cleavage sites for certain restriction endonucleases. EcoRI and SacI sites were conserved. Only the repeat units of *H. bulbosum* contained a HindIII site. Greater polymorphism was observed in BamHI cleavage sites, resulting in five basic BamHI restriction maps. In Figure 3, the BamHI cleavage sites have been numbered, although only sites 1 and 2 are clearly homologous throughout. The maps (Fig. 4) show some consistency with the classification of *Hordeum* species as proposed by Bothmer and Jacobsen (1985) and shown in Table 1. This relationship is most consistent with species in the section *Hordeum* (Table 1). Map type A, with sites 1 and 2 only, is identical to the maps of wheat and rye (Gerlach and Bedbrook 1979; Appels et al. 1980). Maps B, C and D each have a third BamHI site in a unique location giving distinctive BamHI digestion fragments. Our barley samples, as well as three plants from each of five additional Canadian 2-row barley cultivars (S. J. Molnar, unpublished results), all conform to map C. Map type E is a composite of related maps with a minimum of four BamHI sites, similar to that reported for barley (Appels et al. 1980).

Intraspecific variation was detected in the positions of BamHI sites in the repeat units of accessions of *H. leporinum,* yet no variation in the mapped positions was detected for three accessions of *H. parodii,* or amongst the 16 accessions of *H. spontaneum.* It therefore seems to be a reasonable explanation that the flexibility of repeat unit length in *H. spontaneum* is due to fluctuations in the number of IGS sub-repeat units which do not affect map type C. The contrast between the repeat unit length uniformity of the accessions of *H. bulbosum* and variability in those of *H. spontaneum* was most striking. *H. bulbosum* is a perennial and obligate outbreeding species (due to self-incompatibility). Since repeat unit length variants are inherited as codominant Mendelian alleles at independent NOR loci (Ellis et al. 1984; Saghai-Maroof et al. 1984; Polans et al. 1986), the apparent homogeneity of the rDNA repeat units in *H. bulbosum* is likely to be a consequence of its breeding system, which minimizes homozygosity and discourages the establishment of unique variants within the population. The converse is true for inbreeding species, like *H. spontaneum,* where variants would be expected to become fixed and propagated by their breeding system which produces homozygosity.

H. bulbosum has been regarded as closely related to *H. vulgare* on the strength of meiotic data (Kasha and Sadasivaiah 1971), and the same genome (I) has been assigned to both species (Bothmer et al. 1986). Their chloroplast genomes are also closely related to each other (Kataoka et al. 1987). However, the karyotypes of *H. vulgare* and *H. bulbosum* differ appreciably (Hsiao et al. 1986); also there is only one satellited chromosome per haploid complement in *H. bulbosum,* whereas there are two in *H. vulgare* (Rajhathy et al. 1964). The results of our analysis of rDNA repeat units suggest that *H. bulbosum* is more closely related to *H. murinum, H. glaucum* and *H. leporinum* than it is to *H. vulgare.*

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