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# Analysis of genome-specific sequences in *Phleum* species: identification and use for study of genomic relationships

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**Abstract** *Sau*3AI "shot gun" cloning and colony hybridization with total genomic probes were used to isolate genome-specific sequences in *Phleum* species. The total DNA isolated from diploid species *P. alpinum* and *P. bertolonii* was partially digested with *Sau*3AI and cloned using pUC19 as a vector to an *E. coli* strain DH5αmcr. A partial genomic DNA library consisting of 3030 colonies for the genome of *P. alpinum* and one consisting of 3240 colonies for the genome of *P. bertolonii* were constructed. Twelve hundred and thirty colonies from the DNA library of *P. alpinum* and 1320 from that of *P. bertolonii* were respectively blotted to membrane filters and hybridized to the total genomic probes from these two species. Eight clones specific to *P. alpinum* and 13 specific to *P. bertolonii* were isolated through colony hybridization and further dot-blot hybridization. Most of these clones may carry highly or moderately repetitive sequences. Three sequences specific to *P. alpinum* and 3 specific to *P. bertolonii* were used as probes to hybridize the *Eco*RI-digested DNA samples from four species, *P. alpinum, P. bertolonii, P. pratense* and *P. montanum*, on Southern blot. The results from these hybridization experiments showed that all 3 *P. bertolonii*-specific probes and 2 of the 3 *P. alpinum*-specific probes hybridized to the DNA of *P. pratense*, thus confirming the conclusion of the close relationships between the cultivated timothy and its two wild relatives that was drawn in our previous study using the C-banding technique.

**Key words** Genome-specific sequences · DNA hybridization · Repetitive sequences · Phylogeny · *Phleum*

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## Introduction

DNA hybridization with genome-specific probes is a new molecular approach for genome studies. DNA sequences specific to a particular genome can serve as markers to identify a particular genome type (Zhao et al. 1989) or to trace genome phylogenetic relationships (Hosaka et al. 1990; Crowhurst and Gardner 1991). The hybridization of genome-specific probes to isolated DNA or in situ hybridization to chromosomes is believed to be a reliable approach for the examination of phylogenetic relationships of plant species. It is also effective when used to discriminate the polyploid nature of plants or to test models of progenitor-derivator relationships. As a consequence, it has become a useful tool in studies of genetics, evolution and systematics of plants. Genome-specific, highly repeated sequences have been isolated from the following genera of higher plants: *Triticum* (Metzlaff et al. 1986; Rayburn and Gill 1986), *Hordeum* (Junghans and Metzlaff 1988), *Secale* (Bedbrook et al. 1980; Guidet et al. 1991), *Brassica* (Hosaka et al. 1990), *Oryza* (Zhao et al. 1989), *Beta* (Schmidt et al. 1990), *Avena* (Fabijanski et al. 1990), *Actinidia* (Crowhurst and Gardner 1991) and *Solanum* (Pehu et al. 1990). They have been used as probes for identifying somatic cell hybrids (Saul and Potrykus 1984; Pehu et al. 1990), for detecting hybrid addition lines (Schmidt et al. 1990) and for studying genome evolution (Rayburn and Gill 1985, 1987; Hosaka et al. 1990; Anamthawat-Jónsson et al. 1990; Fabijanski et al. 1990). Genome-specific se-

**Table 1** List of species studied, their accession number, somatic chromosome number and source

| Species | Accession | | |
|---|---|---|---|
| | no. | 2n | Source |
| *P. pratense* | M35 | 42 | Jardin Botanique National de Belgique, Meise |
| | M46 | 42 | Agriculture Canada, Ste-Foy Research Station, Quebec |
| | M103 | 42 | The University of Guelph, Ontario |
| | M104 | 42 | The University of Guelph, Ontario |
| *P. alpinum* | M27 | 14 | Botanischer Garten und Botanisches Museum, Berlin-Dahlem |
| | M39 | 14 | Muséum National d'Histoire Naturelle, Paris |
| *P. bertolonii* | M33 | 14 | Botanischer Garten und Botanisches Museum, Berlin-Dahlem |
| | M40 | 14 | Muséum National d'Histoire Naturelle, Paris |
| *P. montanum* | M29 | 28 | Botanischer Garten und Botanisches Museum, Berlin-Dahlem |

quence analysis has contributed to genome studies in higherplants, especially to genome diagnosis in plant breeding research (Schmidt et al. 1990).

Timothy (*Phleum pratense* L.) is an important forage crop, widely grown in cool and temperate regions of the world, including North America and Europe. At least ten species have been recognized in the genus *Phleum*. Only *P. pratense* is of marked economic importance. The cultivated form of timothy has been recognized as a hexaploid (2n=6×=42)(Gregor 1931; Gregor and Sansome 1930). Earlier studies of timothy addressed two hypotheses about the genome constitution and the origin of *P. pratense*. One considered that it originated as an allohexaploid from the interspecific hybridization between diploid species *P. bertolonii* and a tetraploid form of *P. alpinum* (Gregor 1931; Gregor and Sansome 1930; Müntzing 1935; Müntzing and Prakken 1940; Stebbins 1950). The other hypothesis considered it to have originated as an autohexaploid that most likely was derived from diploid *P. bertolonii* (Nordenskiöld 1941, 1945). Although the second hypothesis seems to be generally accepted, no definite conclusion has been made because of a lack of conclusive evidence from both cytological and genetic studies. Our previous study using Giemsa C-banding technique suggested that the hexaploid karyotype of *P. pratense* may consist of two genomes, A and B, with a genomic formula of AAAABB, the A genome coming from *P. alpinum* and the B genome donated by *P. bertolonii* (Cai and Bullen 1991). To verify this primary conclusion, a molecular study with genome specific probes has been initiated in *Phleum* species. This paper presents the process of isolation of genome-specific sequences from *Phleum* species and the results of genome analysis using some of the isolated genome-specific sequences as probes.

## Materials and methods

### Plant materials

Nine accessions of plants from four species, *P. pratense* (four accessions), *P. alpinum* (two accessions), *P. bertolonii* (two accessions) and *P. montanum* (one accession), were used in this study. All these accessions are listed in Table 1.

### Isolation of plant total DNA

The grass seeds were germinated and grown in a greenhouse at the Macdonald Campus of McGill University for 2 weeks. Plant tissue was harvested and DNA was extracted as described by Ausubel et al. (1989). The extracted DNA was precipitated with 3 vol of 95% chilled ethanol, spooled out with a glass rod and resuspended in 10 ml TE buffer. The DNA was extracted with an equal volume of phenol followed by phenol: chloroform: isomyl alcohol (24: 24: 1) and precipitated again with 2.5 vol of ethanol. The DNA resuspended in TE buffer was purified by CsCl gradient (5.15 g/8 ml)-ethedium bromide ultracentrifugation in a Beckman 41 STi rotor at 38 000 *g* for 48 h.The purified DNA was quantified in a Beckman DU-40 UV Spectrophotometer.

### Construction of partial genomic DNA libraries and colony hybridization

The total DNA isolated from *P. alpinum* (M39) and *P. bertolonii* (M33) was digested with *Sau*3AI at 37°C for 40 min and then purified with the Gene Clean II Kit (Bio 101) as suggested by the manufacturer.The *Sau*3AI-digested fragments of plant DNA were ligated to *Bam*HI-digested, dephosphorylated pUC19 with a insert-vector ratio of 2:1 (Sambrook et al. 1989). The ligated DNA mixture was used to transform *E. coli* strain DH5αmcr. The recombinant colonies were identified on the agar plates with Ampicillin and X-gal.

The colonies bearing *Phleum* genomic DNA libraries were blotted to Hybon-N membranes (Amersham) on which the DNA was denatured and fixed as suggested by the manufacturer. The total DNA from *P. alpinum* (M39) and *P. bertolonii* (M33) was used as total genomic probes. The total genomic probes were prepared by completely digesting the DNA with *Sau*3AI at 37°C for 1h and purified with the Gene Clean II. The probes were labeled with [α-$^{32}$P]-dCTP by nick translation using the Nick Translation Labelling System (BRL). The specific activity of the labeled DNA was calculated based on data obtained from the counting of 3 µl of labeled labeled DNA mixture in a LS7500 scintillation counter (Beckman). The labeled probes were hybridized to the blotted colonies using the following procedure: the membranes were prehybridized at 42°C in a solution containing 50% formamide, 0.25 *M* $NaHPO_4$ (pH 7.2), 0.25 *M* NaCl, 7% (w/v) SDS, 1 m*M* EDTA and 10% (w/v) PEG-3000 for 1 h, followed by hybridization overnight in the same but fresh new solution to which the labeled probes were added (5 ng/ml, about $10^6$ cpm/ml). Subsequently the membranes were washed three times for 15 min, first in 2×SSC+0.1% SDS at room temperature, then in 0.5×SSC+0.1% SDS at the same temperature and finally in 0.1×SSC+0.1% SDS at 60°C. After this the filters were immediately exposed to Hyperfilm™-ECL (Amersham).

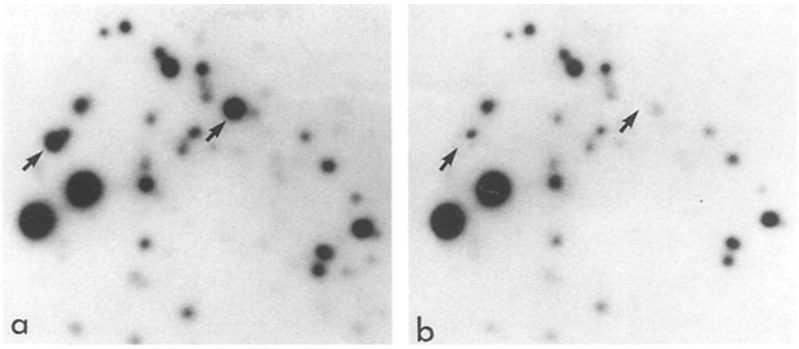


**Fig. 1a, b** Comparison of the hybridizations of colonies from the library of *P. alpinum* to [$^{32}$P]-labeled total DNA probes from *P. alpinum* (*a*) and from *P. bertolonii* (*b*). The arrows point to the colonies showing hybridization signals specific to the genomic probe from *P. alpinum*

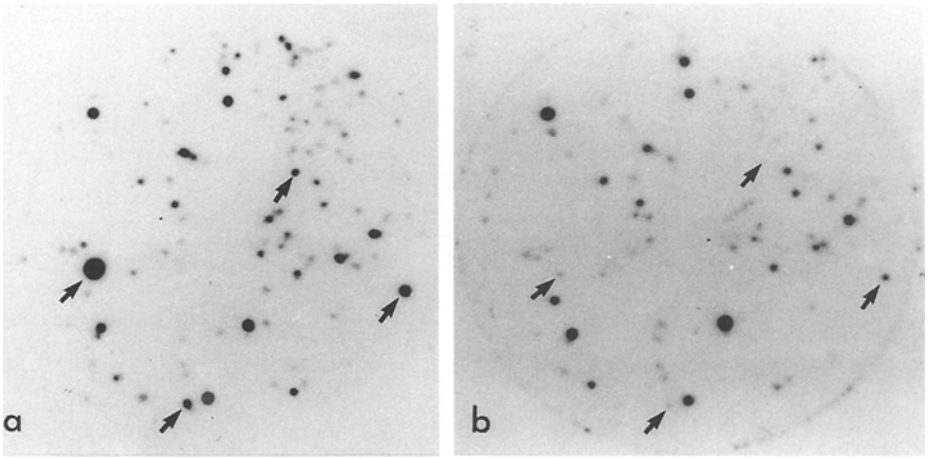


**Fig. 2a, b** Comparison of hybridizations of colonies from the library of *P. bertolonii* to the total DNA probes from *P. bertolonii* (*a*) and from *P. alpinum* (*b*). The arrows point to the colonies showing hybridization signals specific to the genomic probe from *P. bertolonii*

Dot-blot analysis

All the clones isolated from colony hybridization were amplified and subjected to dot-blot analysis. The amplified recombinant plasmids were isolated with the alkaline lysis method of Birnboin and Doly (1979) and dot-blotted to Zeta-Probe membranes (BioRad) using the Bio-Dot Microfiltration Apparatus (BioRad) as suggested by the manufacturer. The probes and the hybridization solution used for this experiment were the same as those used in colony hybridization except for the concentration of formamide. To assess the genome specificity of the isolated clones, the dot-blotted plasmids were hybridized and washed under the following stringencies: hybridization in 50% formamide and washing at 55°C; hybridization in 50% formamide and washing at 65°C; hybridization in 60% formamide and washing at 55°C; and hybridization in 60% formamide and washing at 65°C. All hybridizations were performed at 42°C, and the membranes were washed by the same procedure as that used in colony hybridization but with the temperature changed in the last step. To determine whether sequence overlapping is involved between the clones, the dot-blotted plasmids were hybridized to various labeled probes from the isolated clones under the same hybridization condition as in colony hybridization except that the temperature of the last step wash was 65°C.

Southern-blot analysis

Plant DNA was digested with *Eco*RI (5 units/μg) at 37°C for 1 h and separated in 0.8% agarose gel in TAE buffer (Sambrook et al. 1989). Subsequently the DNA samples were transferred to a Zeta-Probe membrane in a transfer cell (BioRad) and then denatured and fixed as suggested by the manufacturer. Six genome-specific clones were used to probe the Southern-blotted DNA under the same hybridization conditions as used in colony hybridization.

## Results

### Identification and analysis of genome-specific sequences

*Cloning of Phleum DNA and screening of the genomic libraries*

About 80% of the DNA fragments generated after *Sau*3AI partial digestion of the total genomic DNA from *P. alpinum* and *P. bertolonii* ranged in size from 200 bp to 3 kb. These DNA fragments were randomly inserted into pUC19 at the *Bam*HI site. By transforming *E. coli* strain DH5αmcr, we obtained a high frequency of recombinant colonies. The colonies were subsequently blotted to the Hybon-N membranes and hybridized to the [$^{32}$P]-labeled total genomic probes from *P. alpinum* and *P. bertolonii*, respectively. Hybridization signals were compared between self-hybridization and cross-hybridization, and any colonies showing signals only in self-hybridization but not in cross-hybridization were recognized as potential genome-specific clones. The total genomic probe from *P. alpinum* and that from *P. bertolonii* were respectively hybridized to a total of 1230 recombinant colonies from the genomic library of *P. alpinum*. A total of 920 colonies gave detectable signals upon self-hybridization with labeled total genomic probe from *P. alpinum*. The rest of the colonies showed no detectable signals either in self-hybridization or in cross-hy-

**Table 2** Insert size, inferred copy-number range and stringency of genome specificity of the isolated clones specific to *P. alpinum*

| Clone | Size of insert (kb) | Range of copy number | Stringency of signal specificity[a] |
|---|---|---|---|
| pPa07 | 0.85 | High | *** |
| pPa08 | 0.15 | Middle | **** |
| pPa11 | 2.50 | High | ** |
| pPa16 | 4.55 | Middle | * |
| pPa24 | 0.75 | Low | **** |
| pPa33 | 4.15 | High | * |
| pPa42 | 2.05 | Low | **** |
| pPa47 | 0.90 | High | * |

[a] DNA hybridization was carried at 42°C in the following stringencies: ****, hybridization in 50% formamide and washing at 55°C; ***, hybridization in 50% formamide and washing at 65°C; **, hybridization in 60% formamide and washing at 55°C; *, hybridization in 60% formamide and washing at 65°C

**Table 3** Insert size, inferred copy-number range, and stringency of genome specificity of the isolated clones specific to *P. bertolonii*

| Clone | Size of insert (kb) | Range of copy number | Stringency of signal specificity[a] |
|---|---|---|---|
| pPb03 | 2.90 | High | **** |
| pPb05 | 1.60 | Low | ** |
| pPb08 | 3.25 | High | *** |
| pPb27 | 3.20 | High | **** |
| pPb43 | 4.90 | High | **** |
| pPb68 | 0.65 | High | **** |
| pPb69 | 1.70 | Middle | **** |
| pPb70 | 3.15 | Middle | * |
| pPb75 | 1.10 | Low | *** |
| pPb78 | 9.30 | Low | * |
| pPb81 | 0.18 | Middle | **** |
| pPb82 | 1.90 | High | **** |
| pPb88 | 0.20 | High | *** |

[a]DNA hybridization was carried at 42°C in the following stringencies: ****, hybridization in 50% formamide and washing at 55°C; ***, hybridization in 50% formamide and washing at 65°C; **, hybridization in 60% formamide and washing at 55°C; *, hybridization in 60% formamide and washing at 65°C

bridization. The signal intensity was very high in 20% of the signalled colonies, moderate in 35% of them and low in 45% of them.

In a comparison of hybridized signals between self-hybridization and cross-hybridization, 24 colonies were found to be specific to self-hybridization. The signal specificity in some colonies can be seen in Fig. 1. As indicated by the arrows in Fig. 1, 2 colonies show intensive signals to the total genomic probe from *P. alpinum* (Fig. 1a) but show no signals to the genomic probe from *P. bertolonii* (Fig. 1b). These colonies may be specific to the genome of *P. alpinum* and were isolated for further analysis.

In screening 1320 colonies in the genomic library of *P. bertolonii*, 1035 colonies gave hybridization signals upon self-hybridization. Of the hybridized colonies 25% showed the signals with high intensity, 36% with middle intensity and 39% with low intensity. Hybridization specificity could be readily identified in some colonies between self-hybridization and cross-hybridization. Figure 2 shows the hybridization results of some colonies from the genomic library of *P. bertolonii* probed by labeled total DNA from *P. alpinum* and that from *P. bertolonii* respectively. The arrows indicate 4 colonies that show intensive signals to the genomic probe from *P. bertolonii* (Fig. 2a) but no signal or very weak signals to the genomic probe from *P. alpinum* (Fig. 2b), suggesting their possible specificity to *P. bertolonii*. Through colony hybridization, a total of 34 colonies primarily recognized to be specific to the genome of *P. bertolonii* were isolated.

### *Dot-blot analysis for genome specificity*

All colonies isolated from the genomic libraries were subject to dot-blot analysis to determine whether these clones were really specific to a genome and also to determine at which level of stringency their signal specificity can be identified. The dot blot was respectively hybridized with the total genomic probe from *P. alpinum* and the genomic probe from *P. bertolonii*. A total of 8 of the isolated clones were confirmed to be specific to the genome of *P. alpinum* (Table 2). Among these, 3 clones, pPa08, pPa24 and pPa42, showed signal specificity at the lowest stringency, namely, hybridization in 50% formamide followed by washing at 55°C. Under this stringency, pPa08 showed no signal and pPa24 and pPa42 gave extremely weak signals when hybridized to the genomic probe from *P. bertolonii*, while in contrast they all showed intensive signals when hybridized to the genomic probe from *P. alpinum*. When the washing stringency was raised by increasing the temperature to 65 °C, all signals with pPa24 and pPa42 were completely lost in cross-hybridization. Another 3 clones, pPa16, pPa33 and pPa47, showed their signal specificity only at the highest stringency, namely, hybridization in 60% formamide followed by washing at 65°C. This implies their low sequence differentiation between these two species. Finally, pPa07 showed the signal specific to *P. alpinum* at the lower hybridization stringency (in 50% formamide) but higher washing stringency (65°C), and pPa11 showed the same feature at the higher hybridization stringency but lower washing stringency.

Among the clones isolated from the genome of *P. bertolonii*, 13 were confirmed to be specific to this genome by dot-blot analysis (Table 3). Of these clones 7 showed their signal specificity identifiable at the lowest stringency. Upon hybridization in 50% formamide and washing at 55°C, 5 clones, pPb03, pPb68, pPb69, pPb81 and pPb82, gave signals unique to the total genomic probe from *P. bertolonii*. Another 2, pPb27 and pPb43, showed highly intensive signals in self-hybridization but very weak signals in cross-hybridization. Under this stringency, no other clones showed their genome specificity. Upon increasing the washing stringency to the higher level (65°C), however, another 3 clones, pPb08, pPb75 and pPb88, showed hybridization exclusively to the genomic probe from *P. bertolonii*. For the remaining 3 clones, signal specificity could be identified only at the higher hybridization stringency.

**Table 4** Cross-hybridization of *P. alpinum*-specific clones

| | Target DNA | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pPa07 | pPa08 | pPa11 | pPa16 | pPa24 | pPa33 | pPa42 | pPa47 |
| Probe | | | | | | | | |
| pPa07 | +++ | – | – | – | ++ | – | – | – |
| pPa08 | – | +++ | – | – | – | – | – | ++ |
| pPa11 | – | – | +++ | – | – | – | – | – |
| pPa24 | – | – | – | – | ++ | – | – | – |
| pPa42 | – | – | ++ | – | – | ++ | – | - |

+++ high intensive signal · ++ moderate intensive signal · – no signal)

**Table 5** Cross-hybridization of *P. bertolonii*-specific clones

| | Target DNA | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pPb03 | pPb05 | pPb08 | pPb27 | pPb43 | pPb68 | pPb69 | pBb70 | pPb75 | pPb78 | pPb81 | pPb82 | pPb88 |
| Probe | | | | | | | | | | | | | |
| pPb03 | +++ | – | – | – | – | +++ | – | – | – | – | – | – | – |
| pPb08 | – | – | +++ | – | – | – | – | – | – | – | – | – | – |
| pPb27 | – | – | – | +++ | – | – | ++ | – | – | – | – | – | – |
| pPb43 | – | – | – | – | +++ | – | – | – | – | – | – | – | – |
| pPb68 | +++ | – | – | – | – | +++ | – | – | – | – | – | – | – |
| pPb82 | – | – | – | – | – | – | – | – | – | – | – | +++ | – |

+++ high intensive signal · ++ moderate intensive signal · – no signal)

Among these, 1 clone, pPb05, showed its hybridization specificity to *P. bertolonii* at the lower washing stringency and the other 2, pPb70 and pPb78, showed their specificity only in the higher washing stringency.

*Analysis of genome-specific clones*

The sizes of plant DNA inserts in the genome-specific clones (Tables 2 and 3) were determined by comparing *Eco*RI and *Hin*dIII-digested plasmids with standards after electrophoresis on 1.0% agarose gels.To determine whether the same or overlapping sequences were carried among some of these selected clones, the dot-blotted plasmid DNA was cross-hybridized to various labeled insert probes from some of these clones. Within the group of *P. alpinum*-specific clones, all 8 target DNAs were hybridized to the labeled insert probes from 5 clones. Cross-hybridization signals were found between pPa07 and pPa24, between pPa08 and pPa47 and between pPa16 and pPa33. This indicates that overlapping sequences were involved in the inserts among these three pairs of plasmids (Table 4). In the cross-hybridization within the group of *P. bertolonii*-specific clones, all 13 isolated plasmids were dot-blotted as targets and hybridized to 6 labeled insert probes. Sequence overlapping was confirmed between pPb03 and pPb68 and between pPb27 and pPb69 (Table 5).

Analysis of genomic relationships using genome-specific probes

In order to confirm genomic relationships between hexaploid *P. pratense* and the two diploid species *P. alpinum* and *P. bertolonii,* 3 *P. alpinum*-specific clones, pPa07, pPa08 and pPa11, and 3 *P. bertolonii*-specific clones, pPb27, pPb43 and pPb68, were used as probes against the Southern blots to screen the DNA samples from four *Phleum* species, *P. pratense*, *P. alpinum*, *P. bertolonii* and *P. montanum*. Among these species, tetraploid *P. montanum* ($2n= 4\times= 28$) seemed to have a karyotype differentiated from the polyploid genome in *P. pratense* and the diploid genome in *P. alpinum* and in *P. bertolonii* (Q. Cai and M. R. Bullen, unpublished), and it was therefore used for these analyses as a control to highlight the genome specificity of the selected probes. The Southern blots were hybridized in 50% formamide following by washing at 60°C. Under this stringency, 2 of the *P. alpinum*-specific probes, pPa08 and pPa11, revealed hybridization signals to the DNA samples from *P. alpinum* and *P. pratense* (Fig. 3a,b) and the other probe, pPa07, gave the signal only in *P. alpinum*. None of these probes revealed hybridization signals to the DNA samples from the other species. This result suggests that the DNA from *P. pratense* is closely related to the *P. alpinum* DNA. A possible explanation for the lack of hybridization between *P. pratense* DNA and pPa07 is that the related sequence may have been highly differentiated between these two closely related species.

In the Southern blot hybridizations with *P. bertolonii*-specific probes, besides *P. bertolonii* DNA, the DNA samples from *P. pratense* exhibited strong hybridization signals to the probes, but no DNA from the other two species, *P. alpinum* and *P. montanum,* gave signals. Two Southern blots probed respectively by pPb43 and pPb68 are shown in Fig. 3c, d. Among the 3 *P. bertolonii*-specific probes, 2 probes, pPb27 and pPb43, seemed to produce signals with a ladder pattern in the *Eco*RI-digested DNA samples. This

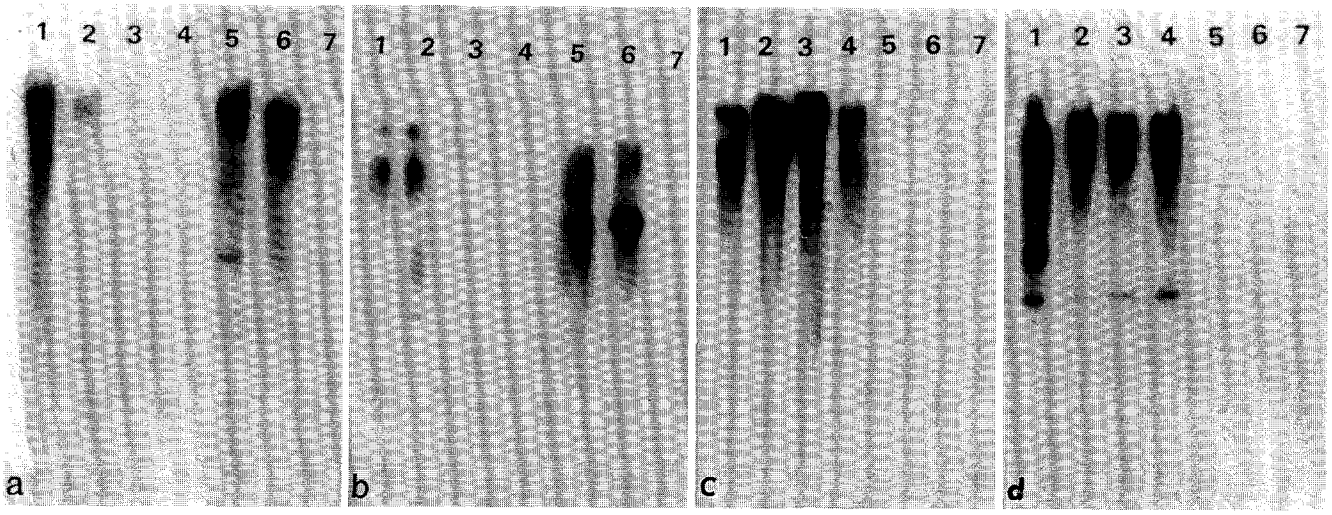


**Fig. 3a–d** Southern blot hybridization of the total DNA from four *Phleum* species, *P. pratense* (lanes 1, 2: M46, M103), *P. bertolonii* (lanes 3, 4: M33, M40), *P. alpinum* (lanes 5, 6: M39, M27) , and *P. montanum* (lane 7: M29), using genome-specific clones as probes. *Eco*RI-digested total DNA (5 μg) was loaded onto each lane, and the Southern-blotted DNA was probed by the *P. alpinum*-specific clones: pPa08 (*a*) and pPa11 (*b*), and the *P. bertolonii*-specific clones: pPb43 (*c*) and pPb68 (*d*). The exposure time of the hybridized membrane was 20 h

ladder pattern was clearer in *Sau*3AI-digested samples (Cai 1991). A ladder pattern was expected for tandemly repeated, interspersed sequences (Zhao et al. 1989; Pehu et 1990).

## Discussion

Successful construction of species- or genome-specific clones by "shot gun" cloning of *Sau*3AI-digested plant DNA and colony hybridization with total genomic probes has been reported in other genera of plants, e.g. *Triticum* (Metzlaff et al. 1986), *Hordeum* (Junghans and Metzlaff 1988) and *Beta* (Schmidt et al. 1990). Our paper describes DNA cloning and the isolation of genome-specific sequences in the genus *Phleum*. By screening 1230 recombinant colonies from the genome of *P. alpinum* and 1320 from the genome of *P. bertolonii* through colony hybridization and further dot-blot analysis using total DNA from these two species as probes, we could identify 8 clones specific to *P. alpinum* and 13 specific to *P. bertolonii*. The frequency of genome-specific clones detected in this work is much lower than that reported by Saul and Potrykus (1984) and Metzlaff et al. (1986) and slightly lower than that reported by Pehu et al. (1990). The low frequency of genome-specific clones identified in this study may imply a relatively low level of differentiation between *P. alpinum* and *P. bertolonii*. This seems to be in agreement with the results from the earlier studies by interspecific hybridization and cytological observation of hybrids (Nordenskiöld 1945), which concluded that *P. bertolonii* might be partially homologous to *P. alpinum*. Almost all of the species- or genome-specific sequences reported for the other plants were repetitive sequences. Repetitive DNA sequences are an ubiquitous feature of eukaryotic genomes and comprise a high, varying proportion of the genomes in most plants (Flavell et al. 1974; Walbot and Cullis 1985; Crowhurst and Gardner 1991). It has been concluded that repetitive DNA evolves rapidly between genomes presumably through processes involving amplification, divergence and transposition (Hake and Walbot 1980; Singer 1982; Zhao et al. 1989). Therefore it is considered to be an important source of probes that are species specific or genome-specific. Three classes of relative sequence abundance in the clones isolated from *Phleum* species were deduced from the signal intensities in dot-blot hybridization. High copy-number sequences were deduced from high-intensive signals, and moderate and low copy-number were inferred from middle- and low-intensive signals. Although the larger inserts as target DNA may enhance the signal, the differences in signal intensities scored among the clones isolated from the *Phleum* species may mostly be due to the copy-number differences of the sequences since no obvious correspondence between signal intensities and insert sizes was found in dot-blot hybridization. Among the clones listed in Table 2 and 3, only 5 may have low abundant sequences and all the rest were deduced to carry highly or intermediately repetive sequences. However, no precise lengths of repeat units and no definite copy numbers have been determined. The interspersed arrangement pattern of two sequences pPb27 and pPb43 was inferred from the results of Southern blot analysis. More studies are required to further characterize these sequences.

This study has provided the first molecular evidence for genome relationships in *Phleum*. Three *P. alpinum*-specific clones and 3 *P. bertolonii*-specific clones were used as probes to hybridize to the DNA from four species, *P. alpinum, P. bertolonii, P. pratense* and *P. montanum*, on Southern blots. All 3 *P. bertolonii*-specific probes and 2 of the 3 *P. alpinum*-specific probes were shown to hybridize to the DNA of *P. pratense*, thus confirming that the polyploid genome in *P. pratense* is closely related to the genome of

*P. alpinum* and that of *P. bertolonii*. This result coincides with those obtained in our previous study by means of C-banding (Cai and Bullen 1991). Therefore, it can be concluded that *P. pratense* is an allohexaploid containing two genomes, of which one may have come from *P. alpinum* and the other most likely donated by *P. bertolonii*. In contrast to the case of *P. pratense*, neither *P. alpinum*-specific probes nor *P. bertolonii*-specific probes were shown to hybridize to DNA from *P. montanum*, suggesting the genome distinctiveness between this species and the two diploid species, *P. alpinum* and *P. bertolonii*. It should be pointed out that although *P. pratense* may share a common genome with *P. alpinum*, the genome seems to have differentiated between these two species since pPa07 from *P. alpinum* cannot hybridize to *P. pratense* DNA. The C-banded karyotype of *P. pratense* (Cai and Bullen 1991) seems to suggest that the genome formula in *P. pratense* was AAAABB. However, no evidence obtained in the present study can be considered to support this conclusion. A possible way to clarify the genome formula in this species is to *in situ* hybridize genome-specific probes to its chromosomes. This work has been attempted in our laboratory but has not succeeded as yet.

The genome-specific sequences isolated in this study have proven to be effective molecular markers in simple hybridization experiments to verify the close relationships between the cultivated timothy and two wild species, *P. alpinum* and *P. bertolonii*. The genome analysis with these probes provides concrete evidence leading to a conclusion about the genome constitution and evolution in timothy. These sequences may also be used as probes to test whether some other species in this genus have close relationships to the hexaploid species *P. pratense*. Moreover, they may also be used as molecular markers in practical breeding to identify interspecific hybrids involving *P. alpinum* or *P. bertolonii*.

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