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The distribution, organization and evolution of two abundant and widespread repetitive DNA sequences in the genus *Hordeum*

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Abstract The genomic organization and chromosomal distributions of two abundant tandemly repeated DNA sequences, dpTa1 and pSc119.2, were examined in six wild *Hordeum* taxa, representing the four basic genomes of the genus, by Southern and fluorescence in situ hybridization. The dpTa1 probe hybridized to between 30 and 60 sites on the chromosomes of all five diploid species studied, but hybridization patterns differed among the species. Hybridization of the pSc119.2 sequence to the chromosomes and Southern blots of digested DNA detected signals in *Hordeum bulbosum*, *Hordeum chilense*, *Hordeum marinum* and *Hordeum murinum* 4*x*, but not in *Hordeum murinum* 2*x* and *Hordeum vulgare* ssp. *spontaneum*. A maximum of one pSc119.2 signal was observed in the terminal or subterminal region of each chromosome arm in the species carrying this sequence. The species carrying the same I-genome differed in the presence (*Hordeum bulbosum*) or absence (*Hordeum spontaneum*) of pSc119.2. The presence of pSc119.2 in the tetraploid cytotype of *Hordeum murinum*, but its absence in the diploid cytotype, suggests that the tetraploid is not likely to be a simple autotetraploid of the diploid. Data about the inter- and intra-specific variation of the two independent repetitive DNA sequences give information about both the interrelationships of the species and the evolution of the repetitive sequences.

Key words Barley · *Hordeum* · In situ hybridization · Phylogeny · Tandemly repeated DNA sequence

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Introduction

Genes show extensive conservation over wide taxonomic distances so that, at the DNA level, homologous coding sequences can be isolated from many species in a taxonomic grouping (e.g. in the grasses, Sasaki 1996). In contrast, the single-copy sequences flanking many stretches of simple sequence repeats (microsatellites) are often species-specific and, within the Triticeae, the great majority of such markers are unique to one genome (Waugh et al., personal communication). Repetitive DNA sequences – DNA motifs that are repeated hundreds or thousands of times in the genome – make up the majority of most plant genomes and examples both with a widespread distribution and with high species (or genome) specificity have been reported (see Schmidt and Heslop-Harrison 1998).

As well as genome-specific dispersed and localized sequences, many sequences with a more-widespread genome distribution have been isolated. These are of value for studying the occurrence and mechanisms of both sequence and species evolution. Tandemly repeated sequences have been isolated and characterized from the Triticeae, and are investigated here in the genus *Hordeum*. pSc119.2 was among the first tandemly repeated DNA sequences to be isolated from cereals (Bedbrook et al. 1980), representing several percent of the rye genome. (It was originally cloned as a chimera which was separated by McIntyre et al. 1990.) Sequences homologous to this 118-bp repeat have been found widely in the Triticeae, and even outside (Katsiotis et al. 1997, in *Avena*), but notably Southern and in situ hybridization cannot detect its presence in *Hordeum vulgare*, although it is present in many other *Hordeum* species (Gupta et al. 1989). Vershinin et al. (1996) were able to show its presence by PCR analysis in low copy number in *Hordeum vulgare* and showed details of its higher-order structure in rye (Vershinin et al. 1995;Vershinin and Heslop-Harrison 1998). Another subtelomeric tandemly repeated sequence (the HvRT family sequence) also shows a characteristic, but different, species-specific distribution among

Hordeum species; this sequence is present in high copy number in *Hordeum vulgare* and *Hordeum murinum*, but is undetectable in *Hordeum marinum* (Belostotsky and Ananiev 1990).

The dpTa1 sequence family, originally isolated from wheat, represents a further tandem repeat found in many Triticeae species, including the genus *Hordeum*, by Southern hybridization (Vershinin et al. 1994). Sequences homologous to this 340-bp sequence have been isolated from other species: pAs1 from *Aegilops squarrosa* (Rayburn and Gill 1986), pHcKB6 from *Hordeum chilense* (Anamthawat-Jónsson and Heslop-Harrison 1993) and both pHvMWG2315 (Busch et al. 1995) and pHv14A (Tsujimoto et al. 1997) from cultivated barley. The chromosomal distribution of these dpTa1 homologous sequences has been studied in some *Hordeum* species by in situ hybridization (Busch et al. 1995; Bustos et al. 1996; Lima-Brito et al. 1996; Tsujimoto et al. 1997), and it is located at multiple intercalary and sub-terminal sites on many chromosomes.

In the present study, the chromosomal distribution of the pSc119.2 and dpTa1 sequences was examined in diploid and tetraploid *Hordeum* species that represent the four basic genomes (H, I, X and Y) in the genus (Bothmer et al. 1995) by both in situ hybridization to chromosomes and Southern hybridization to digested DNA, in order to investigate the evolution of both the species and the tandemly repeated sequences.

Materials and methods

Plant materials, DNA and probes

Accessions used in this study are given in Table 1. Here, we refer to *H. vulgare* ssp. *vulgare* as *H. vulgare* or barley, and *H. vulgare* ssp. *spontaneum* as *Hordeum spontaneum*; together they are referred to as *H. vulgare sensu lato*. In the present paper, because we are discussing the whole genus, genome designations by Bothmer et al. (1995) are followed except that individual barley chromosomes are designated by their homoeologous groups followed by the letter H (Linde-Laursen et al. 1997) and superscripts are used for other species.

DNA was isolated using standard methods. The following labelled probes were employed:

dpTa1, containing a 340-bp tandem repeated sequence unit of DNA isolated from *Triticum aestivum* (Vershinin et al. 1994). The DNA was labelled by PCR with digoxigenin-11-dUTP (Amersham).

pTa71, containing a 9-kb *Eco*RI fragment of the 18S-25S rDNA isolated from *T. aestivum* (Gerlach and Bedbrook 1979) and recloned in pUC19. The DNA was labelled by nick translation with either biotin-11-dUTP (Sigma) or digoxigenin-11-dUTP.

pTa794, containing a 410-bp *Bam*HI fragment of the 5S rDNA isolated from the embryos of *T. aestivum* (Gerlach and Dyer 1980) and cloned in pBR322. The DNA was amplified and labelled by PCR with digoxigenin-11-dUTP.

pSc119.2, containing a 120-bp tandem repeated sequence unit of DNA isolated from rye, *Secale cereale* (Bedbrook et al. 1980), subcloned by McIntyre et al. (1990). The DNA was directly labelled by nick translation with rhodamine-4-dUTP (Amersham) or labelled by PCR with digoxigenin-11-dUTP.

These labelled sequences were used in various combinations as probes for double-target in situ hybridization.

In situ hybridization

Techniques for metaphase preparation and in situ hybridization followed Schwarzacher et al. (1992). Briefly, seeds were germinated, root-tips excised and transferred to ice water at 0°C for 24 h to accumulate metaphases before fixation in 3:1 (v/v) 100% ethanol:acetic acid, enzyme digestion and squashing on glass slides in a drop of 45% acetic acid. The hybridization mixture consisted of 50 ng/slide of each probe, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulphate), 30 ng/µl of autoclaved salmon sperm DNA and 2 × SSC (hybridization stringency of 76%). The hybridization mixture was denatured at 70°C for 10 min, cooled on ice for 5 min, loaded onto the slides and covered with a plastic coverslip. A programmable temperature controller was used to denature the probes and the chromosomes for 10 min at 80°C and the hybridization was then carried out overnight at 37°C in a humid chamber inside an incubator. Post-hybridization washes were 2 × SSC for 5 min at 42°C followed by two 5-min high-stringency washes in 20% formamide in 0.1 × SSC at 42°C. The slides were then washed twice in 2 × SSC for 5 min at 42°C, and once in 2 × SSC for 5 min at room temperature. Under these conditions, probe-target combinations with more than 85% similarity over extended regions remain stably hybridized.

Hybridization sites were detected using anti-digoxigenin conjugated to FITC (Fab fragment, Boehringer) and streptavidin conjugated to Cy3 (Sigma), respectively. To detect both digoxigenin and biotin hybridization sites, the slides were transferred into detection buffer (4 × SSC, 0.2% Tween 20) for 5 min at room temperature, blocked with 5% (w/v) bovine serum albumin (BSA) in detection buffer for 5 min and then incubated at 37°C for 1 h in a solution of 10 µg/ml of anti-digoxigenin FITC, 5 µg/ml of streptavidin-Cy3 in 5% (w/v) BSA. The preparations were then stained with DAPI (4',6-diamidino-2-phenylindole) and mounted. Slides were examined on a Leitz epifluorescence microscope using ap-

Table 1 List of the *Hordeum*, *Triticum* and *Aegilops* species studied

Genome	Species	Ploidy	Accession	Figure
I	*H. vulgare* L. ssp. *vulgare*	2*x*	'Betzes'	
I	*H. vulgare* ssp. *spontaneum* (C. Koch) Thell	2*x*	OUH602	5
I	*H. bulbosum* L.	2*x*	6R52 × J1R132	4
H	*H. chilense* Roemer & Schultes	2*x*	John Innes line 1	1
X	*H. marinum* Hudson ssp. *marinum*	2*x*	H515	3
Y	*H. murinum* ssp. *glaucum* (Steude) Tzvelev	2*x*	John Innes line 71	2
YY	*H. murinum* L. ssp. *murinum*	4*x*	Norwich local population	6
A	*T. monococcum* L.	2*x*	KU104-2	
SS	*Ae. speltoides* Tausch	4*x*	KU51 (induced tetraploid)	
D	*Ae. squarrosa* L.	2*x*	RL5271	
ABD	*T. aestivum* L. em Thell	6*x*	'Chinese Spring'	

propriate filters. Photographs were taken using Fujicolor Super HG 400 and Fuji Superia 400 color print film, digitized to CD-ROM and printed from Adobe Photoshop using only processing functions that affect all pixels equally. The lengths of individual chromosomes were based on ten measurements and the idiograms of karyotype were prepared from four measurements of each band.

Southern hybridization

The genomic organization of the pSc119.2 sequence was also analyzed by Southern-blot hybridization. DNA was digested with *Taq*I and size-separated on a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer. The fractionated DNA fragments were transferred to Hybond-N^+ membrane (Amersham). The insert of the pSc119.2 clone was amplified by PCR and used for labelling. Probe labelling, hybridization, washing and detection were carried out using the enhanced chemiluminescence (ECL) direct labelling and detection system (Amersham), following the manufacturer's instruction. Stringencies were 78% for hybridization and 86% for washing, which are similar to those used for in situ hybridization.

Results

In situ hybridization

In situ hybridization of dpTa1 detected signals on the chromosomes of all five diploid *Hordeum* species studied (Figs. 1-5 parts b green signals). This agrees with the results of Southern hybridization by Vershinin et al. (1994). The intensity and distribution of the dpTa1 signals differed among the species. The clearest banding patterns, with up to 60 discrete bands, were observed in *H. chilense*, *H. murinum* 2*x* and *H. marinum*, while more dispersed patterns were seen in the two I-genome species *H. spontaneum* and *Hordeum bulbosum*. Hybridization signals of pSc119.2 were detected in *H. chilense*, *H. marinum*, *H. bulbosum* and *H. murinum* 4*x*, but not in *H. murinum* 2*x* and *H. spontaneum* (Figs. 1-4 parts c red signals, Fig. 6 b green signals). pSc119.2 sites were exclusively subterminal or terminal, mostly on short chromosome arms. In Figs. 5 c and 6 b, red in situ hybridization signals show 18S-25S rDNA sites.

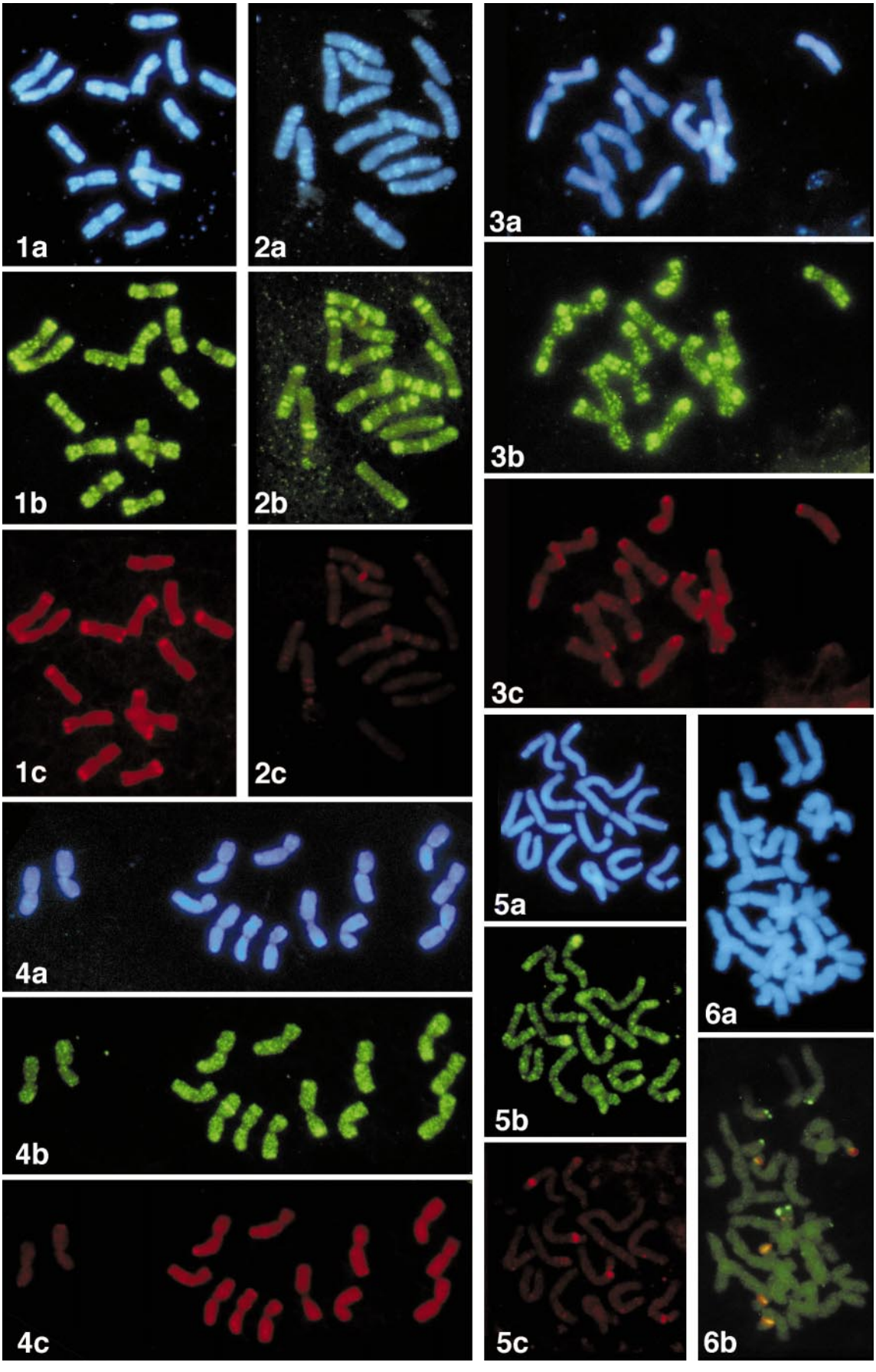
Karyotypes based on the dpTa1 and pSc119.2 sequences of five *Hordeum* taxa are shown in Fig. 7. The chromosome morphologies (see Figs. 1-6 parts a) were generally in agreement with those of previous studies (Linde-Laursen et al. 1989; Cabrera et al. 1995). The chromosomes of *H. chilense* and *H. spontaneum* were assigned to homoeologous groups on the basis of chromosome morphology and similarity to previous karyotypes. In *H. chilense*, in situ hybridization patterns of two dpTa1 homologous sequences were reported: pAs1 (Cabrera et al. 1995; Bustos et al. 1996) and pHcKB6 (Lima-Brito et al. 1996). The present dpTa1 karyotype is very similar to the pAs1 karyotype of the same accession by Cabrera et al. (1995), but we detected a few more sites. For pSc119.2, the *H. chilense* accession studied in the present study had two extra pairs of sites, in addition to the five pairs in the accession studied by Bustos et al. (1996); the additional signals were at the end of the short arm of chromosome $2H^{ch}$ and in the subterminal region of the short arm of chromosome $7H^{ch}$. This indicates intraspecific polymorphisms of the pSc119.2 sequence among *H. chilense* accessions. The *H. bulbosum* accession studied here had a maximum of six terminal pSc119.2 signals, as reported for different accessions by Xu et al. (1990). The chromosomes of *H. bulbosum* could not be identified even by combining dpTa1 and pSc119.2 signals because of the small number of bands and the heterozygous karyotype of this outcrossing species.

The present dpTa1 karyotype of *H. marinum* differed from the pAs1 karyotype of *H. marinum* accessions reported by Bustos et al. (1996). With the exception of the chromosomes with 18S-25S rDNA or 5S rDNA sites, it is difficult to estimate homologous chromosomes between the two studies. The number and distribution of telomeric pSc119.2 signals also differed between the two studies indicating intraspecific polymorphisms. Bustos et al. (1996) reported considerable variation in the distribution of both sequences between the two subspecies of *H. marinum* (ssp. *marinum* and ssp. *gussoneanum*), and this variation is reflected by the difference from our results.

To our knowledge, this is the first report of in situ hybridization of dpTa1-homologous sequences to *H. murinum* 2*x*. The present dpTa1 karyotype of *H. murinum* 2*x* shows no similarity to the (partial) pAs1 karyotype of *H. murinum* 4*x* reported by Bustos et al. (1996). In *H. murinum* 4*x*, we detected two pairs of pSc119.2 signals (agreeing with Bustos et al. 1996), but the signals were located on different chromosomes between these two studies, suggesting intraspecific polymorphisms.

Southern hybridization

Southern hybridization of pSc119.2 to *Taq*I digests detected abundant and strongly homologous sequences in *H. chilense*, *H. marinum*, *H. bulbosum* and *H. murinum* 4*x* (in decreasing order of signal intensity), but not in *H. vulgare*, *H. murinum* 2*x* and *H. spontaneum* (Fig. 8). These results agreed with those of in situ hybridization (Figs. 1–6), indicating that the sensitivity of both techniques with respect to the ability to detect sequences is similar. Gupta et al. (1989) found weak hybridization of the chimeric clone pSc119 for Southern hybridization to DNA from *H. murinum* 2*x*, but this may be from the unrelated sub-sequence pSc119.1. Of the species from other genera included, *Triricum monococcum* did not show signals, and the other four species showed the expected ladder pattern typical of a tandem repeat. The enzyme revealed polymorphisms in genomic organization between the species, with different patterns of bands. In particular, the organization in *H. bulbosum* is different since no clear ladder pattern is seen.


1a
2a
3a
1b
2b
3b
1c
2c
3c
4a
5a
6a
4b
5b
6b
4c
5c

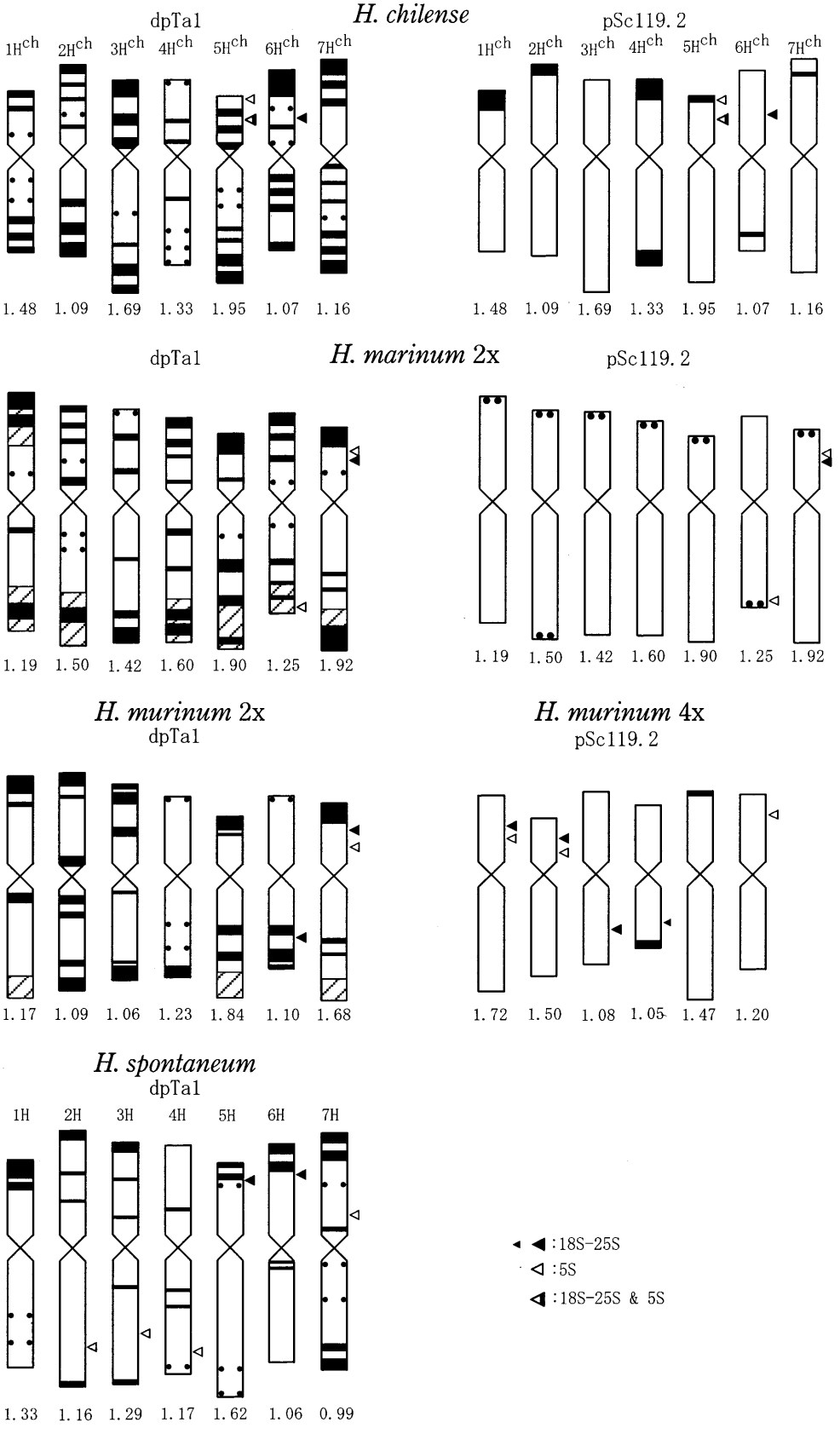


Fig. 7 Karyotypes of four diploid and one tetraploid *Hordeum* taxa probed with dpTa1 and pSc119.2. In *H. chilense* and *H. spontaneum*, chromosomes are arranged according to homoeologous groups. In *H. marinum* and *H. murinum* 2*x*, chromosomes without satellite are arranged in decreasing order of the length. In *H. murinum* 4*x*, only chromosomes with pSc119.2, 18S-25S rDNA or 5S rDNA sites are illustrated. *Blocks and pairs of dots* indicate strong and weak signals, respectively. *Hatched areas* indicate more dispersed signals. *Numbers* under the chromosomes are arm ratios. Locations of 18S-25S rDNA and 5S rDNA sites are cited from Taketa et al. (1999)

◀ **Figs. 1-6** In situ hybridization to root-tip metaphase cells from six *Hordeum* taxa. *Blue* DAPI staining (parts a) shows the chromosomal DNA. In Figs. 1–5, *green* in situ hybridization signals show dpTa1 sites (parts **b**). In Figs. 1–4 *red* in situ hybridization signals show pSc119.2 sites (parts **c**). (**1**) *H. chilense*, (**2**) *H. murinum* 2*x*, (**3**) *H. marinum*, (**4**) *H. bulbosum*, (**5**) *H. spontaneum*. *Red* in situ hybridization signals show major 18S-25S rDNA sites. (**6**) *H. murinum* 4*x*, an incomplete metaphase cell having 27 chromosomes; one chromosome with a major 18S-25S rDNA site is missing. *Red* in situ hybridization signals show 18S-25S rDNA sites overlayed with *green* signal of pSc119.2 sites

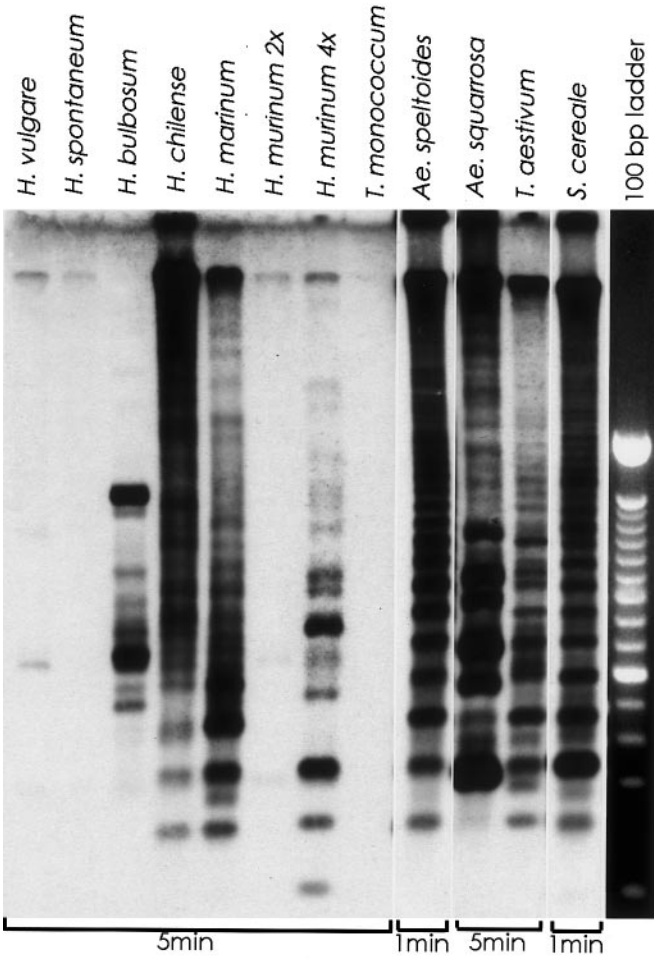


Fig. 8 Southern hybridization of pSc119.2 to *Taq*I-digested DNA. The species names are indicated at the top of the figure. The amount of total genomic DNA loaded on each lane was 0.3 µg for *Ae. speltoides*, *T. aestivum* and *S. cereale*, and 1.5 µg for the other nine species. For better visualization of hybridization patterns, the figure represents a composite of different exposure times as indicated

Discussion

Distribution of dpTa1 and pSc119.2

Both dpTa1 and pSc119.2 represent abundant, tandemly repeated DNA sequence families that are located at discrete sites on multiple individual chromosomes from many species of the tribe Triticeae. No 'function' has been ascribed to either sequence, although Galasso et al. (1997) suggested the importance of sub-terminally located repetitive sequences in speciation in *Dasypyrum*. pSc119.2 may be one such sequence that plays an important role in the differentiation of either genome or species in the genus *Hordeum*. In the absence of alternative data, it is considered that both pSc119.2 and dpTa1 families amplify or reduce in copy number by mechanisms including slippage replication and unequal crossing over, while homogenization following sequence changes may lead to divergence of the sequences between species or chromosomes.

There are notable differences in the genomic organization and inferred evolutionary behavior between the two sequences, evident in the *Hordeum* species studied here and in comparison with work on the same sequences in other Triticeae species. In *Hordeum* species (Figs. 1–7), as in rye (the species of origin of the clone), pSc119.2 locates mainly near the ends of the short arms, with a few sub-terminal long arm and intercalary sites (Vershinin et al. 1995; the true telomeric sequence, similar to TTTAGGG, is terminal, Schwarzacher and Heslop-Harrison 1991); in wheat, the same sequence shows more intercalary sites. The second sequence studied here, dpTa1, shows a much wider chromosomal distribution than pSc119.2, with between 30 and 60 discrete hybridization sites per haploid (*x*) genome in *Hordeum* (Figs. 1–7), near the ends of most chromosome arms and at multiple intercalary sites on every chromosome. Both sequences show extensive inter-species polymorphisms and sometimes intra-species polymorphisms. We now have extensive data about the chromosomal distributions of the two sequences so that more general conclusions about their evolution can be made, taking into account the differences in species or accessions and in situ hybridization techniques (and, for dpTa1, variants of the same family with 80% or more similarity; Busch et al. 1996) used by different authors.

Comparisons of in situ hybridization patterns

The pSc119.2 karyotypes of *H. chilense*, *H. marinum* and *H. murinum* 4*x* (Fig. 7) show some differences from those of Bustos et al. (1996). Because the clone and in situ hybridization technique were the same, these differences show the occurrence of intraspecific polymorphisms. A high level of intraspecific polymorphisms are known in the C-banding patterns of wild *Hordeum* species (Linde-Laursen et al. 1995), no doubt reflecting the variation in repetitive DNA sequences.

In barley, the chromosomal distribution of three dpTa1 homologous sequences has been reported: pAs1 (Brandes et al. 1995), pHvMWG2315 (Busch et al. 1995) and pHvA14 (Tsujimoto et al. 1997). The present dpTa1 karyotype of *H. spontaneum* is most similar to the pHvA14 karyotype of the barley cultivar Betzes (Tsujimoto et al. 1997), with a notable difference in the strength of the terminal site on 1HS. Tsujimoto et al. (1997) have found that two variants of dpTa1, (pAs1 from *Aegilops squarrosa*, Rayburn and Gill 1986, and pHv14A from barley) sharing 87.3% homology, show different hybridization characteristics to barley chromosomes: pAs1 did not stably hybridize, while pHv14A was stable and showed a distinct hybridization pattern. We found dpTa1 showed more dispersed hybridization to barley (Fig. 4) than the other *Hordeum* species studied. The differences indicate divergence of the target sequences as well as variation in copy number. Despite these differences, the distributions of dpTa1 homologous sequences were similar between homoeologous chromo-

somes of *H. chilense* and *H. spontaneum* (Fig. 7). It is notable that homoeologous group-4 chromosomes of both species have fewer dpTa1 signals. A chromosome pair with fewer signals was also found in *H. murinum* 2*x*, but not in *H. marinum*, although the homoeology of chromosomes to other species is unknown. Similar distributions of pAs1 homologous sequences were found between *Ae. squarrosa* and *H. chilense* or *H. vulgare* (Cabrera et al. 1995; Tsujimoto et al. 1997). Therefore, similarities in dpTa1 hybridization patterns may suggest relationships of species, but exceptions are found where the family seems to have become 'active' and diversified in sequence, copy number and chromosomal distribution at a rate similar to that of the speciation process.

Evolution of *H. murinum* 2*x* and 4*x*

Both in situ and Southern hybridization (Figs 1–8) indicate that pSc119.2 is abundant in *H. murinum* 4*x*, but essentially absent (though the chimeric but related clone pSc119 is in much reduced copy number; Gupta et al. 1989) in *H. murinum* 2*x*. Metaphase-I pairing analysis has suggested that the tetraploid cytotype is an autotetraploid with two sets of chromosomes from *H. murinum* 2*x* (Bothmer et al. 1987). However, the present results support the alternative view that *H. murinum* 4*x* is an alloploid involving *H. murinum* 2*x* (pSc119.2 essentially absent) and an unidentified diploid species (pSc119.2 abundant). We consider it unlikely that pSc119.2 has shown extensive secondary amplification in the tetraploid, or been lost more recently in the diploid and one genome of the tetraploid. The allopolyploid origin of the tetraploid cytotype is also suggested by the C-banding pattern (Linde-Laursen et al. 1989), isozyme analysis (Jørgensen 1986) and in situ hybridization (Bustos et al. 1996). The source of the genome carrying pSc119.2 is not clear. However, analysis of restriction fragment patterns in digests of DNA from various species, perhaps in combination with the nucleotide sequence from clones of the pSc119.2 family from the species, might suggest candidate diploid ancestors. It is notable that the *Taq*I restriction fragment pattern (Fig. 8) from *H. murinum* 4*x* is unlike that of the other species examined.

Phylogeny of the genus *Hordeum*

Abundant sequences homologous to clone pSc119.2 were found in many *Hordeum* species including *H. bulbosum*, the closest relative to *H. vulgare sensu lato* (Gupta et al. 1989; Xu et al. 1990; Bustos et al. 1996; the present study), but the sequence is hardly detectable in *H. vulgare sensu lato* (Xu et al. 1990) and *H. murinum* 2*x*. Morphologically, *H. vulgare sensu lato*, *H. bulbosum* and *H. murinum* are classified in the section *Hordeum* (Bothmer et al. 1995) although it is generally accepted that *H. vulgare sensu lato* and *H. bulbosum* are most closely related to each other (carrying the same I-genome) while *H. murinum* (Y-genome) is less closely related (Bothmer et al. 1995). A simple model where extensive loss of the pSc119.2 sequence family occurs only once would not be entirely consistent with this phylogeny, although the detailed positions of the I- and Y-genome species are not certain. It is possible that the same mechanisms leading to deletion of the family have occurred in some species with both I- and Y-genomes. Extensive differences in 5S and 18S–25S rDNA sites detected by in situ hybridization of wheat rDNA clones (Leitch and Heslop-Harrison 1992; Pedersen and Linde-Laursen 1994; Taketa et al. 1999) and C-banding patterns (Linde-Laursen et al. 1995) suggest the involvement of more complicated changes in repetitive sequences during the evolution of these species.

In the *Hordeum* species studied, the in situ hybridization patterns of dpTa1 and pSc119.2 are different from the C-banding (e.g. Linde-Laursen et al. 1995) and the in situ hybridization with a labelled GAA-satellite sequence (Pedersen et al. 1996) patterns. Therefore, these sequences, in combination with genomic in situ hybridization, should provide useful cytological and molecular markers for monitoring alien chromosome segments in interspecific crossing and substitution programs in this genus, as used in the crosses between *H. vulgare* and *H. bulbosum* (Xu et al. 1990; Pickering et al. 1994). In general terms, the study of the distribution and evolutionary changes in the sequences suggests that the two are evolving independently and have different constraints on their diversification in the *Hordeum* genus. How this relates to the mechanisms that define their amplification and characteristic chromosomal sites, and the constraints on copy number evolution, are unknown. Examination of these sequences in further species and additional accessions of the same species at both chromosomal and sequence levels will certainly add to our understanding of species relationships and phylogeny in the genus.

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